

## **General Approach to Toxicology Cases**

### **1 Scope**

This procedure provides general guidelines for the analysis of toxicology cases in the Chemistry Unit of the FBI Laboratory. These guidelines are based on recommendations set forth in a number of reference documents such as:

- *Society of Forensic Toxicologists (SOFT)/American Academy of Forensic Sciences (AAFS) Forensic Toxicology Laboratory Guidelines*
- *Drug Abuse Handbook*
- *Tietz Handbook of Clinical Chemistry*
- *Poison Detection in Human Organs*
- *Introduction to Forensic Toxicology*
- *Principles of Forensic Toxicology, Handbook of Analytical Toxicology*
- *FBI Laboratory Quality Assurance Manual*
- *FBI Laboratory Operations Manual*
- *Chemistry Unit Quality Assurance and Operations Manual*
- *American Society of Crime Laboratory Directors / Laboratory Accreditation Board (ASCLD/LAB) Accreditation Manual*
- *International Organization of Standardization (ISO) / International Electrotechnical Commission (ISO/IEC) 17025 Requirements*
- *ASCLD/LAB-International Supplemental Requirements*
- *American Board of Forensic Toxicology, Inc. (ABFT) Laboratory Accreditation Checklist*

### **2 Equipment/Materials/Reagents**

Not applicable.

### **3 Standards and Controls**

Not applicable.

## **4 Sampling and Sample Selection**

The most common toxicology specimens, blood, vitreous humor and urine, are liquid. Before sampling, containers will be inverted, swirled or vortexed to ensure homogeneity. If a blood sample is clotted, clots may have to be destroyed before sampling. This can be performed using a ground glass clot buster or with a disposable plastic clot buster. When blood samples are very viscous and cannot be pipeted accurately, they may be sampled by weight. When this occurs, the weight will be documented in case notes to the nearest 0.01 gram. When blood must be weighed for a quantitative examination, this will be considered in the uncertainty calculations. Additionally, concentrations will be reported in w/w instead of in w/v.

Blood samples may be received in multiple tubes. When blood is collected at the same time, from the same location, it may be considered the same item number upon initial check-in. These items will be further marked A, B, etc. in the Chemistry Unit, and each chemist will document in the case notes which tube was sampled from for each examination.

Tissue, gastric, and food samples are not always homogenous. Therefore, they are typically homogenized in a blender before analysis. It is not always feasible to homogenize the whole item, and often, some will be kept in the original form should additional testing be required. To prepare a homogenate, a portion is removed and weighed to the nearest gram. An equal amount of water (by weight) is added before blending to create a 1:1 (also called a two-fold) homogenate. Homogenates will be sampled by weight to the nearest 0.01 gram, and quantitative results will be reported in w/w.

When the total amount of a drug or poison in a gastric sample or food sample is needed for interpretative reasons, the whole item will be homogenized before analysis.

## **5 Procedure**

### **5.1 Sample Collection and Receipt**

The proper selection, collection, and submission of biological and other specimens for toxicological analyses is of paramount importance for scientifically sound interpretation of analytical results. While there are recommended minimum amounts of specific specimens desired to accomplish routine toxicological examinations (Tables 1 and 2), specimen amount is often limited. In these cases, the type and amount of specimen submitted may dictate the analyses that are performed.



For the analysis of blood for certain drug classes (e.g., cocaine, ethanol, cyanide), it can be advantageous if the specimen is mixed with a preservative in order to enhance stability of the analytes. This can be accomplished by using specimen tubes containing preservatives. For most toxicology cases, the preferred collection tube is a grey-top Vacutainer<sup>®</sup>, containing a mixture of sodium fluoride and potassium oxalate. If a blood specimen is not properly preserved, it is important to know what effect that this may have on the analyte(s) of interest.

Each specimen should be identified as to type. For blood, the anatomical site of collection should be stated. Specimens collected from a living person should be labeled with the time and date of collection. Specimens should always be labeled with the donor's name.

Table 1: Minimum Specimen Requirements for Postmortem Cases

Specimen:	Amount:
Heart Blood <sup>1</sup>	10 mL
Peripheral Blood <sup>1</sup>	5 mL
Urine, Gastric Contents, Vitreous Humor, and Bile <sup>2</sup>	All
Kidney <sup>2</sup>	50 g
Brain and Liver <sup>2</sup>	100 g

<sup>1</sup>Blood is the preferred specimen for most postmortem analyses.

<sup>2</sup>For routine postmortem cases, all specimens are not required. Testing of blood and vitreous humor or blood and urine is often sufficient.

Table 2: Minimum Specimen  
Requirements for Human Performance Cases

Specimen:	Amount:
Blood <sup>3</sup>	10 mL
Urine	20 mL

Biological specimens should be submitted to the FBI Laboratory under proper seal and with appropriate warning labels. FBI cases should be submitted individually and under a single communication. Specimens should be submitted in a refrigerated or frozen condition. Upon receipt of the evidence, a chain-of-custody will be started. For more detail on these procedures, consult the *FBI Laboratory Quality Assurance Manual*, the *FBI Laboratory Operations Manual*, and the *Chemistry Unit Quality Assurance and Operations Manual*.

After assignment of a toxicology case, an Examiner or Chemist will inventory the evidence to document the type of specimen(s) received, any labeling present on the specimen containers, the specimen amounts, and any damage to or leaks from the containers. Additionally, the specimen containers will be labeled with the item number and the Laboratory number.

## 5.2 Specimen Storage

Due to the nature of biologicals, specimens are kept refrigerated or frozen when not under active examination. A refrigerator and a freezer are located in the main Chemistry Unit Evidence Storage Room.

## 5.3 Analytical Schemes for Toxicology Testing

Forensic toxicological examinations are conducted on a variety of specimens for a wide range of drugs and other substances. Toxicological examinations begin with a review of the case history. Professional judgment is used to determine the sequence of assays that will be performed for a given case. Generally, cases of suspected drug-related homicide will include a blood ethanol analysis and a standard drugs-of-abuse screen with confirmation of any relevant findings. Fatalities involving motor vehicle drivers will usually require a blood ethanol determination and a more comprehensive screen for recreational and prescription drugs. Relevant positive findings are

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<sup>3</sup>Blood is the *only* specimen that permits determination of whether an individual may have been under the influence of a drug or chemical at the time of collection.

usually quantitated in the blood. Suspected drug-facilitated sexual assault (DFSA) cases may call for routine screens of urine for recreational drugs, alcohol, and other central nervous system depressants (e.g., benzodiazepines and barbiturates), but targeted screens for popular DFSA drugs (e.g., GHB, flunitrazepam, chloral hydrate) may also be necessary. Suspected poisoning cases without an alleged poison may call for a review of medical records to guide the analytical scheme. These cases may require an extensive general unknown approach which may include screening for volatile chemicals, cyanide, pesticides, etc.

When vitreous humor is the only specimen submitted in a post mortem investigation, testing will typically be limited to ethanol and other low molecular weight volatiles.

#### 5.4 Analytical Schemes for Screening Tests

Most analytical schemes begin with the use of screening protocols for classes of drugs or poisons. Commonly employed toxicological screening techniques include, but are not limited to, immunoassays, solvent extractions, solid phase extractions, headspace analysis, thin layer chromatography, color spot tests, or the use of selective detectors on gas chromatographs. Table 3 contains a list of analytes commonly screened for in the FBI Laboratory using immunoassay, headspace gas chromatography, and common drug screens.

Table 3: Analytes Routinely Screened for in Blood and Urine in Toxicology Cases

<i>Headspace gas-chromatography/mass spectrometry</i>			
ethanol	acetone	methanol	isopropanol
<i>Immunoassay</i>			
11-nor-9-carboxy $\Delta^9$ THC			
<i>UPLC/HRMS (ultra performance liquid chromatography/high resolution mass spectrometry)</i>			
alprazolam	flunitrazepam	oxymorphone	benzoylecgonine
$\alpha$ -hydroxyalprazolam	flurazepam	noroxycodone	cocaine
$\alpha$ -hydroxymidazolam	lorazepam	oxycodone	cocaethylene
$\alpha$ -hydroxytriazolam	midazolam	normorphine	ecgonine methyl ester
clonazepam	oxazepam	morphine	
7-aminoclonazepam	phenazepam	hydromorphone	
chlordiazepoxide	temazepam	norcodeine	
diazepam	zolpidem	6-acetylmorphine	
nordiazepam	zaleplon	dihydromorphone	
desalkylflurazepam	zopiclone		
7-aminoflunitrazepam			
<i>Acid/Neutral Drug Screen</i>			
acetaminophen	citalopram	lidocaine	phenobarbital

amobarbital	cyclobenzaprine	meprobamate	phenytoin
bupropion	diphenhydramine	methadone	propoxyphene
butalbital	ibuprofen	mirtazapine	secobarbital
carbamazepine	ketamine	naproxen	theophylline
carisoprodol	lamotrigine	pentobarbital	
<i>Alkaline Drug Screen</i>			
7-OH-quetiapine**	diltiazem	methamphetamine	oxymorphone
amitriptyline	diphenhydramine	methylone	paroxetine
amphetamine	doxepin	metoprolol	phencyclidine
brompheniramine	doxylamine	mirtazapine	phenylephrine**
buprenorphine	duloxetine	morphine	promethazine**
bupropion	ecgonine methyl ester	norbuprenorphine**	propoxyphene
chlorpheniramine	ephedrine	norchlorcyclizine**	propranolol
chlorpromazine	fentanyl	nordoxepin	pseudoephedrine
citalopram	fluoxetine	norfentanyl**	quetiapine
clomipramine	hydrocodone	norfluoxetine	scopolamine
clozapine	hydromorphone	norhydrocodone**	sertraline
cocaethylene	hydroxyzine	norketamine**	strychnine
cocaine	imipramine	normeperidine	tapentadol
codeine	ketamine	noroxycodone**	tetrahydrozoline**
cyclobenzaprine	MBDB	norpheniramine**	thioridazine
desmethylocyclobenzaprine**	MDA	norpropoxyphene	tramadol
desipramine	MDEA	norquetiapine**	trazodone
desmethylocitalopram**	MDMA	norsertraline	trimipramine
desmethyloclozapine**	MDPV	nortriptyline	venlafaxine
desmethyltapentadol**	meperidine	norvenlafaxine**	verapamil
dextromethorphan**	mephedrone	oxycodone	ziprasidone
dextrorphan	methadone		zolpidem

\*\*These analytes have either only been validated in urine or are only recovered at meaningful concentrations in urine.

The purpose of screening techniques is to rule out the presence of analytes that are detectable by these techniques, or to indicate when further testing may be warranted. Screening techniques should have minimum detection limits for analytes of interest that will include therapeutic concentrations for drugs and lethal concentrations for chemicals. The selection of the screening technique(s) utilized will depend upon the case history, the available specimen, current technology, and the Examiner's professional judgment. If the goal of the analysis is to screen a blood sample

for common drugs and metabolites, the following screening techniques might be applicable: ethanol analysis, immunoassays, UPLC/HRMS screen, alkaline drug screen, acid/neutral drug screen. If the goal of the analysis is to screen a blood sample for poisons, the following analytes might be targeted: volatiles, cyanide, metals, pesticides, and carbon monoxide. Detailed instructions for these assays may be found in the Toxicology Subunit Standard Operating Procedures Manual.

### **5.5 Analytical Schemes for Confirmatory Tests**

As a general matter of scientific and forensic principle, the detection of drugs and other toxins are confirmed (whenever possible) by a second technique based on a different chemical principle. Generally, the confirmatory test for the target analyte is more specific than the first assay. The confirmatory test will always include analysis of positive and negative controls for the analyte of interest.

When a screening technique indicates the possible presence of a drug or chemical in one biological specimen (e.g., urine), confirmation of the identity of the analyte in a second specimen from the same individual (e.g., blood) is acceptable, as is confirmation of a second aliquot of the same specimen.

Whenever possible and practical, the use of mass spectrometry is recommended for the confirmation. An immunoassay will never be used to confirm the results of another immunoassay since analytes that cross-react with one assay may cross-react in the second assay.

### **5.6 Analytical Schemes for Quantitations**

Quantitation is performed on analytes that are important to a case (as determined by the case history, specimen volume, and the derived interpretive value used in assessing the toxicological significance). For a more detailed discussion on toxicological quantitations see the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

Table 4 contains a list of analytes that have been validated for quantitation in blood samples.

Table 4: Analytes Routinely Quantitated in Blood in Toxicology Cases

6-acetylmorphine	codeine	ketamine	oxycodone
7-aminoclonazepam	cyclobenzaprine	lorazepam	oxymorphone
7-aminoflunitrazepam	desalkylflurazepam	MDA	paroxetine
acetaminophen	desipramine	MDEA	PCP
acetone	desmethyflunitrazepam	MDMA	pheniramine
$\alpha$ -hydroxyalprazolam	dextromethorphan	MDPV	phenobarbital
$\alpha$ -hydroxymidazolam	diazepam	meperidine	phenytoin
$\alpha$ -hydroxytriazolam	diphenhydramine	mephedrone	propoxyphene
alprazolam	doxepin	meprobamate	propranolol
amitriptyline	doxylamine	methadone	pseudoephedrine
amphetamine	duloxetine	methamphetamine	salicylic acid
benzoylecgonine	EDDP	methanol	secobarbital
butalbital	ephedrine	methylone	sertraline
carbamazepine	ethanol	midazolam	temazepam
carisoprodol	ethylene glycol	mirtazapine	THC
chlordiazepoxide	fentanyl	morphine	THC-COOH
chlorpheniramine	flunitrazepam	nordiazepam	THC-OH
chlorpromazine	fluoxetine	nordoxepin	tramadol
citalopram	flurazepam	norfentanyl	trazodone
clomipramine	gabapentin	norfluoxetine	triazolam
clonazepam	hydrocodone	normeperidine	trimipramine
cocaethylene	hydromorphone	norpropoxyphene	venlafaxine
cocaine	imipramine	nortriptyline	verapamil
	isopropanol	oxazepam	zolpidem

## 6 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 7 Measurement Uncertainty

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty

will be estimated and reported following the Chemistry Unit's *Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13).

## 8 Limitations

Not applicable.

## 9 Safety

Chemists and Examiners will follow all safety guidance provided in the *FBI Laboratory Safety Manual*. When opening a blood tube or other biological specimen container, the possibility of aerosolizing the contents exists. In order to prevent unwanted contact with the specimen, several different measures may be taken in addition to wearing appropriate personal protective equipment.

- Specimens may be opened and pipetted in a chemical fume hood.
- Specimens may be opened and pipetted behind a bench top shield.

## 10 References

*Chemistry Unit Quality Assurance and Operations Manual.*

*FBI Laboratory Operations Manual.*

*FBI Laboratory Quality Assurance Manual.*

*FBI Laboratory Safety Manual.*

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for the Toxicological Analysis of Product Tampering Investigations* (Tox 105); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Procedures for Estimating Uncertainty in Reported Quantitative Measurement* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*SOFT/AAFS Forensic Toxicology Laboratory Guidelines.* Society of Forensic Toxicologists, Inc.

and the American Academy of Forensic Sciences, Toxicology Section. 2006.

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Rev. #	Issue Date	History
6	09/28/15	In Sections 1 and 10, updated the reference to the ABFT Checklist. Removed calibration Section (previous Section 4) and renumbered subsequent sections. In Section 4, removed reference to "Qs and Ks". Removed hair from Table 2. Table 3 was updated to account for the replacement of most immunoassays with a UPLC/HRMS method. Updated text in 5.4 to account for newly validated UPLC/HRMS method. Updated Table 4 to account for newly validated analytes. Removed Reagent SOP from Section 10.
7	03/01/16	In Table 2, updated minimum volume of urine. Added guidance for vitreous humor only cases to Section 5.3. Clarified wording in Section 5.4. In Section 9, added guidance for safely opening and pipetting biological specimens.

**Approval**

  
Redacted - Signatures on File

## **Guidelines for the Toxicological Investigation of Drug-Facilitated Crimes**

### **1 Scope**

This procedure serves as a general guideline for the toxicological investigation of drug-facilitated crimes (DFC) in the Chemistry Unit of the FBI Laboratory. DFC investigation may include cases in which a sexual assault is alleged to have occurred, commonly known as drug-facilitated sexual assault (DFSA). It is important to note that no two cases are alike, each having its unique circumstances and history which affect the direction of the toxicological investigation. However, the following serves as a suggested approach in the analysis of submitted specimens. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **2 Background**

One of the more difficult problems for analytical and forensic toxicologists investigating these crimes is the number of different drugs that have been used to commit DFC. Table 1 lists the common symptoms that victims of this crime have reported. Many different drugs can cause the symptoms listed in Table 1. Table 2 lists examples of drugs that have been reportedly used in DFC.

Table 1: Symptoms Described by Victims of DFC

Confusion	Reduced Inhibition
Drowsiness	Impaired Memory
Dizziness	Reduced Blood Pressure
Nausea	Decreased Heart Beat
Lack of Muscle Control	Loss of Consciousness
Impaired Judgment	

Table 2: Example Drugs Reportedly Used to Commit DFC

Ethanol	Barbiturates
GHB, GBL, and 1,4-Butanediol	Zolpidem
Benzodiazepines	Ketamine
	Opiates
Clonazepam	Sedative Antihistamines
Lorazepam	Hallucinogens
Alprazolam	Sedative Antidepressants
Triazolam	Chloral Hydrate
Chlordiazepoxide	Sedative Muscle Relaxants
Diazepam	Scopolamine
Temazepam	Herbal Sedatives
Flunitrazepam	

It is very important for the examiner to get a complete history of the case. Table 3 lists information that should be obtained and evaluated before beginning the toxicological analysis.<sup>1</sup>

Table 3: Questions to ask investigators of DFC cases

1	What symptoms did the victim describe?
2	How long was the victim's memory impaired?
3	What specimens were collected?
4	How much time passed between the alleged drugging and the collection of the specimen(s)?
5	Did the victim consume any ethanol? How much?
6	Did the victim take any drugs (recreational, herbal, prescription, or over-the-counter)? How much?
7	How many times did the victim urinate prior to the collection of a urine specimen?
8	What drugs does the suspect(s) have ready access to?
9	What are the suspect's hobbies / occupation?

After obtaining such a history, it may steer the toxicological investigation in a particular direction. This may help alleviate the time-consuming analyses typically required in a DFC investigation.

<sup>1</sup> Allow the criminal investigation to drive the toxicological investigation, not *vice versa*. The *DFC Information Collection Worksheet* (Appendix A) may be useful in gathering this information.

## **3 Specimens**

### **3.1 The Best Specimen - Urine**

The more quickly a specimen can be obtained, the better the chances of detecting a drug in a biological specimen. This is very important in cases of DFC. As a general rule, a urine specimen (~50 mL) should be obtained within 120 hours (5 days) of the alleged drugging. Urine specimens allow for a longer window of detection of drugs commonly used in these crimes. Therefore, urine is usually considered "the best" specimen for most DFC cases. Analytes such as GHB and ethanol are rapidly excreted from the body, so testing for these compounds will not usually be performed if more than 12 hours have elapsed between the incident under investigation and the collection of the urine specimen.

### **3.2 Blood Specimens**

In addition to the urine specimen, a blood specimen (~10 mL) should be obtained if the drugging occurred within the prior 24 hours. Blood may be tested if more than 24 hours elapse between the incident and the collection time to be able to better interpret the significance of positive urine findings, or if target drugs are known to have longer half lives. These specimens should be properly preserved so as to prevent putrefaction. Sodium fluoride and/or potassium oxalate are the preferred preservatives. It is recommended that, when possible, blood specimens only be analyzed for drugs or metabolites identified in the related urine specimen.

### **3.3 Hair Specimens**

Hair would appear to be a logical specimen choice following a suspected DFC, particularly when there has been a delay in reporting the incident. However, there are many limitations as to the usefulness of hair specimens in these cases.

The first limitation is that most published methods for drug testing of hair samples are designed to detect chronic drug use in an individual. Until recently, drugs that have been identified in hair are those that are generally consumed in relatively high doses. Many of the drugs listed in Table 2 are low-dose formulations. Thus, there is very little drug available to incorporate into the hair. Additionally, most of these cases involve a one-time exposure to the drug.

The next issue is that hair is not conducive to comprehensive drug screens. Testing for a few drugs or drug classes may consume the entire available hair sample. In addition, hair cannot be used to screen for the most commonly encountered drug used to facilitate rape -- alcohol. Therefore, it is imperative to have a good idea as to what the likely drugging agent(s) is/are prior to the hair analysis.

Of course, hair length should also be considered. Human hair grows at a rate of approximately one centimeter per month. If the victim has short hair or there is a delay of months in collection of the hair sample, evidence of the drugging may have been removed with his/her last hair cut. It takes about two weeks for drugs to begin to appear in the hair above the scalp. Hair should be cut by isolating an approximately pencil-width section of head hair from the victim using rubber bands, ensuring that the end closest to the scalp is clearly marked. The hair should be cut close to the scalp and placed into a paper envelope.

### **3.4 Other Specimens**

As Table 1 indicates, nausea and vomiting are common symptoms of many of the drugs related to DFC. These drugs take time to completely absorb into the blood stream after ingestion. When a victim vomits shortly after ingestion of a drug, the substance may not have had an opportunity to completely absorb into the blood stream. Thus, the vomitus may contain a significant amount of the drugging agent so collection and analysis of this specimen should be assessed in these cases.

If the vomitus has dried prior to collection, the article that contains the vomitus should be submitted to the laboratory for testing. If it has not dried, it should be carefully transferred into a clean container, such as a urine collection cup.

There are other stains that may be discovered in these cases that should be collected for toxicological testing. Occasionally, the victim may release his/her bladder and a urine stain may be present on bedding materials or clothing. Also, there may be stains on items as a result of sweat as well as vaginal or rectal bleeding. If these stains are large enough, these items may also be analyzed for many of the same drugs and metabolites that would be found in a traditional urine or blood specimen. Analyzing an unstained portion of the stained material is recommended to aid with interpretation of results.

Other important evidence may include beverages, cups, plates, or containers in which the drug is suspected to have been delivered. When analysis of these items reveals a DFC drug, biological specimen analyses should be targeted toward these findings.

## **4 Analysis**

### **4.1 Routine Drugs**

Obtaining answers to the questions in Table 3 narrows down the likely candidates of drugs to search for in a given case. Whenever a likely candidate is uncovered, that drug and/or its primary biotransformation product should be targeted in a sensitive analysis.

Unfortunately, many DFC investigations do not reveal a likely drug. Then the toxicological investigation must incorporate screens for "routine" DFC drugs, as the case history dictates. Table 4 contains drugs that should be considered as part of a routine DFC screen in the FBI Laboratory.

Table 4: Routine DFC Drugs

Ethanol	Opioids:**
Gamma Hydroxybutyrate	Heroin
Gamma Butyrolactone**	Morphine
1,4-Butanediol**	Codeine
Benzodiazepines:**	Hydrocodone
Alprazolam	Hydromorphone
Bromazepam	Oxycodone
Chlordiazepoxide	Fentanyl
Chlorazepate	Methadone
Estazolam	Barbiturates:
Flurazepam	Amobarbital
Midzolam	Butalbital
Nitrazepam	Phenobarbital
Triazolam	Pentobarbital
Secobarbital	
Zolpidem**	Sedative Antidepressants:**
Amphetamines:	Amitriptyline
Amphetamine	Desipramine
Methamphetamine	Citalopram
Methylenedioxymethamphetamine	Sedative Antihistamines:
Cocaine**	Diphenhydramine
Marijuana**	Brompheniramine
Cyclobenzaprine	Chlorpheniramine
Carisoprodol**	Doxylamine
Ketamine**	
Scopolamine	

\*\*Screens for these drugs should include major metabolites in addition to the parent drug.

## 4.2 Non-routine Drugs

At times the toxicological investigation may warrant a more comprehensive analysis. It may become necessary to perform targeted analyses for some non-routine DFC drugs. Table 5 lists the drugs that should be considered as part of a non-routine DFC screen in the FBI Laboratory.

Table 5: Non-routine DFC Drugs

Anticonvulsants:**	Sedatives :	Hallucinogens :
Chloral Hydrate	Clonidine	p-Methoxyamphetamine
Ethchlorvynol	Zolazepam	Tiletamine
Valproic Acid		

\*\*Screens for these drugs should include major metabolites in addition to the parent drug.

### 4.3 Results

The percentage of positive findings in cases of DFC is not high. There are a number of factors that may contribute to the low number of findings. First, victims often do not come forward immediately. Every hour that passes between the drugging and the collection of specimen decreases the chance of detecting many of the drugs used in these crimes.

Next is the fact that many laboratories are relying on analytical techniques that are not sensitive enough to determine if a person was drugged. While these techniques are adequate to determine if an individual overdosed or was driving under the influence of one of these drugs, they are far from adequate when addressing whether a victim was drugged to the point of unconsciousness many hours to days prior to the collection of a specimen. Improving methodologies to lower the detection limits for certain drugs should result in an increase in the number of positive findings in these cases. The Society of Forensic Toxicologists Drug-Facilitated Crimes Committee has recommended maximum detection limits for common DFC drugs and metabolites in urine sample.

Another cause for the lack of a "positive" finding may be that an inappropriate specimen is taken (e.g., blood with no urine).

These factors should be taken into consideration when reviewing results performed both in the FBI Laboratory and from other laboratories.

### 5 Equipment/Materials/Reagents

Not applicable.

### 6 Standards and Controls

Not applicable.

## **7 Sampling**

Not applicable.

## **8 Procedure**

Not applicable. This document is meant to serve only as a guideline for analyzing evidence from suspected DFC cases. Follow appropriate Toxicology SOPs for specific procedures.

## **9 Calculations**

Not applicable.

## **10 Measurement Uncertainty**

Not applicable.

## **11 Limitations**

Not applicable.

## **12 Safety**

Not applicable.

## **13 References**

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*FBI Laboratory Safety Manual.*

*Chemistry Unit Quality Assurance Manual.*

*FBI Laboratory Operations Manual.*

*FBI Laboratory Quality Assurance Manual.*

Rev. #	Issue Date	History
1	03/11/11	In Section 3.1, updated the urine specimen collection time to 120 hours and that GHB and ethanol testing are typically capped at 12 hours post incident. Removed comments in Section 3.3 that stated hair testing in DFSA cases as a technique of the future. In Section 4.1, added a comment about recommended controls and validation. Also added high resolution mass spectrometry to 4.1. Updated Tables 4 and 5 with drugs routinely screened for and those considered non-routine. Added SOFT DFSA Fact Sheet to references. Removed all references to DFSA Positive Control from Sections 5, 6 and 7. Added Appendix A, the DFSA Information Collection Worksheet and referenced in a footnote in Section 2.
2	08/20/18	Removed footers. Removed Calibration (formerly Section 7), and renumbered. Rephrased "Measurement Uncertainty". Retitled document to align with current SOFT language. Updated language in Sections 1, 4.4, 13, and information sheet (form) to reflect SOFT language where appropriate. Replaced DFSA with DFC throughout document. Updated 3.2 to clarify blood testing. 3.4: added sweat as a matrix and added language about testing unstained portions. 13: removed references to specific TOX SOPs. 1: Updated scope statement for personnel. 3.3: removed non-metric units of measurement and changed collection to diameter specification. 2: updated language. 8: removed "subunit". Reformatted Table 3. Updated 3.1 urine volume to 50mL. Deleted 4.1 and renumbered. Updated Table 4. Added sentence to Section 4.3

### **Approval**

Redacted - Signatures on File

Toxicology  
 Technical Lead:

Date: 08/17/2018

Chemistry Unit Chief:

Date: 08/17/2018

### **QA Approval**

Quality Manager:

Date: 08/17/2018

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## Preparation of Chemical Reagents

### 1 Scope

This procedure provides instructions for the preparation and storage of all reagents used in the various standard operating procedures of the Chemistry Unit's Toxicology Subunit. This document does not provide information for materials used directly as obtained from the manufacturer. Neither does it provide instructions for the preparation of calibrators, controls, or analytical standards. Prepared reagents are listed, in alphabetical order, in section 6, "Procedure" and materials needed for preparation of these reagents are listed in section 2, "Equipment/Materials/Reagents." Refer to the *Chemistry Unit Procedure for Verification of Reagents, Kits, Solvents and Standards* (CUQA 9) for guidance in labeling and testing the reliability of reagents.

### 2 Equipment/Materials/Reagents

- a. Electronic balance
- b. pH paper in various ranges
- c. Ultrasonicator
- d. Vacuum filtration apparatus (1 liter size) with 0.5  $\mu$ m PTFE filter membranes
- e. Miscellaneous routine laboratory glassware and supplies
- f. Acetic acid, glacial (17 M) (ACS grade)
- g. Acetonitrile (HPLC grade)
- h. Ammonium acetate (reagent grade)
- i. Ammonium hydroxide, concentrated (15 M) (ACS grade)
- j. Calcium chloride (reagent grade)
- k. Chloramine T (reagent grade)
- l. Chloroform (GC<sup>2</sup> grade and HPLC grade)
- m. o-Cresol (reagent grade)

- n. Cupric sulfate pentahydrate (reagent grade,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ )
- o. Curcumin (reagent grade)
- p. Diethyl ether (high purity grade)
- q. Dimethylsulfoxide (ACS grade)
- r. Diphenylamine (reagent grade)
- s. Ethyl acetate (HPLC grade)
- t. Ethyl alcohol (200 proof and pharmaceutical grade)
- u. Ferric chloride hexahydrate (reagent grade,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ )
- v. Ferric nitrate nonahydrate (reagent grade,  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ )
- w. Gold(III) chloride hydrochloride trihydrate (reagent grade,  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ )
- x. Heptafluorobutyric acid (aka HFBA) (reagent grade)
- y. Hexamethonium hydroxide solution, 0.1 M (obtained from Fluka Chemical Company)
- z. Hexane (UV grade)
- aa. Hydrochloric acid, concentrated (12 M) (ACS grade)
- bb. Indigo Carmine (reagent grade)
- cc. Iodine (reagent grade)
- dd. Isopropanol (2-propanol) (HPLC grade)
- ee. Magnesium nitrate (high purity grade I)
- ff. Mercuric chloride (reagent grade,  $\text{HgCl}_2$ )
- gg. Methanol ( $\text{GC}^2$  grade and HPLC grade)
- hh. Methylene Chloride (dichloromethane) (HPLC grade)
- ii. Nitric acid, concentrated (16 M) (ACS grade and Optima grade)

- jj. Palladium matrix modifier solution (0.1% obtained from High-Purity Standards, Inc.)
- kk. PIC reagent (methanesulfonic acid) (reagent grade)
- ll. PICB-8 reagent (octanesulfonic acid) (low UV grade obtained from Waters Corp.)
- mm. Potassium cyanide (reagent grade)
- nn. Potassium ferricyanide (reagent grade)
- oo. Potassium hydroxide (reagent grade)
- pp. Potassium iodide (reagent grade)
- qq. Potassium phosphate, monobasic (ACS grade,  $\text{KH}_2\text{PO}_4$ )
- rr. Pyromellitic acid (reagent grade)
- ss. Saponin (reagent grade)
- tt. Silver nitrate (reagent grade)
- uu. Sodium acetate trihydrate (reagent grade)
- vv. Sodium bicarbonate (reagent grade)
- ww. Sodium borate (sodium tetraborate decahydrate) (ACS grade,  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ )
- xx. Sodium chloride (reagent grade)
- yy. Sodium dithionite (reagent grade)
- zz. Sodium hydroxide (ACS grade)
- aaa. Sodium phosphate, dibasic heptahydrate (ACS grade,  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ )
- bbb. Sodium phosphate, monobasic monohydrate (ACS grade,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )
- ccc. Sulfuric acid, concentrated (18 M) (ACS grade)
- ddd. Tetramethylammonium hydroxide (ACS grade)

- eee. Toluene (HPLC grade)
- fff. Triethanolamine (reagent grade)
- ggg. Trifluoroacetic acid (98+% purity)
- hhh. Water, Deionized (18+MΩ grade)

### **3 Standards and Controls**

Not applicable.

### **4 Calibration**

Not applicable.

### **5 Sampling**

Not applicable.

### **6 Procedure**

Unless specifically noted otherwise, all reagents can be prepared in larger or smaller total volumes, as needed, by appropriate scaling of component volumes and masses. Listed grades or qualities of chemicals are the minimum acceptable levels. Unless specifically noted otherwise, a higher quality of the same chemical may be substituted.

- a. 50 mM Acetic Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.25 mL glacial acetic acid. Mix well and bring to 85 mL with deionized water. Store in glass at room temperature. Stable 3 months.
- b. 0.1 M Acetic Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.5 mL glacial acetic acid. Mix well and bring to 85 mL with deionized water. Store in glass at room temperature. Stable 6 months.

- c. Dilute (~ 1.5 M) Acetic Acid:  
Mix 2 mL glacial acetic acid with 20 mL deionized water and shake to combine. Store in glass at room temperature. Stable 3 months.
- d. 1:1 Acetonitrile:Water:  
Combine 100-mL HPLC grade acetonitrile with 100 mL deionized water and mix well. Store in glass at room temperature. Stable 6 months.
- e. 0.1 M Ammonium Acetate:  
Add 3.85 g ammonium acetate to a 500-mL volumetric flask containing 300 mL deionized water. Mix well to dissolve, and bring to volume with deionized water. Store in refrigerated in glass. Stable 2 months.
- f. 0.24 M Ammonium Hydroxide:  
Add 1.6 mL concentrated ammonium hydroxide to 50 mL deionized water in a 100-mL graduated cylinder. Fill to the 100-mL mark with deionized water and mix well. Store in glass at room temperature. Stable 1 month.
- g. 2 M Ammonium Hydroxide:  
Add 10 mL concentrated ammonium hydroxide to 50 mL deionized water in a 100-mL graduated cylinder. Fill to the 75-mL mark with deionized water and mix well. Store in glass at room temperature. Stable 1 month.
- h. 5% (w/v) Calcium Chloride Solution:  
Dissolve 1 g calcium chloride in 20 mL deionized water. Store in glass at room temperature. Stable 1 year.
- i. CE (Capillary Electrophoresis) Run Buffer – Anions:  
To a 1000-mL volumetric flask, add 500 mL deionized water, 612 mg pyromellitic acid, 280 mg sodium hydroxide, 238 mg triethanolamine, and 7.5 mL 0.1M hexamethonium hydroxide solution. Mix well to dissolve, and bring to volume with deionized water. Store refrigerated in plastic. Stable 1 week.
- j. 0.5% (w/v) Chloramine T:  
To a 100-mL volumetric flask, add 80 mL deionized water and 0.5 g chloramine T. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 6 months.
- k. 4:1 Chloroform:Methanol:  
Combine 40 mL GC<sup>2</sup> grade chloroform with 10 mL GC<sup>2</sup> methanol. Mix well. Store in brown glass at room temperature. Stable 1 month.



- l. 1% (by volume) o-Cresol:  
Place 1 mL o-cresol in a 100-mL volumetric flask and fill to the mark with deionized water. Mix well and allow to stand for at least 24 hours before use. Store refrigerated in brown glass. Stable 6 months.
- m. 5% (w:v) Cupric Sulfate Solution:  
Dissolve 1.56 g cupric sulfate pentahydrate in 20 mL deionized water. Store in glass at room temperature. Stable 1 year.
- n. Curcumin Solution (saturated in ethanol):  
Add curcumin to 10 mL 200 proof ethanol in a test tube with mixing until no more will dissolve. Centrifuge at low speed for 5 min and transfer the supernatant. Store in glass at room temperature. Stable 1 year.
- o. Cyanmethemoglobin Reagent (Drabkin's Solution):  
To a 1000-mL volumetric flask containing 500 mL deionized water, add 200 mg potassium ferricyanide, 50 mg potassium cyanide, and 1 g sodium bicarbonate. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in brown glass. Stable 4 months.
- p. Diphenylamine Reagent (0.5% w:v in sulfuric acid):  
Dissolve 0.5 g diphenylamine in 100 mL concentrated sulfuric acid. Store in glass with a PTFE-lined cap at room temperature. Stable 1 year.
- q. 80% (by volume) Ethanol:  
Measure 80 mL pharmaceutical grade ethanol into a 100-mL volumetric flask. Bring to volume with deionized water and mix well. Store in glass at room temperature. Stable for 6 months.
- r. 1:1 Ether:Toluene:  
Combine 50 mL HPLC grade toluene with 50 mL diethyl ether. Mix well. Store in glass at room temperature. Stable 1 month.
- s. 5% (w/v) Ferric Chloride Solution:  
Dissolve 1.67 g ferric chloride hexahydrate in 20 mL deionized water. Store in glass at room temperature. Stable 1 year.
- t. 0.5% (w/v) Gold Chloride Solution:  
Dissolve 130 mg gold(III) chloride hydrochloride trihydrate in 20 mL deionized water. Store in brown glass at room temperature. Stable 1 year.

- u. 0.1% (w/v) Heptafluorobutyric Acid:  
Add 0.5 g HFBA to 400 mL deionized water in a 500-mL volumetric flask and mix well. Bring to volume with deionized water. Store in glass at room temperature. Stable 3 months.
- v. 2 mM Hydrochloric Acid:  
In a 100-mL volumetric flask, combine 80 mL deionized water with 16  $\mu$ L concentrated hydrochloric acid and mix well. Bring to volume with deionized water. Store in glass at room temperature. Stable 6 months.
- w. 0.1 M Hydrochloric Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.8 mL concentrated hydrochloric acid. Bring to 96 mL with deionized water and mix well. Store in glass at room temperature. Stable 6 months.
- x. 0.96 M Hydrochloric Acid:  
To a 100-mL volumetric flask, add 80 mL deionized water. Add 8 mL concentrated hydrochloric acid and mix well. Bring to volume with deionized water. Store in glass at room temperature. Stable 6 months.
- y. 1 M Hydrochloric Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water. Add 8 mL concentrated hydrochloric acid and mix well. Bring to 96 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- z. 6 M Hydrochloric Acid (~ 50% v:v):  
To a 25-mL graduated cylinder containing 10 mL deionized water, add 12 mL concentrated hydrochloric acid and mix well. Bring to 24 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- aa. 0.01% (w/v) Indigo Carmine Reagent:  
Dissolve 10 mg indigo carmine in 100 mL deionized water. Store in glass at room temperature. Stable 1 year.
- bb. Iodine Test Solution:  
Dissolve 0.4 g iodine and 0.6 g potassium iodide in 20 mL deionized water. Store in glass at room temperature. Stable 6 months.
- cc. LC (Liquid Chromatography) Mobile Phase – Alkaline#1 / Cocaine (95:5:0.03 methanol:water:ammonia):  
Combine 950 mL HPLC grade methanol and 50 mL deionized water. Mix well and vacuum filter through a 0.5  $\mu$ m PTFE membrane. Add 0.3 mL concentrated ammonium

- hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 1 month.
- dd. LC Mobile Phase – Alkaline#2 (5:95:0.03 methanol:water:ammonia):  
Combine 25 mL HPLC grade methanol and 475 mL deionized water. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Add 0.15 mL concentrated ammonium hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 1 month.
- ee. LC Mobile Phase – Benzodiazepines (60:40:0.03 methanol:water:ammonia):  
Combine 300 mL HPLC grade methanol and 200 mL deionized water. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Add 0.15 mL concentrated ammonium hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 1 month.
- ff. LC Mobile Phase – Rodenticide #1 (0.1% acetic acid in water):  
Vacuum filter 500 mL deionized water through a 0.5 µm PTFE membrane. Add 0.5 mL ACS grade glacial acetic acid. Store in glass at room temperature. Stable 1 month.
- gg. LC Mobile Phase – Rodenticide #2 (0.1% acetic acid in methanol):  
Vacuum filter 500 mL Optima grade methanol. Add 0.5 mL ACS grade glacial acetic acid. Vacuum filter through a 0.5 µm PTFE membrane. Store in glass at room temperature. Stable 1 month.
- hh. LC Mobile Phase – Mivacurium #1 (acetonitrile):  
Measure out 1000 mL HPLC grade acetonitrile and vacuum filter through a 0.5 µm PTFE membrane. Store in glass at room temperature. Stable indefinitely.
- ii. LC Mobile Phase – Mivacurium #2 (5 mM octanesulfonic acid):  
Quantitatively transfer the contents of one vial of PICB-8 reagent into a 1000-mL volumetric flask and bring to the mark with deionized water. Vacuum filter through a 0.5 µm PTFE membrane. Store in glass at room temperature. Stable 1 month.
- jj. LC Mobile Phase – Mivacurium MSMS (40:60:0.015 acetonitrile:water:methanesulfonic acid):  
Combine 200 mL HPLC grade acetonitrile, 300 mL deionized water, and 75 µl PIC reagent. Vacuum filter through a 0.5 µm PTFE membrane, and verify 2<pH<3.5. Store at room temperature in brown glass. Stable 1 month.
- kk. LC Mobile Phase – Succinylmonocholine #1 (92:8:0.1 water:methanol:PIC reagent):  
Combine 460 mL deionized water, 40 mL HPLC methanol, and 0.5 mL PIC reagent. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Store at room temperature in brown glass. Stable for 1 month.

- ll. LC Mobile Phase – Succinylmonocholine #2 (80:15:4.75:0.25 0.1% HFBA:0.1 M ammonium acetate:acetonitrile:isopropanol):  
Combine 400 mL 0.1% heptafluorobutyric acid, 75 mL 0.1 M ammonium acetate, 23.75 mL HPLC grade acetonitrile, and 1.25 mL HPLC grade isopropanol. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Store in glass at room temperature. Stable 1 month.
- mm. 1:1 Methanol:Dilute Hydrochloric Acid:  
Combine 2 mL GC<sup>2</sup> grade methanol with 2 mL 1 M hydrochloric acid, and mix well. Store in glass at room temperature. To be prepared fresh.
- nn. 95:5 Methanol:Water:  
Combine 95 mL HPLC grade methanol with 5 mL deionized water and mix well. Store in glass at room temperature. Stable 12 months.
- oo. 1:1 Methanol:Water:  
Combine 50 mL HPLC methanol with 50 mL deionized water and mix well. Store in glass at room temperature. Stable 12 months.
- pp. Nitric Acid, Dilute (33% by volume):  
Mix 5 mL concentrated nitric acid with 10 mL deionized water and shake to combine. Store in glass at room temperature. Stable 1 year.
- qq. 0.2% (by volume) Nitric Acid:  
To a 1000-mL Nalgene volumetric flask containing 600 mL deionized water, add 2 mL Optima grade concentrated nitric acid. Bring to volume with deionized water and mix well. Store in plastic at room temperature. Stable 1 year.
- rr. Opiates Extraction Solvent (90:10 chloroform:isopropanol):  
Combine 50 mL HPLC grade isopropanol and 450 mL HPLC grade chloroform and mix well. Store at room temperature in brown glass. Stable 1 month.
- ss. Palladium / Magnesium Nitrate Matrix Modifier for AAS (Atomic Absorption Spectroscopy):  
To a 100-mL Nalgene volumetric flask containing 50 mL deionized water, add 15 mg magnesium nitrate and 25 mL palladium matrix modifier solution. Bring to volume with deionized water and mix well. Store at room temperature in Nalgene container. Stable 5 years.

- tt. 11.8 M Potassium Hydroxide:  
To a 100-mL Nalgene volumetric flask add 66 g potassium hydroxide and 50 mL deionized water. Mix well to dissolve and bring to volume with deionized water. Store at room temperature in Nalgene container. Stable 1 year.
- uu. 5% (w/v) Potassium Phosphate Buffer (pH 4.5):  
To a 100-mL volumetric flask, add 80 mL deionized water. Add 5 g monobasic potassium phosphate and mix well to dissolve. Bring to volume with deionized water, and verify  $4.0 < \text{pH} < 5.0$ . Store refrigerated in glass. Stable 1 month.
- vv. 10% (w/v) Silver Nitrate Solution:  
Dissolve 2 g silver nitrate in 20 mL deionized water. Store at room temperature in an opaque container. Stable 1 year.
- ww. 0.1 M Sodium Acetate Buffer (pH 7):  
To a 250-mL volumetric flask, add 3.4 g sodium acetate trihydrate and 200 mL deionized water. Mix well and adjust to  $6.5 < \text{pH} < 7.5$  by slow addition of 1 N hydrochloric acid. Bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.
- xx. Sodium Acetate Buffer with 5% Methanol:  
Add 5 mL HPLC grade methanol to a 100-mL volumetric flask and bring to volume with 0.1 M sodium acetate buffer (pH 7). Store refrigerated in glass. Stable 2 months.
- yy. 1.1 M Sodium Acetate Buffer (pH 5.5):  
To a 100-mL volumetric flask, add 14.95 g sodium acetate trihydrate, 60 mL deionized water, and 2.2 mL glacial acetic acid. Mix well to dissolve, and bring to volume with deionized water. Verify  $5 < \text{pH} < 6$ . Store refrigerated in glass. Stable 2 months.
- zz. 0.1 M Sodium Borate Buffer (pH 9):  
To a 100-mL volumetric flask add 3.8 g sodium borate and bring to volume with deionized water. Sonicate for 15 minutes to assist dissolution, and verify  $8.5 < \text{pH} < 9.5$ . Store refrigerated in glass. Stable 3 months.
- aaa. Saturated Sodium Chloride Solution (~ 35% w/v):  
To a 500-mL volumetric flask, add 450 mL deionized water and 175 g sodium chloride. Gently heat with continuous stirring for at least one hour. Remove the stirbar, fill to volume with deionized water, and mix by inversion. A small amount of undissolved solid should remain in the bottom of the flask. Store in glass at room temperature. Stable for one year.

- bbb. 0.287 M Sodium Dithionite Reducing Agent:  
To a 50-mL volumetric flask, add 40 mL deionized water and 2.96 g sodium dithionite. Mix well to dissolve and bring to volume with deionized water. Store in glass at room temperature. Stable 1 month.
- ccc. 0.1 M Sodium Hydroxide:  
To a 100-mL Nalgene volumetric flask, add 60 mL water and 0.4 g sodium hydroxide. Mix well to dissolve and bring to volume with deionized water. Store in Nalgene containers at room temperature. Stable 1 year.
- ddd. 5 M (20% w/v) Sodium Hydroxide:  
To a 100-mL Nalgene volumetric flask, add 60 mL water and 20 g sodium hydroxide. Mix well to dissolve and bring to volume with deionized water. Store in Nalgene containers at room temperature. Stable 1 year.
- eee. 0.1 M Sodium Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify  $5.8 < \text{pH} < 6.1$ , adjusting pH by addition of 0.1 M dibasic sodium phosphate (increases pH) or 0.1 M monobasic sodium phosphate (decreases pH) as necessary. Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.
- fff. 0.1 M Sodium Phosphate, Dibasic:  
To a 100-mL volumetric flask, add 2.7 g sodium phosphate dibasic heptahydrate and 80 mL deionized water. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 1 month.
- ggg. 0.1 M Sodium Phosphate, Monobasic:  
To a 100-mL volumetric flask, add 1.4 g sodium phosphate monobasic monohydrate and 80 mL deionized water. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 1 month.
- hhh. SPE (Solid Phase Extraction) Alkaline / Cocaine Elution Solvent (78:20:2 methylene chloride:isopropanol:ammonia):  
Combine 20 mL HPLC grade isopropanol with 2 mL concentrated ammonium hydroxide and mix well. Add 78 mL HPLC grade methylene chloride and mix well. Store in glass at room temperature. To be prepared fresh.
- iii. SPE Benzodiazepines Elution Solvent (49:1 ethyl acetate:ammonia):  
Combine 49 mL ethyl acetate with 1 mL concentrated ammonium hydroxide and mix well. Store in glass at room temperature. To be prepared fresh.

- jjj. SPE Benzodiazepines Wash Solvent (20% acetonitrile in 0.1 M phosphate buffer):  
Combine 80 mL 0.1 M phosphate buffer (pH 6) with 20 mL HPLC grade acetonitrile and mix well. Store in glass at room temperature. Stable 1 month.
- kkk. SPE THC Elution Solvent aka Rodenticide Wash Solvent (95:5 hexane:ethyl acetate):  
Combine 95 mL hexane with 5 mL ethyl acetate and mix well. Store in glass at room temperature. Stable 3 months.
- lll. SPE THC-COOH Elution Solvent aka Rodenticide Elution Solvent (75:25:1 hexane:ethyl acetate:acetic acid):  
Combine 75 mL hexane with 25 mL ethyl acetate and 1 mL glacial acetic acid. Mix well. Store in glass at room temperature. Stable 1 month.
- mmm. 5 N Sulfuric Acid:  
To a 100-mL graduated cylinder containing 70 mL deionized water, slowly add 12.5 mL concentrated sulfuric acid. Mix well and bring to 90 mL with deionized water. Store in glass at room temperature. Stable 1 year.
- nnn. 5% (by volume) Sulfuric Acid:  
To a 100-mL volumetric flask containing 80 mL deionized water, slowly add 5 mL concentrated sulfuric acid. Mix well and bring to volume with deionized water. Store in glass at room temperature. Stable 1 year.
- ooo. 1 M Sulfuric Acid with 1.5% (w/v) Saponin:  
Add 80 mL deionized water and 1.35 g saponin to a 100-mL graduated cylinder and mix well to dissolve. Slowly add 5 mL concentrated sulfuric acid. Bring to the 90-mL mark with deionized water and mix well. Store in glass at room temperature. Stable 1 year.
- ppp. THC-COOH Extraction Solvent (7:1 hexane:ethyl acetate):  
Combine 70 mL hexane with 10 mL ethyl acetate and mix well. Store in glass at room temperature. Stable 3 months.
- qqq. TMAH Reagent:  
Dissolve 0.25 g tetramethylammoniumhydroxide in 1 mL deionized water. Add 20 mL dimethylsulfoxide and mix well. Store refrigerated in brown glass. Stable 1 year.
- rrr. 0.04% (by volume) Trifluoroacetic Acid (TFA):  
To a 100-mL volumetric flask, add 90 mL deionized water and 40 µl trifluoroacetic acid. Mix well and bring to volume with deionized water. Store in glass at room temperature. Stable 3 months.

- sss. Trinder's Reagent:  
Add 400 mg mercuric chloride, 400 mg ferric nitrate nonahydrate, and 0.1 mL concentrated hydrochloric acid to 5 mL deionized water in a 10-mL volumetric flask. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 6 months.

## **7 Instrumental Conditions**

Not applicable.

## **8 Decision Criteria**

Not applicable.

## **9 Calculations**

Not applicable.

## **10 Uncertainty of Measurement**

Not applicable.

## **11 Limitations**

Not applicable.

## **12 Safety**

Follow standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.



### 13 References

Shugar, G. J.; Ballinger, J.T. *Chemical Technicians' Ready Reference Handbook*, 3<sup>rd</sup> Ed.; McGraw-Hill: New York, NY, 1990.

*FBI Laboratory Safety Manual.*

*Toxicology Subunit SOP Manual.*

*Chemistry Unit Procedure for Verification of Reagents, Kits, Solvents and Standards* (CUQA 9); FBI Laboratory Chemistry Unit Quality Assurance Manual.

Rev. #	Issue Date	History
0	1/30/06	New document.
1	6/21/06	Added new reagent (saturated sodium chloride), and removed several (insulin ELISA reagents).
2	10/27/06	Added new reagents (mobile phases for rodenticides).
3	10/21/09	Updated expiration dates of 0.1 M acetic acid, LC Mobile Phase – Mivacurium #1, 95:5 Methanol:Water, 1:1 Methanol:Water, 0.1 M Sodium Acetate Buffer and 0.1 M Sodium Phosphate Buffer.

**Approval**

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## **Guidelines for Comparison of Mass Spectra**

### **1 Introduction**

Many of the analytical procedures used in the General Chemistry and Toxicology Subunits rely on mass spectrometry to help establish identification of individual chemical entities within a sample. In order to ensure consistency and reproducibility in compound identification, it is desirable to have guidelines for the comparison of known and unknown mass spectra.

### **2 Scope**

This document provides guidelines to help determine what constitutes a match between known and unknown mass spectra. Various critical characteristics of a mass spectrum are defined, and procedures for using these characteristics to evaluate matching between spectra are laid out. Note that this document provides guidelines for the matching of mass spectra, and does not directly address compound identification. A good quality mass spectral match will normally be only one element in establishing the identity of an unknown substance. These protocols are intended for application to full scan, tandem, and selected ion monitoring (SIM) mass spectra acquired in electron impact (EI), chemical (CI), electrospray (ESI), and atmospheric pressure chemical (APCI) ionization modes. Other mass spectral techniques are beyond the scope of this document. Specific mass spectral comparison guidelines in individual analytical standard operating procedures (SOPs) will override any guidelines set forth in this document.

### **3 Principle**

The spectrum of a given unknown of interest is compared to the known spectrum of a target analyte. The unknown spectrum should contain all the significant ions present in the known spectrum, and should not contain any unexplained significant ions not seen in the known spectrum. The relative intensities (hereafter referred to as "ion ratios") of several selected characteristic ions should match in both spectra, to within defined tolerances. These guidelines draw heavily on technical document 2003IDCR from the World Anti-Doping Agency, the 2003 recommendations of the American Society for Mass Spectrometry's Measurements and Standards Committee, and the 2002 National Committee for Clinical Laboratory Standards Approved Guideline for GC/MS (gas chromatography/mass spectrometry) Confirmation of Drugs.

### **4 Specimens**

Not applicable.

## **5 Equipment/Materials/Reagents**

Not applicable.

## **6 Standards and Controls**

Not applicable.

## **7 Calibration**

The calibration of all mass spectrometers should be verified regularly per the appropriate instrument protocols in the *Instrument Operations and Support Subunit SOP Manual*.

## **8 Sampling**

Not applicable.

## **9 Procedure**

Provided below are procedures for defining and determining critical characteristics of a mass spectrum to be used in establishing whether or not two spectra match. A glossary of terms is provided in Appendix 1.

### **9.1 Averaging and Background Subtraction of Mass Spectra**

In many samples, it may be necessary to correct mass spectra of interest for the presence of ions resulting from sample background, instrument background, or partially coeluting sample components. The necessary background-subtracted spectrum will usually be generated by averaging not more than five spectra across the peak of interest and then subtracting the average of a number of background spectra equal to not more than twice the number of sample spectra. The background spectra may come before and/or after the sample spectra, and should all be selected from outside the region integrated for determination of ion ratios. This background-subtracted spectrum will be used to establish the list of significant ions and the base peak for that spectrum.

### **9.2 Determination of "Significant Ions" in a Mass Spectrum**

Any ion signal greater than 15% of the most intense ion signal in a background-subtracted mass spectrum will normally be considered a *significant ion*. An ion that would otherwise be

considered significant may be excluded if it can be demonstrated that the ion arises from, or is significantly disturbed by, an uncorrectable chemical interference. Such interferences will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

### 9.3 Determination of "Diagnostic Ions" in a Mass Spectrum

**Diagnostic ions** are those ions in a mass spectrum that are characteristic of the chemical compound under investigation. Determination of diagnostic ions depends upon knowledge of the chemical structure of a component under investigation, and may therefore only be determined from mass spectra of known standards. There is not a universally accepted standard for determining diagnostic ions, however, the following recommendations should be considered.

Adduct ions will normally be excluded, except that one pseudomolecular adduct ion may be considered diagnostic. Isotopomers will be excluded unless they are characteristic of a specific chemical composition. Normally this will be limited to chlorine and bromine isotopomers, but other possibilities may arise. Ions resulting purely from a derivatizing or complexing reagent will normally be excluded from the list of diagnostic ions. For example, the  $m/z$  73 ion of a trimethylsilyl derivative may not be chosen as a diagnostic ion. Normally the (pseudo)molecular ion for a compound will be considered diagnostic, unless the intensity for that ion is less than 5% of the intensity for the base peak in the background-subtracted spectrum of the component in question.

### 9.4 Determination of the "Base Peak" in a Mass Spectrum

The **base peak** for the mass spectrum of a known standard is the most intense signal for a diagnostic ion in the background-subtracted spectrum. For the purpose of determining ion ratios, the base peak in an unknown mass spectrum will be taken as the base peak of the standard spectrum to which it is being compared, even if a different diagnostic ion shows higher intensity in that spectrum.

In instances where it can be demonstrated that the nominal base peak signal is significantly disturbed by an uncorrectable chemical interference, the second most intense diagnostic ion present in the spectrum may be used as the base peak. Such interference will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

### 9.5 Method for Calculating Ion Ratios

Ion ratios will normally be determined by integrating reconstructed ion traces for each diagnostic ion present in a given component. All integrations of reconstructed ion traces from a given component should have comparable stop and start points. Ion ratios are then calculated by dividing the area for each ion trace by the area for the trace of the base peak ion, and expressing the

result as a percentage. In instances where the reconstructed ion traces produce non-integratable data, it is acceptable to substitute ion abundances from the background subtracted spectrum of the compound of interest for the integrated areas from reconstructed ion traces. This will normally happen in situations where multiple sorts of mass spectral data are simultaneously acquired in a single analytical run, resulting in discontinuous data streams for the various individual mass spectral experiments.

## **10 Instrument Conditions**

Not applicable.

## **11 Decision Criteria**

Provided below are guidelines for establishing a match between a known mass spectrum and that of an unknown spectrum. Note that some analytical SOPs include detailed criteria for the evaluation of mass spectra of individual target analytes. Such specific instructions will supercede the guidelines provided below.

In almost all cases, unknown spectra should be matched against known spectra obtained from contemporaneously analyzed reference material. Exceptions are discussed in section 11.5 of these guidelines. When assessing spectra for a targeted analyte from multiple unknown samples in a single analytical run, it is acceptable to compare each unknown spectrum to the known spectrum resulting from a different contemporaneously analyzed reference sample. The mass spectra of many chemical entities are known to vary with analyte load. It is acceptable to dilute and reanalyze a sample containing a high level of a suspected target compound in order to be able to more appropriately match its spectrum to a lower concentration sample.

### **11.1 For Full Scan Mass Spectra**

In order to establish a match between known and unknown mass spectra in the full scan mode, both of the following criteria should be met:

- a. Every significant ion present in the known spectrum should be present in the unknown spectrum, and vice-versa.
- b. All ion ratios for diagnostic ions in the unknown spectrum should match those observed in the known spectrum within the tolerances shown in Table 1 or Table 2. If these limits would produce an acceptable lower bound of less than 1% for a given ion ratio, the lower limit will be set at 1%. Ion ratios for specific diagnostic ions may be excluded from consideration if they meet any of the following criteria:

1. The ion ratio for that ion in the known spectrum is less than 5% (less than 10% for CI, ESI, or APCI spectra).
2. The signal-to-noise ratio of the reconstructed ion trace for that ion in the unknown spectrum is less than 3.
3. It can be shown that the signal for that ion in either the known or the unknown spectrum is significantly disturbed by an uncorrectable chemical interference. Such interference will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

If there are more than four diagnostic ions in the known spectrum, then only the ratios for four diagnostic ions (three ratios) need to be evaluated in order to establish a scientifically valid match between the spectra. For compounds with a molecular weight less than 80 AMU, or less than 8 atoms, only three diagnostic ions (2 ratios) need be evaluated to establish a scientifically valid match. The selected ions will normally include the base peak and the (pseudo)molecular ion, unless those ions meet one of the three exclusion criteria given above. If fewer than three diagnostic ions are available for evaluation, the spectra may still be matched, but information derived from such a match is limited. Scan range should be chosen to provide an adequate "buffer space" around the diagnostic and significant ions of the substance in question.

Table 1: Ion Ratio Matching Tolerances for EI Mass Spectra

If the ion ratio in the known spectrum is:	>50%	$\geq 25\%$ and $\leq 50\%$	<25%
Then the ion ratio in the unknown spectrum should be within:	10% absolute	20% relative	5% absolute

Table 2: Ion Ratio Matching Tolerances for CI, ESI, and APCI Mass Spectra

If the ion ratio in the known spectrum is:	>60%	$\geq 40\%$ and $\leq 60\%$	<40%
Then the ion ratio in the unknown spectrum should be within:	15% absolute	25% relative	10% absolute

## 11.2 For SIM Mass Spectra

Selected ion monitoring experiments can allow for the detection of very low levels of analyte in complex sample matrices, at the cost of reducing the information content of that experiment. Ions for a SIM experiment must be based upon a known full scan spectrum of the species of interest collected on the instrument to be used for the SIM experiment. Four diagnostic ions will normally be selected (three ions for compounds with a molecular weight less than 80 AMU or less than 8 atoms; see 11.4 for another exception), and, if possible, all should be significant as well as diagnostic. The base peak will normally be one of the chosen ions, and the (pseudo)molecular ion should be included if it has an ion ratio greater than 5% in the known full scan spectrum. In order to establish a match between a known SIM spectrum and an unknown SIM spectrum, all resulting

ion ratios should meet the tolerances specified in Table 1 or Table 2, as appropriate.

### **11.3 For Tandem Mass Spectrometry (MS/MS)**

Tandem mass spectrometry can lend a great deal of additional specificity to mass spectral experiments by greatly increasing the confidence that the ions in a given spectrum are all associated with a single substance. Due to the nature of most collision-induced dissociation processes, however, ion ratios in tandem mass spectra tend to be much less stable, and much more dependent on analyte load, than is true for classic electron impact mass spectra.

Tandem mass spectra tend to be much "cleaner" than full scan mass spectra, with fewer extraneous ions. Therefore, the limit for determination of significant ions in a tandem mass spectrum is lowered to 10% (from 15%) of the most intense observed ion in the background subtracted spectrum. The high probability of ion association in tandem mass spectrometry means that nearly all ions of reasonable intensity observed in an MS/MS experiment should be considered diagnostic, with the exception of ions resulting purely from the loss of an adduct.

Due to the physical processes involved in the precursor ion isolation and fragmentation events in an ion trap mass spectrometer, tandem mass spectra acquired on such an instrument will occasionally show an "ion-splitting" artifact for a precursor ion returned in a product ion mass spectrum. This is evidenced by the presence of two ions, separated by a fraction of an AMU, at the nominal mass of the precursor ion in the product ion spectrum. In instances where this phenomenon is observed, the response for the affected ion should be taken as the total of the response for both components of the "split" ion signal.

#### **11.3.1 Product Ion Experiments**

When conducting product ion experiments, the selection of a precursor ion is critical to obtaining useful and reliable information. In most cases, the (pseudo)molecular ion of the species under consideration should be selected, if available. It is also acceptable to use a diagnostic isotopomer of the (pseudo)molecular ion, if one is available. If the (pseudo)molecular ion is not available, or is not suitable for some reason, then the selected precursor ion should be both significant and diagnostic in the full scan mass spectrum of the substance under consideration. With product ion spectra, it is also important to ensure that the observed fragment spectrum is, in fact, emerging from the selected precursor ion. For this reason, one of the two following criteria should normally be met for a product ion spectrum:

- a. The precursor ion should be observed in the product ion spectrum with an ion ratio of at least 5%.
- b. If full scan mass spectral data are collected concurrently with the product ion spectra, the full scan spectrum of the component of interest should show no ions within 1.5 AMU of the precursor ion with greater than three times the intensity of the precursor ion.



In order to establish a match between a known product ion spectrum and the product ion spectrum of an unknown, both of the following criteria should be met:

- a. Every significant ion present in the known spectrum should be present in the unknown spectrum, and vice-versa.
- b. All ion ratios for diagnostic ions in the unknown spectrum should match those observed in the known spectrum to within the tolerances shown in Table 3. If these limits would produce an acceptable lower bound of less than 1% for a given ion ratio, the lower limit will be set at 0.5%. Ion ratios for specific diagnostic ions may be excluded from consideration if they meet any of the following criteria:
  1. The ion ratio for that ion in the known spectrum is less than 5%.
  2. The signal-to-noise ratio of the reconstructed ion trace for that ion in the unknown spectrum is less than 3.
  3. It can be shown that the signal for that ion in either the known or the unknown spectrum is significantly disturbed by an uncorrectable chemical interference. Such interference will normally be demonstrated by showing that a reconstructed ion trace for the ion in question is not coincident with the traces for other ions associated with the component of interest.

If there are more than three diagnostic ions in the known spectrum, then only the ratios for three diagnostic ions (two ratios) need to be evaluated in order to establish a scientifically valid match between the spectra. The three selected ions should include the base peak and the precursor ion (if present), unless those ions meet one of the three exclusion criteria given above. If only a single diagnostic ion is observed in the product ion spectrum, spectra may still be matched, but information derived from such a match is limited.

Table 3: Ion Ratio Matching Tolerances for MS/MS Product Ion Spectra

If the ion ratio in the known spectrum is:	>40%	≤40%
Then the ion ratio in the unknown spectrum should be within:	25% relative	10% absolute

### 11.3.2 Precursor Ion and Neutral Loss Experiments

The practical information content for precursor ion and neutral loss MS/MS experiments is generally low, but circumstances may still arise in which one of these techniques can provide critical additional information about a given substance. For precursor ion experiments, a match between a known and an unknown spectrum may be established if all significant ions present in the known spectrum are present in the unknown spectrum, and vice-versa. For neutral loss experiments, a match between a known and an unknown spectrum may be established if all significant transition pairs present in the known spectrum are present in the unknown spectrum and vice-versa.

### 11.3.3 Selected Reaction Monitoring (SRM) Experiments

SRM analysis shares many features, advantages, and limitations with SIM analysis, but benefits from the added specificity afforded by tandem mass spectrometry. Two or three diagnostic ion transitions may be chosen for an SRM experiment. Generally, transitions should share a common precursor ion, although it is appropriate to use multiple precursor ions if all are part of a diagnostic isotope cluster in the full scan spectrum of the substance in question. It is desirable that the chosen precursor ion be the (pseudo)molecular ion of the substance in question. If this is not possible, or not practical, then the chosen precursor ion should be both significant and diagnostic in the full scan spectrum of the substance in question. In order to establish a match between a known SRM spectrum and an unknown SRM spectrum, the ion ratio of the unknown should be within  $\pm 10\%$  (relative) of the ion ratio of the known when only two transitions are monitored. When three transitions are monitored, both resulting ion ratios should meet the tolerances specified in Table 3.

### 11.3.4 Higher Order ( $MS^n$ ) Tandem Mass Spectrometry

Tandem mass spectra of order higher than 2 are beyond the scope of this document. There is little to no discussion of this subject in the various published technical guidelines, and the technique is rarely practiced in forensic and regulatory settings. When used, higher order tandem mass spectra will be addressed on a case-by-case basis, usually as a part of method validation. The criteria for product ion MS/MS in section 11.3.1 may be used as a starting point for such evaluation.

## 11.4 Exact (Precise) Mass Measurement Techniques

Exact mass measurement can provide a significant additional level of information content in a mass spectrum. The use of exact mass measurement techniques does not, however, allow other aspects of the mass spectrum under consideration to be disregarded. As such, mass spectra obtained using exact mass techniques should still meet all of the matching criteria for the appropriate mass spectral techniques given above, but different standards may be used in selecting diagnostic ions, and more confidence can be placed in matches based upon a limited set of diagnostic ions.

Ions in an unknown spectrum will be considered to be an exact mass match to those in a known spectrum if the measured masses agree to within 0.005 AMU. For a SIM experiment, only three ions, instead of four, need be monitored, and show appropriate ion ratio agreement, if all three ions meet this exact mass match criterion. When determining diagnostic ions, any isotopomer of a (pseudo)molecular ion may be considered diagnostic if it meets this exact mass match criterion. One additional adduct ion, beyond the pseudomolecular ion, may also be considered diagnostic if it meets this exact mass match criterion.

## 11.5 Matching to Library Spectra

While mass spectral libraries (either commercial compendia or collections generated in-house) can be invaluable tools in helping to direct examinations and suggest possible targets for further investigation, there are limitations to their use. Most commercial libraries do not clearly indicate the instrumentation the spectra were acquired on, or at what level of sample loading. In-house library data may have been acquired on the same instrumentation used to obtain a given unknown spectrum, but it is very difficult to ensure that long-term drift in instrument performance has not compromised the reproducibility of those library spectra.

Despite these limitations, there may arise rare instances in which it is necessary to compare an unknown spectrum to a library entry, for example if a standard of the substance in question cannot be readily obtained, or for purposes of screening to direct further investigation. In cases where such matching is attempted, all criteria for the appropriate type of mass spectrometry, given above, should still be observed, with one significant change. In these instances, ion abundances for the determination of ion ratios will be measured as the intensity of the ion in the spectrum, rather than as the integrated area of a reconstructed ion trace. For the unknown spectrum, all criteria regarding averaging and background subtraction from section 9.1 should still be observed.

## 12 Calculations

$IR_x = (A_x/A_b) \times 100$ , where:

$IR_x$  = the percent ion ratio for ion x

$A_x$  = the integrated area of the reconstructed ion trace for ion x

$A_b$  = the integrated area of the reconstructed ion trace for the base peak ion

(Ion abundances from background subtracted mass spectra may be substituted for integrated areas under certain circumstances detailed in section 9.5.)

## 13 Uncertainty of Measurement

Not applicable.

## 14 Limitations

This procedure, while extensive, is not intended to be exhaustive. Known limitations for specific analytes will be documented in individual analytical SOPs.

## 15 Safety

Not applicable.

## 16 References

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*Instrument Operation and Support Subunit SOP Manual.*

Rev. #	Issue Date	History
0	06/21/06	Original Issue
1	08/06/07	Use by General Chemistry Subunit added in Section 1. Emphasized in Section 2 that individual analytical SOPs override this document. In Section 9, changed Appendix 1 to Glossary. In Sections 9.1, 9.3, 11.3, and 14, editorial sentences were removed. Throughout document, changed phrase "relative ion intensities" to "ion ratios". In Section 11, removed "control, standard and calibrator" as examples of lower concentration samples. In Section 11 and 11.3.4, updated reference section number from 12 to 11. In Sections 11.1 and 11.2, added exception for compounds containing less than 8 atoms. Sections 11.1, 11.2, 11.3.1, 11.3.4, 11.4 and 11.5 were reworded. Section 11.3.3 was updated based on newly added reference 118 from FDA. In Section 12, added "percent" to definition of IR <sub>x</sub> . Added a new reference to Section 16. Appendix on Mass Spectra Key Points was deleted.

**Approval**

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## Appendix 1: Glossary of Terms

Adduct Ion – Any ion to which another chemical entity has been attached by a means other than covalent bonding.

Base Peak – The most intense or abundant diagnostic ion in the mass spectrum of a substance.

Diagnostic Ion – Any ion observed in the mass spectrum of a substance that is characteristic of the chemical structure of that substance.

Ion Ratio – The relative abundance or intensity of two ion signals in a mass spectrum.

Isotopomers – Two or more chemical species that differ only in isotopic composition. For example,  $\text{CH}_3\text{OH}$  and  $\text{CD}_3\text{OH}$  are two isotopomers of methanol.

Molecular Ion – A charged intact molecular species, with charge acquired solely through the gain or loss of electrons. Normally denoted as  $\text{M}^+$  or  $\text{M}^-$  for singly charged species.

Precursor Ion – In tandem mass spectrometry, the ion selected for fragmentation. Often referred to as a "parent ion".

Product Ion – In tandem mass spectrometry, an ion resulting from the fragmentation of another ion. Often referred to as a "daughter ion".

Pseudomolecular Ion – A charged molecule in which charge has been acquired through adduction of an ion or through loss of a moiety able to dissociate in solution. Examples include  $(\text{M}+\text{H})^+$ ,  $(\text{M}-\text{H})^-$ ,  $(\text{M}+\text{Na})^+$ , and  $(\text{M}+\text{NH}_4)^+$ .

Reconstructed Ion Trace – A display of the abundance or intensity of a single ion signal as a function of time during an analysis. Often also called an "extracted ion chromatogram" (EIC) or "reconstructed ion chromatogram" (RIC).

Significant Ion – Any ion in the mass spectrum of a substance present above a specified intensity or abundance.

## **Guidelines for the Toxicological Analysis of Product Tampering Investigations**

### **1 Scope**

This procedure serves as a general guideline for the toxicological investigation of suspected product tampering cases in the Toxicology Subunit of the Chemistry Unit of the FBI Laboratory. It is important to note that no two cases are alike, each having its unique circumstances and history which affect the direction of the toxicological investigation. However, the following serves as a suggested approach for the analysis of submitted specimens.

### **2 Background**

The chemical analysis in product tampering cases can be assigned to either the General Chemistry Subunit or the Toxicology Subunit. The Toxicology Subunit of the Chemistry Unit is often involved in product tampering investigations when an alleged victim has come forward and medical personnel have recognized signs and symptoms that may indicate a poisoning. In such cases, the Toxicology Subunit is often asked to examine medical records as part of the analysis process in order to direct the testing in the Laboratory. The Toxicology Subunit might also be asked to analyze the evidence if the product is a very complex mixture that will involve many different clean-up steps before samples may be analyzed by instrumentation.

### **3 Specimens**

Specimens in product tampering investigations may be food items, beverages, personal products, or medical items. In most product tampering investigations, the *control* specimen is extremely important. The control specimen is an item obtained by law enforcement or laboratory personnel that is similar to the questioned product; the control specimen is not suspected of being tampered with. It may be the same item as the item in question, or it may be a similar item, but of a different make, brand, or lot.

## **4 Analysis**

Any approved Chemistry Unit standard operating procedure may be followed. The analysis of unique matrices will often require minor deviations to existing procedures. Minor deviations will be documented and approved according to *FBI Laboratory Practices for Authorizing Deviations*. If case history points to a particular poison or class of poisons, the examiner may choose to begin the analysis with that targeted class. Otherwise, it is recommended to start by documenting the evidence, then proceed to non-destructive analyses, and finally to destructive analyses. The analytical protocol is up to the judgment of the examiner, and may depend on case history, documented illness, amount of sample, visible contamination, and/or other factors.

### **4.1 Preliminary Examinations**

Before the analysis of the evidentiary item(s) begins, it is recommended to review any submitted medical records to determine what class of poisons may or may not be of interest in the investigation. Signs and symptoms observed in the victim(s) may steer the product tampering investigation towards a particular class of poisons. Additionally, the examiner should review results of any clinical testing or other testing performed at the hospital or another facility.

Visual examination can be very important in a product tampering case. Contaminants may be visible in a sample with the naked eye or under magnification (10x magnification is typically sufficient). Holes or other signs of tampering may be visible in packaging. If marks are seen on a package that might have been made with a tool, consult the Firearms and Toolmarks Unit to determine if a Toolmarks examiner needs to examine the evidence. Toolmarks exams are typically performed before chemistry exams.

The questioned sample will be visibly compared to the control specimen, if available, and any observed differences will be documented.

Photographs may be taken of the evidence to document the appearance before chemical testing begins. A questioned sample may also be photographed along with a control sample to demonstrate differences in color, size or texture.

If a unique or identifiable odor is noted during examination, this will be documented.

The volume or mass of an unknown item will be documented before any is consumed in examination. This may prove useful later if the total amount of a contaminant in the entire specimen is estimated based on a measured concentration.



## 4.2 Non-destructive Techniques

Non-destructive techniques are those that can be performed without altering the specimen at all, or by consuming a very small portion of the specimen. Some non-destructive techniques become destructive if the specimen must be homogenized before testing. Commonly used non-destructive techniques are listed below.

- pH testing to determine the presence of a strong acid or base
- Radiography to detect foreign objects or a material of different density embedded in a solid or semi-solid (consult Metallurgy examiners for assistance)
- X-ray Fluorescence Spectroscopy to identify the presence of heavy metals (consult Metallurgy examiners for assistance)
- UV/VIS Spectroscopy to screen for chromophores (non-destructive for liquids only) {see General Chemistry's *Identification of General Unknowns* procedure (GenChem 1) for guidance}
- Direct Analysis in Real Time Mass Spectrometry to identify organic contaminants {see General Chemistry's *Identification of General Unknowns* procedure (GenChem 1) for guidance}
- Scanning Electron Microscopy with Energy Dispersive Spectroscopy to identify elemental composition (see General Chemistry examiners for assistance)
- Raman Spectroscopy to screen a liquid, solid or gas through the packaging {see General Chemistry's *Identification of General Unknowns* procedure (GenChem 1) for guidance; if assistance required, consult General Chemistry examiners)}
- Fourier Transform Infrared Spectroscopy to identify a relatively pure contaminant {see General Chemistry's *Identification of General Unknowns* procedure (GenChem 1) for guidance; if assistance required, consult General Chemistry examiners)}

## 4.3 Destructive Techniques

Destructive techniques are those that will consume a large amount of specimen, or that require homogenization of a solid sample before analysis.

- *Volatile Chemicals by Automated Headspace GC/MS(EI)* (Tox 200) to

identify volatile components

- *Direct Solvent Extraction of Acid/Neutral Drugs* (Tox 201) to identify acidic and neutral contaminants
- *Direct Solvent Extraction of Alkaline Drugs from Biological Fluids* (Tox 202) to identify alkaline contaminants
- *Toxi-Lab Screens* (Tox 206) to detect microgram per milliliter concentrations of many organic components
- *Cyanide Analysis in Biologicals and/or Foodstuffs* (Tox 301) to screen for cyanide
- *Reinsch Analysis for Arsenic, Antimony, Bismuth, and Mercury in Foodstuffs and/or Stomach Contents* (Tox 303) to screen for As, Sb, Bi or Hg
- *Pesticide Analysis in Foodstuffs and/or Stomach Contents* (Tox 304) to screen for pesticides
- *Multi-Element Screen of Urine by Inductively Coupled Plasma Mass Spectrometry* (Tox 310) to screen for metals

#### **4.4 Special Considerations for Chemical Analysis in Product Tampering Cases**

Many of the procedures listed above are designed to look for analytes in concentrations that are expected in biological samples; concentrations applicable to testing food and beverage samples are typically 10-100 fold higher. To account for this, the examiner or chemist may test less sample, dilute the sample before analysis, bring the final extract up in more solvent, etc. For example, the GC/NPD cyanide procedure is designed to look in the range of 0.5-10. This concentration range is applicable to biological fluids, but when analyzing fruit juice, a reasonable target range might be 50-1000 µg/mL. Therefore, the examiner or chemist may choose to dilute the grape juice 1:100 before analysis, and to prepare a control in grape juice, diluting it similarly. This will ensure that the analysis is within a reasonable concentration range for the target specimen, while keeping the analysis within the linear range of the instrument.

The best negative control in a product tampering case is the same product that is known not to have been tampered with. This may be sent in from the field or purchased by laboratory personnel. Sometimes, case samples are not in original packaging, or have been prepared into a meal, so it is not possible to obtain an exact negative control. In that case, the examiner or chemist should make every reasonable effort to obtain the best possible negative control.

The best positive control in a product tampering case is the questioned item itself, spiked to an appropriate concentration of analyte before analysis. When specimen volume allows, this positive control is preferred. For example, if analyzing baby formula for alkaline drugs, the questioned

sample may be diluted 1:10 with deionized water and extracted as is, as well as mixed 1:1 with an appropriate positive control. If all expected analytes are recovered in the spiked item, the examiner or chemist can be confident that most alkaline drugs would have been recovered from this matrix.

Just as in any toxicological analysis, positive results will always be confirmed in a second sampling, when specimen amounts permit. Mass spectrometry following a chromatographic separation is preferred for the identification of most analytes.

Occasionally, quantitation is required for positive findings in a product tampering case. It is recognized that there will probably not be a validated method for most product tampering findings, nor will this particular analyte ever be quantitated in this matrix again. Therefore, a full validation of the method is usually not required before testing of the case sample. However, it is recommended to demonstrate the uncertainty of the quantitation in the matrix by spiking the analyte in an appropriate control and extracting in quadruplicate on three days. If results from the first two days are acceptable, the case sample may be extracted on the third day. If the analyte is expected to be in a matrix, but the typical concentration is unknown, it is advisable to measure the concentration in at least 10 different sources that are known not to have been tampered with. For example, a trace amount of iron may be normally found in a food product. If regulatory levels of iron in that food product cannot be identified, the examiner or chemist may choose to obtain and analyze 10 sources of that food product to determine a range of typical concentrations of iron present in that product. These results may be used to interpret the positive findings in the unknown specimen.

## **5 Equipment/Materials/Reagents**

- a. Digital camera
- b. Microscope capable of 10x magnification
- c. Analytical balance

## **6 Standards and Controls**

Not applicable.

## **7 Calibration**

Quantitation of an identified analyte may be performed if the amount of a contaminant present is required to estimate the dose of a substance received by an individual, or to verify that the amount of an ingredient in a product is not what it should be. Calibrations will be performed according to the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

## **8 Sampling**

Not applicable.

## **9 Procedure**

Not applicable. This document is meant to serve as a guideline for analyzing evidence from suspected product tampering cases. Follow appropriate Toxicology Subunit SOPs for specific procedures.

## **10 Calculations**

Not applicable.

## **11 Uncertainty of Measurement**

Not applicable.

## 12 Limitations

Not applicable.

## 13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 14 References

*FBI Laboratory Practices for Authorizing Deviations*; FBI Laboratory Operations Manual

*Volatile Chemicals by Automated Headspace GC/MS(EI)* (Tox 200); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Identification of General Unknowns* procedure (GenChem 1); FBI Laboratory Chemistry Unit – General Chemistry Subunit SOP Manual

*Direct Solvent Extraction of Acid/Neutral Drugs* (Tox 201); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Direct Solvent Extraction of Alkaline Drugs from Biological Fluids* (Tox 202); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Toxi-Lab Screens* (Tox 206); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual  
*Cyanide Analysis in Biologicals and/or Foodstuffs* (Tox 301); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Reinsch Analysis for Arsenic, Antimony, Bismuth, and Mercury in Foodstuffs and/or Stomach Contents* (Tox 303); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Pesticide Analysis in Foodstuffs and/or Stomach Contents* (Tox 304); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Multi-Element Screen of Urine by Inductively Coupled Plasma Mass Spectrometry* (Tox 310); FBI

Laboratory Chemistry Unit – Toxicology Subunit SOP Manual

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit –  
Toxicology Subunit Manual

*FBI Laboratory Division Safety Manual*

Rev. #	Issue Date	History
0	12/17/08	New document.

**Approval**

  
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## General Approach to Report Writing in Toxicology

### 1 Introduction

Reports issued by the Toxicology group summarize analytical findings, and/or provide interpretation of toxicology results. Due to the wide variety of requests and evidence received, this standard operating procedure is only a general guideline for report writing. It will not always be possible to write a report using only the examples provided here. It is acceptable to use other wording as long as the results of the examinations are accurately communicated, a description of the methodology used to reach the results is included, any known limitations are addressed, and the wording is approved by a second qualified toxicology examiner during the technical review process.

Reporting guidelines specified in individual toxicology standard operating procedures will override any guidance in this procedure.

### 2 Scope

This document provides a guideline for reporting Toxicology results in a consistent manner and applies to Chemistry Unit case working personnel who write toxicology *Laboratory Reports*.

### 3 Equipment/Materials/Reagents

Not applicable.

### 4 Standards and Controls

Not applicable.

### 5 Sampling

Not applicable.



## 6 Procedure

- a. Before preparing a Laboratory report, prepare a **Results Summary**. This summary should include the tests performed, the dates the tests were performed, the identity of the individual performing the tests, the amount of specimen consumed and the results of each test. If applicable, the interpretation of results based on all of the tests performed will be included in the **Results Summary**. An example of a **Results Summary** is included in Appendix 1.
- b. Prepare and format the Laboratory report in accordance with requirements set forth in the *FBI Laboratory Operations Manual*. Prepare a **Results of Examinations** section, a **Limitations** section, and a **Remarks** section.
- c. The **Results of Examinations** section will be used to communicate the results of the toxicology examinations and a brief description of the methodology used. The **Results of Examinations** section will be consistent with the **Results Summary**. Examples of appropriate wording for the **Results of Examinations** section are included in Appendix 2.
  - For screens such as general alkaline or acid/neutral screens, explain that numerous drugs and metabolites were screened for, and give examples of drugs or drug classes that were ruled out.
  - Include the units of quantitative results. When using an abbreviation for the units for the first time in a report, spell out the abbreviation for clarity.
  - Include the uncertainty value associated with any quantitative result, along with the confidence level and *k* factor.
  - Report uncertainty values to a maximum of two significant figures, with any remainder rounded up.
  - Report measured value to the same decimal place. Determine measured value through truncation. (e.g.,  $125.5 \pm 25.5$  becomes  $125 \pm 26$ .)
  - For both positive and negative results, include a description of how the results were obtained (e.g., sample pretreatment, sample extraction and instrumental techniques).
  - Tables may be used to summarize results as long as all applicable elements above are included in the **Results of Examinations** section.
  - Use the word “identified” to report positive results when all of the following apply:
    - Positive results have been obtained in two separate samplings of a biological specimen, or in two specimens from the same person.
    - If screening results or initial testing indicate the presence of a drug, and further testing confirms the presence of the

drug and its metabolite(s), both the drug and its metabolite(s) may be reported as identified. For example, if clonazepam is indicated in an alkaline drug screen, and both clonazepam and 7-aminoclonazepam are confirmed in the benzodiazepine analysis, 7-aminoclonazepam can be reported without an additional confirmatory test for 7-aminoclonazepam. Similarly, if screening results or initial testing indicate the presence of a metabolite, and further testing confirms the presence of the drug and its metabolite(s), the drug and its metabolite(s) may be reported as identified. For example, if 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THC-COOH) is indicated by an immunoassay screen, and both THC-COOH and  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) are confirmed in a single sampling used for the cannabinoid analysis, both  $\Delta^9$ -THC and THC-COOH may be reported without an additional confirmatory test for  $\Delta^9$ -THC.

- Situations may also occur where both a drug and metabolite are indicated in a drug screen, yet only the drug or its metabolites is confirmed in the second test. As long as confirmatory testing is not negative for the drug and/or metabolite, both may be reported as positive. For example, if zolpidem and its metabolite are indicated in an alkaline drug screen, and zolpidem is confirmed via a method that is not designed to look for the metabolite (such as the alkaline drug quantitation), both may be reported without an additional confirmatory test for the zolpidem metabolite.
- The decision criteria were met for the procedure(s) that gave positive results.
- Mass spectrometry has been used as part of the testing procedure.
- Use the word “detected” to report positive results if any of the following apply:
  - Positive results are obtained for a mass spectrometric method for an analyte in one sampling of a biological specimen but there is not enough sample left to perform a second confirmatory analysis.
  - Positive results are obtained for a mass spectrometric method for an analyte in one sampling of a biological specimen but the case scenario does not warrant further analysis.
  - No certified reference material is available for mass spectral

comparison but the mass spectral results compare favorably to a library entry.

- Use the word “inconclusive” to report unconfirmed results if immunoassay screening results are positive but there is insufficient sample remaining for a second confirmatory analysis.
- Use the phrase “not detected” to report negative results when the results of a screening and/or confirmatory procedure are negative.

Additionally, results can be reported as “not detected” if something detected in a screen is not considered to be probative and subsequently the sample(s) are not subjected to confirmatory analysis.

- d. The **Limitations** section will be used to communicate any known limitations of the results, or limitations of the testing based on the evidence received. Examples of appropriate wording for the **Limitations** section are included in Appendix 3.

- When general alkaline and/or acid/neutral screens are performed on a sample, state that many drugs/metabolites were screened for, and that the lab may be contacted if a particular drug or metabolite of interest is not mentioned in the report.
- If testing was limited based on the amount of time between an incident and the specimen collection, include an explanation.
- If testing was limited based on the amount of specimen received, state this.
- The limitations of an ethanol back-extrapolation are stated (see TOX109).
- Additional information may be found in toxicology’s Approved Standards for Scientific Testimony and Report language (ASSTR).

- e. The **Remarks** section will include requirements set forth in the *FBI Laboratory Operations Manual*.

The **Remarks** section may also include facts and interpretations to assist the reader. Examples of appropriate wording for the **Remarks** section are included in Appendix 4.

The following may be included in the **Remarks** section when applicable and appropriate, and when this information may assist the reader of the report.

- Trade names, drug class, uses, side effects, metabolism and/or Federal Schedule of drugs mentioned in the **Results of Examinations** section

- Interpretation of a reported drug concentration
    - When published antemortem blood drug concentrations are used to interpret postmortem blood drug concentrations, this will be noted in the **Remarks** section.
  - An explanation of how to properly collect, mark and preserve toxicology specimens in the future
- f. Maintain copies of approved reports in a central location in order to facilitate consistency over time and among examiners.
- g. When the words “detected” or “inconclusive” are used in the **Results of Examinations** section of a laboratory report, define them in the **Remarks** section as follows:
- An analyte(s) has been reported as *detected* in this report due to insufficient sample volume and a second confirmatory analysis could not be performed for that analyte(s).
  - An analyte(s) has been reported as *detected* in this report since further analyses were not warranted based on the case scenario.
  - An analyte(s) has been reported as *detected* in this report because the Laboratory does not have a certified reference material for comparison.
  - An analyte(s) has been reported as *inconclusive* in this report because preliminary screening results indicated the possible presence of this analyte but confirmatory analysis could not be performed due to insufficient specimen volume.

## 7 Calculations

Not applicable.

## 8 Measurement Uncertainty

Not applicable.

## 9 Limitations

Not every scenario can be anticipated. This document serves as a general guideline only

## **10 Safety**

Not applicable.

## **11 References**

*FBI Laboratory Practices for the Formatting and Content of a Report of Examination - Legacy*,  
FBI Laboratory Operations Manual

*Practices for Preparing Reports of Examination and Retaining Records in Forensic Advantage*  
(FA), FBI Laboratory Operations Manual

*FBI Laboratory Toxicology Approved Standards for Scientific Testimony and Report Language*  
(ASSTR)

Rev. #	Issue Date	History
4	03/01/16	Removed Calibration Section (5) and renumbered subsequent sections. In Section 6.a., clarified that interpretations are needed only when applicable. Changed reference to Lab Ops Manual in Section 6.b. and Section 6.e. in order to be generic and updated references in Section 11 to cover Legacy and Forensic Advantage Reports. In Section 6.c., situations involving reporting of a drug and metabolite were added for clarification. In Section 6.c, clarified wording for rounding of uncertainty values. In Section 6.c, changed reporting option for non-probative, non-confirmed analytes from “detected” to “not detected”. In Sections 6.c and 6.g, removed LCUV example since this testing is no longer performed. Also clarified “not detected” example wording in Section 6.g. In Section 6.e., added a comment about interpreting postmortem blood concentrations based on published antemortem blood concentrations. Renamed Section 8. In Appendices 1-3, replaced references to “Q” numbers with “Item” numbers.
5	04/01/19	Section 2: Updated scope statement. Section 6-d: added some specifics to the limitations wording. Removed “subunit” in multiple instances. In Section 6-c, added bullet point that allows for case scenario related use of “detected”. Section 11: added reference to ASSTR. Removed a statement from Appendix 3.

### **Approval**

Redacted - Signatures on File

Toxicology  
Technical Leader:

Date: 03/28/2019

Chemistry Unit Chief:

Date: 03/28/2019

### **QA Approval**

Quality Manager:

Date: 03/28/2019

## **Appendix 1: Example of a Results Summary**

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## Appendix 2: Example of Appropriate Wording for the Results of Examinations Section of a Toxicology Report

The Item 1 blood was tested with the following results:

<b><u>Analyte</u></b>	<b><u>Result</u></b>	<b><u>Note(s)</u></b>
Ethanol	Item 1 = Ethanol was identified at a concentration of $0.119 \pm 0.017$ gram percent (g%) (99.7% confidence level, k=3).	1
Benzodiazepines	Item 1 = Not detected	2
Cannabinoids ( $\Delta^9$ -THC metabolite)	Item 1 = Not detected	2
Benzoylcegonine (a metabolite of cocaine)	Item 1 = Not detected	2
Over-the-counter, prescription and illicit drugs	Item 1 = Not detected	3

### Notes:

- 1 Analysis was performed using headspace gas chromatography with flame ionization detection (HS-GC/FID) and headspace gas chromatography/mass spectrometry (HS-GC/MS).
- 2 Analysis was performed using immunoassay.
- 3 This analysis consisted of two drug screens. The first screen targeted alkaline drugs such as antihistamines, antidepressants, opioid analgesics, methamphetamine and other stimulants. Analysis was performed using solid phase extraction (SPE) followed by gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). The second screen targeted acidic and neutral drugs such as barbiturates, seizure medications, and some non-steroidal anti-inflammatory drugs. Analysis was performed using liquid/liquid extraction (LLE) followed by GC/MS.



### **Appendix 3: Examples of Appropriate Wording for the Limitations Section of a Toxicology Report**

#### Example of a statement to explain general drug screen limitations:

The FBI Laboratory has performed screening in this case for numerous drugs, some of which are only indicated by a general drug class above. Drug dosages, drug metabolism rates, and laboratory detection limits for drugs and metabolites vary. For questions about whether or not a specific drug or drug metabolite would have been detected in this analysis, please contact the examiner issuing this report.

#### Example of a statement to explain why testing was limited based on time lag between incident and specimen collection:

Due to the time that elapsed between the alleged event and the collection of the Item1 urine specimen, the Item1 urine specimen was not tested for gamma-hydroxybutyrate (GHB) or ethanol, two drugs commonly associated with drug-facilitated assaults. GHB and ethanol are rapidly metabolized and excreted from the body, and are typically not detected beyond eight to twelve hours post-ingestion.

#### Example of a statement to explain that limited specimen precluded a complete toxicological analysis:

Testing in this case was limited to ethanol analysis due to the low volume of specimen Item1 received.

#### **Appendix 4: Examples of Appropriate Wording for the Remarks Section of a Toxicology Report**

##### Example of a drug information statement:

Oxycodone is a schedule II federally controlled substance. Oxycodone is available by prescription by itself, or in combination with other drugs such as acetaminophen or aspirin. Oxycodone is classified as an opioid analgesic, and may be prescribed for severe pain. Common trade names of oxycodone include Oxycontin<sup>®</sup> and Percocet<sup>®</sup>. The reported blood concentration of oxycodone (20 µg/mL) is consistent with reported cases of oxycodone fatalities.

##### Example of a statement on how to properly preserve and mark toxicology specimens in the future:

In the future, when toxicology testing is requested in postmortem investigations, the FBI Laboratory recommends that two grey-top tubes of blood be submitted for analysis, as grey-top tubes contain both a preservative and an anticoagulant. It is also requested that the source of that blood (central or peripheral) is specified, to aid in the interpretation of quantitative results. Additionally, the FBI Laboratory recommends the submission of a second biological specimen such as urine or vitreous humor to allow for thorough screening and to aid in the interpretation of positive results.

## **General Approach to Hair Analysis**

### **1 Introduction**

Drugs and poisons in the bloodstream can be incorporated into the keratin matrix of hair through the root. This can make hair a suitable forensic toxicological specimen for determining a history of drug or poison exposure. Because of the nature of hair as a forensic toxicological specimen, the window of detection after exposure may be wider than that of blood or urine. Since hair grows at an average rate of one centimeter per month, segmental hair analysis can be performed to approximate when the exposure occurred.

### **2 Scope**

This document addresses sample preparation and analysis of hair specimens for the presence of drugs and poisons. Specific procedures for hair analysis can be found in the Toxicology Subunit Manual.

### **3 Principle**

Samples are weighed, pulverized and extracted with methanol, or another appropriate solvent. The initial extracts are screened for the presence of a poison, drug or drug class. Positive specimens are subjected to a second sampling and extraction, when sample size permits. Wash steps may be incorporated to address the concern of exterior contamination. Segmentation and/or quantitation may be performed, when appropriate.

## **4 Specimens**

### **4.1 Specimen Collection**

Note: Although the examiner will not have complete control over the collection of hair samples for analysis, certain suggestions can and should be made to those individuals prior to collecting the specimens to be sent into the Laboratory, such as:

- Hair collectors should wait at least four weeks after an alleged exposure to a drug/poison to collect hair samples for toxicological testing.
- Hair samples collected for analysis should be cut as close to the scalp as possible, from the posterior vertex of the head, just above the neck.
- The diameter of the sample collected should be about the width of a jumbo pencil, or approximately 1 cm. A rubberband or twist tie should be placed securely around the cut end of the hair sample prior to cutting, to mark the proximal end of the bundle.
- A properly labeled, sealed white paper envelope is suggested for packaging the hair sample.
- Two samples should be collected and packaged separately to allow one specimen for testing by the FBI Laboratory, while a second is left untouched.

### **4.2 Specimen Requirements**

Typically, head hair is the specimen of choice for screening. If unavailable, body hair may be analyzed.

By dividing the hair into discrete segments that represent growth over a particular time period (i.e., one centimeter lengths represent one month time periods), the time of an exposure of an individual to a drug may be estimated. This process is called segmental analysis. Of course, the longer the hair, the more historical information may be available. Segmental analysis is only appropriate when the cut end of the hair is known and will require larger amounts of hair to be collected.

## **5 Equipment/Materials/Reagents**

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Scissors
- b. Ruler
- c. Analytical Balance
- d. Mini Beadbeater-8 Cell Disruptor, or equivalent (Biospec Products, Bartlesville, OK)
- e. 2-mL Screw Cap Microtubes
- f. Micro Stir Bars
- g. 2.5-mm Glass Beads
- h. Methanol (HPLC grade, or better)
- i. Vortex Mixer
- j. Heating/Stirring Block
- k. Microcentrifuge
- l. Disposable Syringe Filters (Anotop 25 Plus, 0.2  $\mu\text{m}$ , or equivalent)
- m. Disposable Syringes (2.5 cc, or equivalent)
- n. 22-gauge hypodermic needles
- o. Liquid nitrogen
- p. 12 x 75 mm test tubes, disposable pipettes, and other common laboratory glassware
- q. Deionized water
- r. Evaporator with nitrogen
- s. SPEX 6870 freezer/mill (cryogrinder), or equivalent (SPEX Sample Prep, Metuchen, NJ)

- t. Cryogrinder tubes, or equivalent (SPEX Sample Prep, Metuchen, NJ)

## 6 Standards and Controls

- a. Negative Control Hair:  
Obtain in house. Store at room temperature. Stable indefinitely. A Negative Control sample will be extracted and analyzed with every analysis.
- b. Positive Control Hair (any of the following appropriate controls may be used):
  - 1. Prepared in house following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Typically methanolic solutions are spiked into weighed portions of Negative Control Hair. Prepare fresh.
  - 2. Standard Reference Material<sup>®</sup> 2379 (purchased from the National Institute of Standards & Technology Gaithersburg, MD) is drug-free human hair material that has been fortified with drugs and drug metabolites. It contains the following analytes at the listed concentrations: amphetamine (6.00 ng/mg), benzoylecgonine (4.01 ng/mg), cocaethylene (2.67 ng/mg), cocaine (7.45 ng/mg), methamphetamine (5.20 ng/mg), and phencyclidine (6.24 ng/mg). It can be used as a positive control for the listed analytes. Storage conditions and stability are determined by the manufacturer.
  - 3. Standard Reference Material<sup>®</sup> 2380 (purchased from the National Institute of Standards & Technology Gaithersburg, MD) is drug-free human hair material that has been fortified with drugs and drug metabolites. It contains the following analytes at the listed concentrations: codeine (9.82 ng/mg), morphine (10.54 ng/mg), 6-monoacetylmorphine (2.71 ng/mg), and tetrahydrocannabinol (0.99 ng/mg). It can be used as a positive control for the listed analytes. Storage conditions and stability are determined by the manufacturer.

## **7 Calibration**

Not applicable.

## **8 Sampling**

Not applicable.

## **9 Procedure**

- a. Make note of approximate length of questioned hair sample, color, curvature, condition, and any other pertinent observations.
- b. Thoroughly clean scissors with methanol. Cut a portion of the hair sample into fine snippets. (If segmental analysis is to be performed, cut the hair into 1-2 cm segments and analyze them individually.)
- c. If washing of the hair is required, perform wash using an appropriate organic or aqueous solvent. A series of washes may be desirable. Typically, the last wash will be saved for possible analysis.
- d. Weigh approximately 25 mg of the hair snippets into a microtube. Add 5 glass beads. If sample size permits, two separate aliquots of the hair may be weighed and analyzed. Process control samples in a similar manner.

Alternatively, add a sample of cut hair into a cryogrinding tube. Process control samples in a similar manner.

- e. Pulverize hair in the Beadbeater or cryogrinder.
- f. For cryoground hair, weigh pulverized hair into a microtube.
- g. Add stir bars to microtubes and incubate overnight in methanol or another appropriate solvent while stirring. Apply heat during this process, if appropriate.
- h. Centrifuge microtubes. Remove methanol to a test tube. A hypodermic needle may be used for this process. Methanol extracts can be filtered at this point, if necessary.

- i. Concentrate methanol extracts to dryness. Reconstitute extracts and further purify via liquid or solid phase extraction. (See *Toxicology Subunit Manual* for guidance.)
- j. Analyze final extracts using appropriate instrumentation.

## **10 Instrumental Conditions**

Not applicable.

## **11 Decision Criteria**

Not applicable.

## **12 Calculations**

Not applicable.

## **13 Uncertainty of Measurement**

Not applicable.

## **14 Limitations**

Not applicable.

## **15 Safety**

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.



## 16 References

*Guidelines for Toxicological Quantiations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Society of Hair Testing. *For. Sci. Int.*, 2004, 145, 83-84.

Mandatory Guidelines for Federal Workplace Drug Testing Programs, proposed. Substance Abuse and Mental Health Services Administration, 2006.

Welch, M.J.; Sniegowski, L.T.; Tai., S. *Anal. Bioanal. Chem.*, 2003, 376, 1205-1211.

*FBI Laboratory Division Safety Manual.*

*FBI Laboratory Toxicology Manual.*

Rev. #	Issue Date	History
0	01/25/13	New document that replaces a previous document titled <i>"Extraction and Analysis of Drugs in Hair"</i> .

**Approval**

  
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## **Chemistry Unit (CU)**

# **FBI Approved Standards for Scientific Testimony and Report Language for the Forensic Toxicology Discipline**

### **1 Purpose**

This document provides examples of the scientifically-supported conclusions and opinions approved for reporting examination conclusions and offering expert opinion statements during testimony by Toxicology Examiners within the Toxicology Subunit of the Chemistry Unit. It is noted that these examples are not intended to be all inclusive and may be dependent upon the precedent set by the judge or locality in which a testimony is provided. Further, these examples are not intended to serve as precedent for other forensic laboratories and do not imply that statements by other forensic laboratories are incorrect, indefensible, or erroneous.

### **2 Scope**

This document applies to Chemistry Unit employees who prepare an FBI Laboratory *Report of Examination* (7-1 or 7-1 LIMS) and/or provide expert testimony in the forensic toxicology discipline. This document does not apply to Chemistry Unit employees who provide fact witness testimony.

### **3 Responsibilities**

**3.1** The Examiner will ensure that a *Report of Examination* uses the approved language contained within this document.

**3.2** The Examiner will ensure that his/her toxicology testimony is consistent with the standards contained within this document.

**3.2** The Technical and Administrative Reviewers will ensure compliance of toxicology *Reports of Examination* with the statements contained within this document.

**3.3** The Unit Chief or designee will assess if toxicology testimony complies with the statements contained within this document.

#### **4 Statements Approved for FBI Toxicology Testimony and/or Laboratory Reports**

For more detailed guidance on report writing in the Toxicology Subunit, see the *General Approach to Report Writing in the Toxicology Subunit* standard operating procedure.

- The examiner may report and/or state his/her analytical findings of the presence or absence of a drug, drug metabolite, or poison, as well as quantitative results.
- The examiner may report and/or state the estimated measurement uncertainty associated with the quantitative findings of a drug, drug metabolite, or poison.
- The examiner may report and/or state the pharmacokinetic and pharmacodynamic effects of drugs and poisons based on data published in peer reviewed literature or other authoritative sources.
- The examiner may report and/or state his/her opinion as to the effects of drugs or poisons on the average human. This opinion should be based on the facts of the case, medical information about the individual that the specimens were collected from (e.g., weight, height, disease state, age), current published studies, and/or the examiner's training in the fields of pharmacology, physiology, pathology, clinical chemistry, and/or toxicology.
- The examiner may report and/or state the limitations of his/her examinations and opinion.
- The examiner may report and/or state that a reported blood concentration is within the therapeutic range, toxic range, or consistent with reported fatal concentrations, provided the statement is based on data published in peer reviewed literature or other authoritative sources.
- The examiner may report and/or state that a drug or poison found in a hair specimen is consistent with exposure (either ingestion or environmental) to the drug or poison.
- The examiner may report and/or state the results of segmental analyses of hair samples and interpret those findings based on an average growth rate of 1 cm/month provided he/she acknowledges variation in inter-individual growth rates and assumes proper specimen collection.
- The examiner may report and/or state an extrapolated ethanol concentration in a blood sample collected from a living person.

- The examiner may report and/or state that hair findings indicate the ingestion of a drug or poison if validated wash procedures have been performed that can differentiate between exposure and ingestion and/or if a metabolite that is uniquely associated with ingestion has been identified in the sample.

## **5 Statements Not Approved For FBI Toxicology Testimony and/or Laboratory Reports**

- An examiner may not report or state the dose of a drug or poison given based on analytical findings in post-mortem samples.
- An examiner may not report or state an opinion that suggests his/her interpretation of the effects of a drug or poison can be specified to the individual whose sample was tested.
- An examiner may not report or state an opinion that a drug or poison finding in hair is proof of ingestion of the drug or poison unless a metabolite that is unique to ingestion is also identified and/or validated wash procedures have been performed that can differentiate between exposure and ingestion.
- An examiner may not report or state an opinion that an individual was impaired based on a drug concentration in a urine or hair sample.

## **6 Laboratory Report Reviews**

The content of a Toxicology *Report of Examination* will be reviewed per the *Chemistry Unit Case Record and Review Procedures* standard operating procedure ensuring compliance with the approved statements in this document.

## **7 Testimony Reviews**

Toxicology testimonies will be reviewed following the *FBI Laboratory Practices for Court Testimony Monitoring*. The review will ensure compliance with the statements in this document.

## **8 References**

FBI Laboratory Chemistry Unit Toxicology Subunit Manual, General Approach to Report Writing in the Toxicology Subunit. Latest Revision.

FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual, Chemistry Unit Case Record and Review Procedures. Latest Revision.

ASCLD-LAB-*International* Supplemental Requirement for the Accreditation of Forensic Science Testing and Calibration Laboratories. American Society of Crime Laboratory Directors/Laboratory Accreditation Board, Garner, NC, 2011.

FBI Laboratory Quality Assurance Manual. Latest Revision.

FBI Laboratory Operations Manual. Latest Revision.

Rev. #	Issue Date:	History:
0	05/28/2014	New document.

**Approval**

Redacted - Signatures on File

## **Ethanol and Common Volatiles in Biological Fluids By Headspace GC/FID and GC/MS(EI)**

### **1 Introduction**

This procedure is used to detect, identify and quantitate ethanol and other low molecular weight volatile compounds at low concentrations (<0.5 g%) in biologicals.

### **2 Scope**

This procedure allows for the screening, quantitation, and confirmation of ethanol, methanol, acetone and isopropanol in biological samples and other liquids. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

Sample and a diluent containing internal standard are added to a headspace vial using a pipettor/dilutor. Specimens are qualitatively screened and quantitatively analyzed for ethanol and other low molecular weight volatiles by headspace gas chromatography with flame ionization detection (HS-GC/FID). Specimens are qualitatively confirmed through repeat analysis by headspace gas chromatography/mass spectrometry (HS-GC/MS(EI)). The headspace technique is based on various gas laws which state that when a volatile liquid in solution, such as ethanol, comes into contact with a closed air space, an equilibrium forms between the liquid phase and the headspace. At a constant temperature, the partial pressure of the volatile in the headspace is directly proportional to its concentration in solution. This method affords a means of analyte separation from the biological matrix and produces a ready-made vapor for chromatographic analysis.

### **4 Specimens**

This procedure uses a biological fluid such as: blood, serum, plasma, urine, or vitreous humor. When available, 0.1 mL of specimen is used in this assay. For quantitation, 0.2 mL of specimen is used (2 samplings of 0.1 mL each). Other aqueous liquids may also be analyzed by this procedure, but may require dilution before analysis to ensure that results are within the linear range of this procedure.

### **5 Equipment/Materials/Reagents**

- a. 10-mL disposable headspace vials, magnetic caps, and crimper
- b. Deionized water



- c. Vortex mixer
- d. Routine laboratory glassware and supplies
- e. Agilent Gas Chromatograph / Flame Ionization Detector equipped with a headspace autosampler and a 30 m x 320  $\mu\text{m}$  x 1.2  $\mu\text{m}$  Restek RTX BAC-2 (or equivalent) column
- f. Agilent Gas Chromatograph / Mass Spectrometer equipped with a headspace autosampler and a 30 m x 320  $\mu\text{m}$  x 1.2  $\mu\text{m}$  Restek RTX BAC-1 (or equivalent) column
- g. Hamilton Pipettor/Dilutor (or equivalent) capable of delivering 0.1 mL sample
- h. Laboratory balance
- i. Clot grinder
- j. Chlorine bleach (household grade)
- k. 10% Chlorine Bleach Solution: Add 90 mL of deionized water to 10 mL of chlorine bleach. Mix well and store at room temperature. Stable for one month.

## 6 Standards and Controls

- a. Cerilliant Multicomponent Alcohol Mix Solutions:  
These standards are purchased from Cerilliant in a series of six certified concentrations [(100, 250, 500, 2000, 1000 and 4000  $\mu\text{g/mL}$ ; equivalent to 0.010 g%; 0.025 g%, 0.050 g%; 0.100 g%; 0.200 g% and 0.40 g% (w/v)]. Stability and storage determined by manufacturer.
- b. Cerilliant Ethanol Calibration Solutions: This standard is purchased from Cerilliant at a certified concentration of 0.500 g% (w/v). Stability and storage determined by manufacturer.
- c. Negative Control:  
Deionized water is used as the Negative Control for this procedure. A Negative Control of deionized water is analyzed with every assay.
- d. t-Butanol Internal Standard Stock Solution (1.0 g%):  
Add 1.0 g t-butanol (reagent grade) to about 90 mL deionized water in a 100-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store at room temperature in a tightly sealed glass container. Stable for 6 months.

- e. t-Butanol Internal Standard Solution (0.005 g%):  
Add 5.0 mL of the t-Butanol Internal Standard Stock Solution (1.0 g%) to a 1000-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store at room temperature in a tightly sealed glass container. Stable for 6 months.
- f. Whole Blood Volatiles Controls (Levels 1 and 2):  
Purchased from Cliniqa or another approved supplier. These controls contain ethanol, methanol, acetone, and isopropanol at certified concentrations. Store refrigerated. Stability determined by manufacturer. Both levels are analyzed with every FID/MSD assay. See the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101) for more information.
- g. Characterization of Whole Blood Controls:  
For commercial volatiles controls, each newly acquired lot of control will be analyzed at least 20 times in a minimum of four batches. These batches will also include a previously characterized volatiles control. The initial target value for the new control will be the average of these 20 values, provided that this value is within  $\pm 5\%$  of the nominal value for ethanol (or  $\pm 5$  mg/dL, whichever is greater), and within  $\pm 10\%$  of the nominal value for the other volatiles. After every third analysis of a given lot of volatile control, the accepted target value will be recalculated as the average value from all runs to date, excluding any failed analytical runs. Should the recalculated target value of the control ever exceed  $\pm 5\%$  of the nominal value for ethanol (or  $\pm 5$  mg/dL, whichever is greater), or  $\pm 10\%$  of the nominal value for any of the other volatiles, the control may be degrading and a new lot should be purchased and characterized. The Control Tracking Supervisor will maintain a current database of the historical performance of each lot of volatiles control with the results for that lot.

## 7 Sampling

Not applicable.

## 8 Procedure

- a. Allow specimens, controls, and calibrators to stand at room temperature for approximately 15 minutes. To 10-mL headspace vials, add 0.1 mL of specimen, control, calibrator or blank followed by a 1 mL t-butanol internal standard solution flush using the Hamilton Pipettor/Dilutor using the parameters in Section 10.1.<sup>1,2</sup> (Prepare Positive Controls and specimens in duplicate).

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<sup>1</sup> If sample size is limited, or if the concentration of the unknown is above the linear range of this procedure, the sample may be prediluted before pipetting.

<sup>2</sup> If a questioned sample is clotted and cannot be pipetted accurately, it may be homogenized with a clot grinder before pipetting. Alternatively, 0.1 g of sample may be weighed to the nearest 0.001 g, 1 mL of internal standard will be added manually, and the results will be reported in w/w units. If exactly 0.1 g is not measured, the actual weight will be used in the calculations.

- b. Flush the Hamilton Pipettor/Dilutor with deionized water in between samples.
- c. Immediately cap headspace vials.
- d. Analyze samples by HS-GC/FID and HS-GC/MS(EI) using the conditions specified in Section 9. Typically, one replicate of a case sample and a subset of quality controls are analyzed by HS-GC/MS(EI).
- e. At the conclusion of the work shift, flush the Pipettor/Dilutor with at least three cycles of 10% Chlorine Bleach Solution or an equivalent cleaner, followed by at least three cycles of deionized water.

## 9 Instrumental Conditions

Appendix 1 contains an abbreviated version of the instrumental conditions for this procedure that may be used to verify the instrumental conditions at the bench.

### 9.1 Pipettor/Dilutor Parameters (5000 $\mu$ L / 250 $\mu$ L syringes)

Dilute Method		Syringe Parameters	Left Syringe	Right Syringe	Wash Parameters	
Ratio	1:10	Syringe Fill	10	-	Wash Volume	5000 $\mu$ L
Dilution	1/11	Syringe Aspirate	-	5	Left Fill Speed	6
Left Diluent Volume	1000 $\mu$ L	Syringe Dispense	12	2	Left Dispense Speed	5
Right Air Gap	100 $\mu$ L	Syringe Fill Mode	Auto	Auto		
Right Sample Volume	100 $\mu$ L	Air Gap Mode	Auto			
Final Volume	1100 $\mu$ L	Air Gap Delay	0			

### 9.2 Headspace Sampler Parameters

incubation temperature	60°C	syringe temperature	70°C
incubation time	30 min (FID) 15 min (MS)	injection volume	1.0 mL
agitator speed	250 RPM (FID) 250 RPM (MS)	sample fill rate	0.25 mL/sec (FID) 0.5 mL/sec (MS)
agitation timing	10 sec on 1 sec off	sample fill strokes	5
		sample injection speed	0.5 mL/sec
		syringe flush time	2.0 min (FID)

		4.0 min (MS)
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### 9.3 Gas Chromatograph Parameters (FID)

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature	40°C	type	RTX BAC-2	inlet temp.	200°C
isothermal		length	30 m	injection mode	split
run time	6.0	internal diameter	320 µm	carrier gas	helium
equilibration time	0.2 min	film thickness	1.2 µm		
Flame Ionization Detector Parameters				carrier mode	constant pressure
temperature	250°C	makeup gas	none	pressure	10.2 psi
hydrogen flow	40 mL/min				
air flow	400 mL/min			split ratio	1:1

### 9.4 Gas Chromatograph Parameters (MS)

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature	40°C	type	RTX BAC-1	inlet temp.	200°C
isothermal		length	30 m	injection mode	split
run time	6.0 min	internal diameter	320 µm	carrier gas	ultrapure helium
equilibration time	0.2 min	film thickness	1.8 µm		
				carrier mode	constant flow
				flow	1.23 mL/min
				split ratio	10:1

### 9.5 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	250°C
scan range	27 - 100 m/z	quadrupole temperature	150°C
gain factor	15	solvent delay (nominal)	1.30 ± 0.5 min

## 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of data produced in this assay.

## **10.1 GC/FID Batch Acceptance**

### **10.1.1 Negative Control**

No analytes of interest will be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

### **10.1.2 Positive Control**

Each Positive Control will quantitate within  $\pm 10\%$  of the target value for ethanol, and  $\pm 15\%$  for the other volatiles. See the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101) for more information.

### **10.1.3 Calibrators**

Each calibrator will quantitate within  $\pm 10\%$  of the target value for ethanol, and  $\pm 15\%$  for the other volatiles. See the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101) for more information.

## **10.2 GC/FID Unknown Sample Acceptance**

### **10.2.1 Internal Standard Response**

The peak area for the internal standard will be within three standard deviations of the average of the internal standard area for the calibrators and controls in the batch. If the peak area for a sample is outside of this range, the agreement between sample replicates will be evaluated before reporting a concentration.

### **10.2.2 Chromatography**

The peak of interest will show good chromatographic fidelity, with reasonable peak shape, width, and resolution.

### **10.2.3 Retention Time**

The retention time for the peak of interest will be within  $\pm 2\%$  of that for the corresponding compound in a calibrator or Positive Control sample.

### **10.2.4 Signal-to-Noise**

The signal to noise ratio for the peak of interest will be at least three. The response for the suspect peak will be at least ten fold greater than that for any observed peak at similar retention time in a Negative Control injected just prior to the sample.

## **10.3 GC/MS Batch Acceptance**

### **10.3.1 Negative Control**

No analytes of interest will be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

### **10.3.2 Positive Controls**

Each target analyte (acetone, ethanol, isopropanol, and methanol) will be detected in the Positive Controls.

## **10.4 GC/MS Unknown Sample Acceptance**

### **10.4.1 Chromatography**

The peak of interest will show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample will compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria will be met.

### **10.4.2 Retention Time**

The retention time of the peak is within  $\pm 2\%$  of that for a contemporaneously analyzed calibrator or Positive Control.

### **10.4.3 Signal-to-Noise**

The signal to noise ratio for the peak will be greater than three. The response for the suspect peak will be at least ten-fold greater than that for any observed peak at similar retention time in a Negative Control injected just prior to the sample.

### **10.4.4 Mass Spectrometry**

The mass spectrum of the analyte of interest will match that of a contemporaneously analyzed calibrator or Positive Control. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## **10.5 Reporting a Sample as Positive**

The criteria listed in Sections 10.1- 10.4 must be met in order to report a positive result from this procedure.

## **10.6 Planned Action**

If any of the criteria in 10.1-10.4 are not met then some or all of the following action steps may

be appropriate:

- Not reporting results (qualitative and/or quantitative) from the batch and/or affected case samples
- Reaccession and reanalysis of the batch and/or affected case samples
- Performing instrument maintenance
- Remaking or using new reagents, calibrators, or control materials
- Notifying the TL who will ensure the cause is determined and modifications made to address the issue

## 11 Calculations

A six point calibration is performed for methanol, acetone, and isopropanol (0.010, 0.025, 0.050, 0.100, 0.200 and 0.400 g%). A seven point calibration is performed for ethanol using the same concentrations as the other volatiles with the addition of a 0.500 g% calibrator.

Calibration is linear with  $1/x^2$  weighting. For additional guidance, *Quality Control for Toxicology Examinations* standard operating procedure (Tox 101).

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements from control charts
- accuracy of the pipette used to deliver the sample (or balance if sample was weighed)
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory Report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

a.

Limits of Detection:

Ethanol	0.001 g%
Methanol	0.003 g%
Acetone	0.001 g%
Isopropanol	0.001 g%

b. Linearity:

Ethanol	0.010 – 0.500 g%
Methanol	0.010 – 0.400 g%
Acetone	0.010 – 0.400 g%
Isopropanol	0.010 – 0.400 g%

c. Lower Limits of Quantitation:

Ethanol	0.010 g%
Methanol	0.010 g%
Acetone	0.010 g%
Isopropanol	0.010 g%

d. Accuracy (Bias): (n = 18 per level)

	Low	Mid	High
Ethanol	-1.76%	+0.21%	+1.89%
Methanol	-1.90%	+1.02%	-2.83%
Acetone	+4.42%	+1.28%	-3.35%
Isopropanol	+4.24%	+3.30%	-2.05%

e. Repeatability: (n = 18 per level)

	Low	Mid	High
Ethanol	4.29%	4.04%	3.38%
Methanol	3.68%	5.74%	8.23%
Acetone	3.93%	6.61%	11.82%
Isopropanol	2.34%	2.71%	2.23%

f. Intermediate Precision: (n = 18 per level)

	Low	Mid	High
Ethanol	4.66%	4.33%	3.57%
Methanol	3.68%	6.09%	9.39%
Acetone	4.47%	7.59%	13.59%
Isopropanol	2.42%	3.01%	2.43%

g. Interferences: Ethanol is normally present in the human body at low levels (<0.001% w/v) due to bacterial fermentation in the intestines. Ethanol is also produced as a result of putrefactive processes, either post-mortem or due to poor sample storage. Consequently, caution should be exercised in the interpretation of low positive ethanol results (<0.04% w/v) in post-mortem cases. Severe putrefaction will additionally result in the generation of a wide variety of low molecular weight volatile compounds in biological specimens, including higher weight n-alcohols, aldehydes, sulfides, mercaptans, and alkylamines. Caution will be exercised in the interpretation of any positive volatiles result from extensively decomposed or putrefied specimens.



## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Moffat, A.C., *Isolation and Identification of Drugs*, 2nd ed., Pharmaceutical Press: London, 1986.

Dubowski, K.M., *Manual for Analysis of Ethanol in Biological Liquids*, 1977.

Garriott, James, *Medicolegal Aspects of Alcohol*, 3<sup>rd</sup> ed., Lawyers and Judges Publishing: Tucson, AZ, 1996.

Levine, B.; Smith, M.L.; Smialek, J.E.; Caplan, Y.H. *J For Sci.* 1993, 38, 663-667.

Morris-Kukoski, C.L. et al. *J Chrom B.* 2007, 850, 230-235.

*Quality Control for Toxicology Examinations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
12	12/13/2017	Removed Section 11.1 Back Extrapolation Calculations (moved to new SOP TOX109). Updated Section 6a and 6b to reflect the use of a purchased 0.025g% calibrator and a multicomponent 0.200g% calibrator. Deleted Section 6c and updated numbering. Section 11 was updated to reflect the 0.025 g% calibrator, the 0.200 g% multicomponent calibrator, and specified that $1/x^2$ weighting shall be used. Updated Section 10.4.4, removing the phrase “to within a reasonable degree of scientific certainty”.
13	06/18/2018	Section 1: changed 0.5% w/v to 0.5 g%. Section 3: clarified that samples are also analyzed by GC/MS. Section 4: clarified scope by adding “aqueous”. Section 5-i: removed glass specification. Updated Section 6-a to reflect current calibration scheme. Section 6-f: removed typo, added “approved supplier”, added MSD assay, and updated title for TOX101. Section 6-g added from TOX101. Section 8-a: added room temperature statement. Section 8-d: added reference to Section 9, and clarification that not all samples are analyzed by GC/MS. Footnote 2, page 4: removed glass specification. Updated agitator speed in Section 9.2 to 250 rpm for both FID and MSD (also corrected on instrument bench sheet). Section 9.5: updated solvent delay to “nominal”, and added 0.5 minute variation to account for column maintenance (also updated on instrument bench sheet). Throughout section 10: changed multiple instances of “should” to “will”. Section 10.1.2, 11, and References: updated title for TOX101. Added 10.1.3 for calibrator acceptance. Section 10.2.4 and 10.4.3: updated signal to noise description. Section 10.5: updated numbering to accurately reflect current enumeration. Added Section 10.6 to provide planned action on QC failure. Section 11: changed “analyzed” to “performed”. Updated CUQA-13 title in Section 12 and references. Removed “subunit” from references and header.
14	10/09/18	Increased pipettor-dilutor rinse volume to 5mL for wash efficiency in Section 9.1.

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**Approval**

Toxicology  
Technical Lead:

Date: 10/05/2018

Acting Chemistry  
Unit Chief:

Date: 10/05/2018

**QA Approval**

Quality Manager:

Date: 10/05/2018

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## Direct Solvent Extraction of Acid/Neutral Drugs from Biological Fluids

### 1 Introduction

This procedure detects common acidic and neutral drugs in biological fluids. It is derived from A Gas Chromatographic Screening Procedure for Acidic and Neutral Drugs in Blood published in the *Journal of Analytical Toxicology* by Foerster, et al (1979). While the published procedure using direct solvent extraction of fluids is followed intact to prepare a crude acid/neutral drug isolate, the drug detection method performed on this extract has been modified from gas chromatography-flame ionization detection (GC/FID) to gas chromatography-mass spectrometry (GC/MS). This latter method of detection lends a greater degree of drug specificity and sensitivity than that possessed by the original published procedure.

### 2 Scope

This procedure allows for screening and confirmation of a wide variety of acidic and neutral drugs in biological fluids. Dilutions<sup>1</sup> of food and beverage samples can also be analyzed to screen for acidic and neutral drugs. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

Biological specimens are screened for acidic and neutral drugs. Specimens are mixed with an internal standard (methylphenylhydantoin), adjusted to an acidic pH, and extracted with an ether:toluene mixed solvent. Following centrifugation, the organic solvent is taken to dryness and the residue is partitioned between ethanol and hexane. The ethanol layer is taken to dryness and the extract is reconstituted in a chloroform/methanol mixture prior to analysis by GC/MS.

### 4 Specimens

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate. When available, 0.5 mL of blood or other fluids are used. This procedure may also be used to screen food and beverage samples for acidic and neutral drugs, provided that appropriate controls are simultaneously analyzed. A 0.5 g sample of a food or beverage homogenate or dilution is suggested for analysis.

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<sup>1</sup> Typically, tenfold dilutions of food and beverage samples are appropriate, but case history and the complexity of the matrix may suggest a different dilution.

## 5 Equipment/Materials/Reagents

- a. 16 x 125 mm screw-top tubes with Teflon insert caps
- b. 16 x 100 mm culture tubes with polypropylene snap-tops
- c. 10 x 75 mm and 12 x 75 mm culture tubes with polypropylene snap-tops
- d. N- Hexane (95% or equivalent)
- e. Potassium phosphate, monobasic (ACS grade or equivalent,  $\text{KH}_2\text{PO}_4$ )
- f. Potassium Phosphate Buffer Monobasic (5% w:v, pH 4.5):  
To a 100-mL volumetric flask, add 80 mL deionized water. Add 5 g monobasic potassium phosphate and mix well to dissolve. Bring to volume with deionized water, and verify  $4.0 < \text{pH} < 5.0$ . Store refrigerated in glass. Stable 1 month.
- g. Diethyl ether (High purity grade or equivalent)
- h. Toluene (HPLC grade or equivalent)
- i. Ether:Toluene (1:1 v:v):  
Combine 50 mL HPLC grade toluene with 50 mL diethyl ether. Mix well. Store in glass at room temperature. Stable 1 month.
- j. Chloroform ( $\text{GC}^2$  grade or equivalent)
- k. Methanol (Optima,  $\text{GC}^2$  grade or equivalent)
- l. Chloroform:Methanol ( $\text{CHCl}_3:\text{MeOH}$ ) (4:1 v:v):  
Combine 40 mL chloroform with 10 mL  $\text{GC}^2$  methanol. Mix well. Store in brown glass at room temperature. Stable 1 month.
- m. Ethanol (Pharmaceutical grade or equivalent)
- n. Water (Deionized)
- o. Ethanol 80% (v/v aqueous):  
Measure 80 mL pharmaceutical grade ethanol into a 100-mL graduated cylinder. Bring to volume with deionized water and mix well. Store in glass at room temperature. Stable for 6 months.
- p. Vortex mixer

- q. Centrifuge
- r. Rotator
- s. Evaporator with nitrogen
- t. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
- u. Gas Chromatograph / Mass Spectrometer equipped with a 30 m x 0.25 mm x 0.25  $\mu$ m Rtx-5MS (or equivalent) column
- v. Methylene chloride (Optima grade or equivalent)
- w. 10 cc glass centrifuge tubes (with conical bottom)

## 6 Standards and Controls

- a. Methylphenylhydantoin (MPH) Standard:  
Purchased from Sigma-Aldrich or another approved vendor. Storage and stability determined by manufacturer.
- b. Methylphenylhydantoin Stock Standard (1 mg/mL):  
Add 10.0 mg of methylphenylhydantoin to a 10-mL volumetric flask. Dilute to the mark with methanol and mix well. Store refrigerated in glass. Stable for at least 2 years.
- c. Methylphenylhydantoin Working Internal Standard (30  $\mu$ g/mL):  
Dilute 0.75 mL of the MPH Stock Standard to 25 mL with deionized water. Store refrigerated in glass. Stable for at least 2 years.
- d. Octanoic Acid Standard:  
Purchased from Sigma-Aldrich or another approved vendor. Storage and stability determined by manufacturer.
- e. Octanoic Acid Working Internal Standard (1mg/mL):  
Add 10.0 mg of octanoic acid to a 10-mL volumetric flask. Dilute to the mark with methanol and mix well. Store refrigerated in glass. Stable for at least 2 years.
- f. Negative Control:  
Purchased from Cliniqa or an equivalent supplier, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay. When possible, the Negative Control will be matrix matched.

g. Barbiturate Mix-5:

A mixture of five barbiturates at 250 µg/mL in methanol. Purchased from Cerilliant or another approved supplier. Contains amobarbital, butalbital, pentobarbital, phenobarbital, and secobarbital. This mixture may also be prepared from individual analyte stock solutions if necessary. Storage and stability determined by manufacturer.

h. Positive Control Solution:

In addition to the Barbiturate Mix-5, the following target analytes are obtained from an approved vendor in liquid (1 mg/mL) or solid form. Storage and stability determined by the manufacturer. Solid analytes are dissolved in methanol or another appropriate solvent to prepare 1 mg/mL stock solutions. Analyte stock solutions (1 mg/mL) are added to a 25 mL volumetric flask which is brought to the mark with methanol as described in Table 2 below:

Table 2: Positive Control Solution and Control Preparation

Analyte(s)	Stock Conc. (mg/mL)	Spike Aliquot (µL)	Solution Volume (mL)	Solution Conc. (µg/mL)	Control Spike Aliquot (µL)	Matrix Volume (mL)	Control Conc. (ng/mL)
Barbiturate Mix-5	0.25	250	25	2.5	100	0.5	500
Carbamazepine	1	63	25	2.52	100	0.5	504
Carisoprodol	1	63	25	2.52	100	0.5	504
Ibuprofen	1	300	25	12	100	0.5	2400
Meprobamate	1	63	25	2.52	100	0.5	504
Phenytoin	1	63	25	2.52	100	0.5	504

The Positive Control Solution is stored refrigerated in glass or plastic. Stable for at least two years. (Note: Other drugs or metabolites may be added to this mixture as dictated by case needs with sufficient validation and/or analysis of concurrent controls.)

i. Positive Control:

100 µL of the Positive Control Solution is added to 0.50 mL of the Negative Control Blood or Urine on the day of analysis. Optional: 25 µL of a 1 mg/mL acetaminophen standard can be added directly to the Positive Control as well (yields a 50 µg/mL concentration).

A Positive Control will be extracted and analyzed with every assay. When possible, the Positive Control will be matrix matched. Additionally, the use of the MPH internal standard serves as a qualitative positive control for the individual specimen. Other positive controls preparations may be used as is appropriate.



- j. Acetaminophen Standard (1 mg/mL):  
Purchased as a 1 mg/mL solution in methanol from Cerilliant or another approved supplier. Storage and stability determined by the manufacturer.
- k. Valproic Acid Standard (1 mg/mL):  
Purchased as a 1 mg/mL solution in methanol from Cerilliant or another approved supplier. Storage and stability determined by the manufacturer.
- l. Valproic Acid Working Solution (100 µg/mL)  
Dilute 500 µL of the Valproic Acid Standard (1 mg/mL) to 5 mL in methanol. Storage and stability determined by the manufacturer.
- m. Volatiles Positive Control (20 µg/mL):  
100 µL of the Valproic Acid Working Solution (100 µg/mL) is added to 0.50 mL of the Negative Control.

A Volatiles Positive Control will be extracted and analyzed with every volatiles assay. When possible, the Volatiles Positive Control will be matrix matched. Additionally, the use of the Octanoic Acid internal standard serves as a qualitative positive control for the individual specimen.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To properly labeled 16 x 125 mm screw-top tubes add 0.5 mL of biological fluid, 0.5 g of a prepared food homogenate, 0.5 mL of a prepared beverage dilution, or 1 g of prepared tissue homogenate (1:1 in deionized water). Also prepare Negative and Positive Controls for each matrix being analyzed. Prepare a Volatiles Positive Control if performing volatiles analysis. Bring up to a volume of 1 mL with deionized water.
- b. Add 25 µL of MPH Internal Standard Solution to biological fluid specimens, food homogenates and beverage dilutions.<sup>2</sup> For tissue specimens add 0.5 mL of MPH Internal

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<sup>2</sup>Other internal standards may be substituted or added at relevant concentrations as appropriate,

Standard Solution. This results in MPH specimen concentrations of 1.5 µg/mL and 10 µg/g, respectively. For volatiles analysis, add 25 µL of Octanoic Acid Working Internal Standard Solution (1 mg/mL) to biological fluid specimens, food homogenates and beverage dilutions. (If volatiles analysis *only* is to be performed, only the Octanoic IS needs to be added.)

- c. Add 1 mL of 5% KH<sub>2</sub>PO<sub>4</sub> buffer solution to each specimen. Check pH to ensure pH is between 4 and 6.
- d. Add 5 mL of ether:toluene (1:1) to each tube and extract for 20 minutes on a rotator. Centrifuge 5 minutes. Use a wooden stick to break up any emulsions that develop.
- e. Transfer organic (top) layer to a 16 x 100 mm culture tube.
- f. Evaporate the ether:toluene to dryness under a gentle stream of nitrogen at 50°C. If analysis for volatile acidic drugs (e.g., ethchlorvynol or valproic acid) is desired, pause this evaporation at about 0.5 mL and analyze 1-2 µL by GC-MS / electron impact ionization (positive ion mode) with the instrumental conditions given below.
- g. Transfer dried residue to a 10 cc glass centrifuge tube with two successive 1 mL washes of CHCl<sub>3</sub>:MeOH (4:1). Evaporate this solvent to a dry residue under a gentle stream of nitrogen at 50°C.
- h. Reconstitute the dried residue in 2 mL hexane plus 200 µL 80% ethanol. Vortex for 30 seconds and centrifuge for 5 minutes.
- i. Discard the hexane (top) layer, transfer the ethanol layer to a fresh 12 x 75 tube, and evaporate the ethanol layer to a dry residue under a gentle stream of nitrogen at 50°C.
- j. Reconstitute the residues with 25-50 µL of CHCl<sub>3</sub>:MeOH (4:1) and analyze 1-2 µL by GC-MS/chemical ionization (positive ion mode) and/or GC-MS/electron impact ionization (operating conditions specified in Section 9) after confirming instrument is calibrated and in proper working condition.
- k. Some acidic drugs are known to carryover from run to run. To clean out the GC system, analyze a methylene chloride blank at the beginning of every sequence and after the Positive Control.
- l. Evaluate the data

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with sufficient validation and/or analysis of concurrent controls.

- m. Forensic examiner for the case: review electronic data files for acid/neutral drug screens and record that review by writing “file reviewed”, “peaks checked”, or similar language on the total ion chromatogram along with their initials.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure. Instrumental conditions may be modified to account for particular analytes. Any modifications will be recorded in case notes.

### 9.1 Gas Chromatograph Parameters (Agilent)

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	45°C	inlet temperature	220°C	type	DB-5MS
hold 1	1 min	injection mode	split	length	30 m
ramp 1	25°C/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	150°C	carrier mode	constant flow	film thickness	0.25 µm
hold 2	2 min	carrier flow	1.2 mL/min		
ramp 2 (volatiles)	15°C/min (30°C/min)	split flow	12 mL/min		
temperature 3	280°C	split ratio	10:1		
hold 3	14 min				
total run time (volatiles)	29.87 min (25.53 min)				

### 9.2 Mass Spectrometer Parameters - Agilent (EI)

ionization mode	electron impact (+)	Source/quad temperature	230/150°C
scan mode	full scan	transfer line temperature	280°C
scan range (volatiles)	35 – 500 AMU (35 – 200 AMU)	solvent delay (nominal) (volatiles)	3 min (3.5 min)

## 10 Decision Criteria

### 10.1 Batch Decision Criteria

No analytes of interest will be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch. All analytes should be detected in the Positive Control.

## **10.2 Unknown Sample Decision Criteria**

### **10.2.1 Chromatography**

The peak of interest will show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample will compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **10.2.1.1 Retention Time**

The retention time of the peak will be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

#### **10.2.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio will exceed 3. Further, the baseline signal for the peak of interest will be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

### **10.2.2 Mass Spectrometry**

The mass spectrum of the analyte of interest will match that of a reference standard or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

## **10.3 Planned Action on QC Failure**

Refer to Quality Control for Toxicology Examinations (TOX101) for guidance on action steps in the event of a quality control failure.

## **10.4 Reporting Cut-offs for College of American Pathologists (CAP) T Series and FTC Series:**

See Quality Control for Toxicology Examinations (TOX101) for guidance on estimating the amount of an analyte in a specimen. When analyzing CAP T-Series or FTC specimens, if all decision criteria for an analyte of interest are met, but the concentration of butalbital, carbamazepine, carisoprodol, meprobamate, phenobarbital, phenytoin, and/or secobarbital is estimated to be below 1  $\mu\text{g/mL}$  (or 5  $\mu\text{g/mL}$  for acetaminophen) in two independent analyses, the

analyte will not be reported. Note: the second analysis may be a repeat of this procedure or via another validated procedure. A Positive Control at the Cut-off Level is recommended for the second analysis.

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

- a. Limit of Detection (LOD): Detection limits for common acidic and neutral analytes are listed in Table 2 below. Note: LODs were not evaluated below 100 ng/mL, so true LODs for this method may be lower than what is listed in the table.

Table 3:

Analyte	Blood LOD (µg/mL)	Urine LOD (µg/mL)
Acetaminophen	25	1
Amobarbital	0.1	0.1
Brompheniramine	0.5	>0.5
Bupropion	0.1	0.25
Butalbital	2.5	0.5
Carbamazepine	0.1	0.1
Carisoprodol	0.5	0.1
Citalopram	0.25	0.5
Clozapine	>0.5	0.25
Cyclobenzaprine	0.1	0.1
Diphenhydramine	0.1	0.1
Ibuprofen	5	1
Ketamine	0.1	0.1
Lamotrigine	2.5	1
Lidocaine	0.25	0.25
Meprobamate	0.5	0.25
Methadone	0.25	0.1
Mirtazapine	0.1	0.1
Naproxen	50	2.5
Pentobarbital	0.1	0.1

Phenobarbital	0.1	0.1
Phenytoin	0.25	0.1
Propoxyphene	0.1	0.1
Secobarbital	0.1	0.1
Theophylline	2.5	0.1

- b. Interferences: None known. Grossly decomposed or putrefied samples, as well as samples that have been embalmed, may affect detection limits.
- c. Note: This method is not appropriate for screening for salicylic acid.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Baselt, R.C. and Cravey, R.H. *J Anal Tox*, 1977, 1, 81-103.

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Foerster, E.H., Dempsey, J., and Garriott, J.C. *J Anal Tox*, 1979, 3, 87-91.

Moffat, A.C., *Isolation and Identification of Drugs*, 2nd ed., Pharmaceutical Press: London, 1986.

Winek, C. *Drug and Chemical Blood-Level Data*, 1994.

*Chemistry Unit Validation of Analytical Procedures* (CUQA 11); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Quantitation of Acidic Drugs* (Tox 424); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Quality Control for Toxicology Examinations* (TOX101); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support SOP Manual.

*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
7	04/01/19	Updated Table 2 for clarity and to include 'as-prepared' analyte concentrations. Section 9: added line clarifying that instrument conditions may be modified to target particular analytes, and that the modifications will be recorded in case notes.
8	11/15/19	Updated punctuation in Section 1. Added octanoic internal standard preparation and volatiles positive control preparation to Section 6. Updated positive control concentration in Table 2. Added control preparation step to Section 8.b. Added 10.4 CAP reporting cut-offs. Updated bench sheets.

**Approval**

Redacted - Signatures on File

Acting Toxicology  
Technical Leader:

Date: 11/14/2019

Chemistry Unit Chief:

Date: 11/14/2019



**Appendix 1: Abbreviated version of the Acid/Neutral Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Acid/Neutral Instrumental Conditions for bench use.**

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## **Solid Phase Extraction (SPE) of Alkaline Drugs from Biological Fluids**

### **1 Introduction**

This procedure detects common alkaline drugs. It is derived from "Fentanyl and Analogues in Urine for GC or GC/MS Confirmations" published in the *Solid Phase Extraction Applications Manual* by United Chemical Technologies, Inc. The published procedure for extraction of biofluids is essentially followed intact to prepare a crude basic drug isolate.

### **2 Scope**

This procedure allows for screening and confirmation of a wide variety of alkaline drugs in biological fluids. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

Biological specimens are qualitatively assayed for basic drugs. Specimens are mixed with internal standards, adjusted to a slightly acidic pH, and extracted using Clean Screen DAU solid phase extraction cartridges. Basic drugs are eluted using a mixed solvent system of methylene chloride, isopropanol, and ammonium hydroxide. The eluent is taken to dryness and reconstituted prior to analysis by GC/MS(EI) (gas chromatography/mass spectrometry)(electron ionization) and/or LC/MS(ESI) (liquid chromatography/mass spectrometry)(electrospray ionization).

### **4 Specimens**

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate (1:1). When available, 1 mL of blood, other fluid, or tissue homogenate is used. This procedure may also be used to screen food samples for alkaline drugs, providing that appropriate controls are simultaneously analyzed. A 1 g sample of a food homogenate (1:1) is suggested for analysis.

### **5 Equipment/Materials/Reagents**

- a. Gas Chromatograph/Mass Spectrometer equipped with a 30 m x 0.25 mm x 0.25  $\mu$ m Rtx-5MS (or equivalent) column.
- b. Centrifuge

- c. Evaporator w/ Nitrogen
- d. Vortex mixer
- e. SPE Vacuum or Positive Pressure Manifold
- f. Desiccator
- g. 16 x 100 mm screw-top tubes with Teflon insert caps
- h. 13 x 100 mm culture tubes with polypropylene snap-tops
- i. 12 x 75 mm culture tubes with polypropylene snap-tops
- j. Clean Screen DAU<sup>®</sup> SPE cartridges (regular flow) - 200 milligrams
- k. Water (18mΩ, HPLC, Optima, or UPLC grade)
- l. Methanol (GC<sup>2</sup> grade, HPLC, Optima grade, or better)
- m. 100 mM Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify 5.8<pH<6.1. Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.
- n. Elution Solvent (Methylene Chloride/Isopropanol/Ammonium Hydroxide (78/20/2)):  
Combine 20 mL HPLC grade isopropanol with 2 mL ACS grade concentrated ammonium hydroxide and mix well. Add 78 mL HPLC or pesticide grade methylene chloride and mix well. Store in glass at room temperature. Prepare fresh daily.
- o. 100 mM Acetic Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.5 mL glacial acetic acid. Mix well and bring to 85 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- p. Mid-range pH paper
- q. PTFE (0.5 micron) membrane
- r. HPLC Column (Xterra C-18 MS, 3.0 x 150 mm, 3.5 μm dp; or equivalent)

- s. Mobile Phase 1 (Water with 0.1% Formic Acid ): Combine 500 mL Optima grade water and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable 2 weeks.
- t. Mobile Phase 2 (Acetonitrile with 0.1% Formic Acid ): Combine 500 mL Optima grade acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for 1 month.

## 6 Standards and Controls

- a. Alkaline Screen Internal Standard Working Solution:  
Prepared by adding the following 100 µg/mL solutions (purchased from Cerilliant, or another approved supplier) to a 25-mL volumetric flask and diluting to the mark with methanol:

Analyte	Volume (mL)	Resulting concentration when 50 µL is added to 1.0 mL matrix (ng/mL)
d <sub>5</sub> -fentanyl	1.0	200
d <sub>3</sub> -benzoylecgonine	0.25	50
d <sub>5</sub> -methamphetamine	0.125	25
d <sub>3</sub> -morphine	0.125	25
d <sub>3</sub> -oxycodone	0.125	25
d <sub>3</sub> -ecgonine methyl ester	0.125	25
d <sub>5</sub> -alprazolam	0.100	20

- b. Negative Control:  
Purchased from Cliniq, Dynatek or an equivalent approved supplier, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay. When possible, the Negative Control will be matrix matched.
- c. Alkaline Screen Control Working Solution (5 µg/mL):  
Prepared by adding 0.25 mL of each of the following 1.0 mg/mL standards (purchased from Cerilliant, or another equivalent supplier) to a 50-mL volumetric flask and bringing to the mark with HPLC grade methanol:  
Amitriptyline, amphetamine, cocaine, diphenhydramine, hydrocodone, nordiazepam, tramadol and venlafaxine.  
This mixture is stored refrigerated in glass. Stable for at least one year.
- d. Positive Control (200 ng/mL each analyte):  
Prepared fresh by adding 0.040 mL of the Alkaline Control Working Solution (5 µg/mL) to 1 mL of Negative Control. Alternatively, other alkaline drugs and/or metabolites may be added to an aliquot of Negative Control to prepare controls for specific needs. A

Positive Control will be extracted and analyzed with every assay. When possible, the Positive Control will be matrix matched.

Note: When drugs are indicated in an alkaline drug screen that are not present in the Positive Control, a sample of the reference material (typically ~100 µg/mL for GC/MS or ~1-5 µg/mL for LC/MS) may be analyzed instrumentally without extraction to verify the retention time and spectra of the analyte in the unknown sample. For confirmatory assays, matrix matched positive controls that are extracted alongside the unknown sample when available.

- e. Benzodiazepine Multi-Component Mixture-8 Stock Standard (250 µg/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam): Purchased from Cerilliant Corporation. Storage conditions and stability determined by manufacturer.
- f. Benzodiazepine Multi-Component Mixture-8 Stock Standard Working Solution (5 ug/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam):  
To a 50 mL volumetric flask add 1.0 mL of Benzodiazepine Multi-Component Mixture-8 Stock Standard and dilute to the mark with acetonitrile. Store <0°C in glass or plastic. Stable for at least 2 years.
- g. Amine Mixture-6 Stock Standard (250 µg/mL of amphetamine, methamphetamine, phentermine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), and methylenedioxyethylamphetamine (MDEA)): Purchased from Cerilliant Corporation. Storage conditions and stability determined by manufacturer.
- h. Amine Mixture-6 Stock Standard Working Solution (5 µg/mL of amphetamine, methamphetamine, phentermine, MDA, MDMA, and MDEA):  
To a 50 mL volumetric flask add 1.0 mL of Amine Mixture-6 Stock Standard and dilute to the mark with methanol. Store <0°C in glass or plastic. Stable for at least 2 years.
- i. LC/MS Performance Standard (0.83µg/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam, amphetamine, methamphetamine, phentermine, MDA, MDMA, and MDEA):  
Combine 0.025mL of the Benzodiazepine Multi-Component Mixture-8 Stock Standard Working Solution, 0.025mL of the Amine Mixture-6 Stock Standard Working Solution and 0.100mL of water. Prepare fresh or store mixture under refrigerated conditions. Stable for at least 2 weeks.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To properly labeled 16 x 100 mm screw-top tubes add 1 mL of biological fluid or 1 g of prepared tissue homogenate (1:1 in deionized water). Also prepare Negative and Positive Controls for each matrix being analyzed.
- b. Add 50 µL of the Alkaline Screen Internal Standard Working Solution to biological fluid specimens.<sup>1</sup> For tissue specimens add 100 µL of each Internal Standard Working Solution.
- c. Add 4 mL of 100 mM phosphate buffer. Vortex. Verify pH is  $6.0 \pm 0.5$ .
- d. For blood and tissue specimens: Centrifuge at high speed for 15 minutes. Transfer supernatant to a clean 13 x 100 mm culture tube, leaving solid cellular material behind. Bring volume up to 5 mL with deionized water. Verify pH is  $6.0 \pm 0.5$ .
- e. Pre-rinse SPE extraction cartridge by adding 3 mL of methanol at 1 mL/minute.
- f. Condition cartridge with 3 mL of deionized water followed by 1 mL of 100 mM phosphate buffer at 1 mL/minute. Do not allow sorbent to dry.
- g. Load sample on SPE cartridge at 1-2 mL/minute. Do not allow sorbent to dry.
- h. Wash cartridge with 3 mL of deionized water, 1 mL of 100 mM acetic acid, and 3 mL of methanol (each at 1-2 mL/minute).
- i. Dry cartridge under full vacuum for 3 minutes.
- j. Apply 3 mL of Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm test tubes.
- k. Evaporate to dryness under nitrogen at 40°C.

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<sup>1</sup>Other internal standards may be substituted at relevant concentrations if deemed appropriate.

1. Analyze the extracts using one or both of the instrumental techniques that follow.

1. For GC/MS(EI) analysis, reconstitute the dry residues with 40-50 µL of GC<sup>2</sup> grade methanol and analyze 2 µL following the instrumental parameters given below after confirming that the instrument is in proper working condition.

2. For LC/MS(ESI) analysis, add 100 µL water to the methanol extract prepared above and analyze 10 µL following the instrumental parameters given below after confirming that the instrument is in proper working condition.

## 9 Instrumental Conditions<sup>2,3</sup>

### 9.1 GC-MS (EI)

#### 9.1.1 Gas Chromatograph Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	60°C	inlet temperature	220°C	type	HP5-MS
hold 1	2 min	injection mode	split	length	30 m
ramp 1	35°C/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	280°C	carrier mode	constant flow	film thickness	0.25 µm
hold 2	26.71 min	carrier flow	1.2 mL/min		
total run time	35 min	split flow	12 mL/min		
		split ratio	10:1		
		Injection volume	2 µL		

<sup>2</sup> Instrumental conditions may be modified to account for particular target analytes. Any modifications will be recorded in case notes.

<sup>3</sup> Appendix 2 contains an abbreviated version of instrumental parameters used in this procedure. This checklist may be used by the examiner or chemist performing the procedure.



### 9.1.2 Mass Spectrometer Parameters

ionization mode	electron impact (+)	Source/Quad temperatures	230/150°C
scan mode	full scan	transfer line temperature	280°C
scan range	35 – 500 AMU	solvent delay (nominal)	5 min*

\*This value may be shortened as a column ages and is clipped to ensure that amphetamine elutes after the solvent delay.

### 9.2 LC-MS(ESI)

Mobile Phase Composition	Flow Parameters			Column Parameters	
MP1: Water with 0.1% Formic Acid	total flow		0.3 mL/min	type	Xterra C-18 MS
	time (min)	%MP1	%MP2	length	150 mm
MP2: Acetonitrile with 0.1% Formic Acid	0	90%	10%	internal diameter	3 mm
	5	90%	10%	particle size	3.5 µm
	20	10%	90%	temperature	30°C
	31	90%	10%		
	37	90%	10%		
	total run time		37 min		
Autosampler:	Injection Volume	10 µL	Temperature	15°C	

#### 9.2.1 Mass Spectrometer Parameters<sup>4</sup>

Source	Electrospray (ESI, +)	Segments	5	Events per segment	7
Segment times	#1: 0-12 min; #2: 12-13.5 min; #3: 13.5-14 min; #4: 14-15 min; #5: 15 min - end				

Events #1 and 5	
Mode	full scan MS
Range	100 – 650 m/z
Resolution	FTMS; 15000

Events #2-4		
Full Scan MS/MS	Data Dependent Product Ion	ITMS – unit resolution

<sup>4</sup>Different MS/MS parameters (i.e., higher collision energy or MS<sup>3</sup>) may be used to target specific drugs and metabolites as long as the same parameters are used for all controls and case samples and the method is recorded in the case notes.

MS/MS of 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> most intense ions in Event #1 from segment specific target lists  
 (See Appendix 3 and 4)

Events #6-7

Full Scan MS/MS	Data Dependent Product Ion	ITMS – unit resolution
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MSMS of 1<sup>st</sup> and 2<sup>nd</sup> most intense ions in Event #5

Note: For target analysis, method may be reduced to fewer time segments. Additionally, Scan Events 5-7 may be removed and target ion(s) can be added to or removed from Events 2-4.

## 10 Decision Criteria

### 10.1 LC/MS Performance Standard Decision Criteria

In addition to the performance checks specified in the LC/MS standard operating procedure, a performance standard mix is analyzed through the analytical column to monitor the performance of the column.

#### 10.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each analyte in the performance standard should have reasonable peak shape. Over time, the amines in the performance standard mix may demonstrate wider chromatographic peaks, indicating deterioration of the packing of the analytical column.

The retention times of the 14 analytes from the performance standard should be within  $\pm 0.5$  min of the previous run (same instrument and same column) for benzodiazepines and within  $\pm 0.6$  min for sympathomimetic amines. Minor changes in mobile phase percentage may account for slight retention time shifts. Over time, the amines in the performance standard mix may give longer and longer retention times, indicating deterioration of the packing of the analytical column or a problem with the frit. When the amine peak shape becomes poor or retention times are  $> 0.6$  min longer than the last run, the frit should be replaced. Replacement of the column is also a consideration.

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

#### 10.1.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the 14 analytes in the performance standard will be present. (The following molecular

ions (high resolution ion listed in parentheses) will be present as the base peak for each analyte: clonazepam – 316 (316.048), nitrazepam – 282 (282.087), flunitrazepam – 314 (314.094), lorazepam – 321 (321.019), oxazepam – 287 (287.058), alprazolam – 309 (309.090), temazepam – 301 (301.074), diazepam – 285 (285.079), amphetamine – 136 (136.112), methamphetamine and phentermine – 150 (150.128), MDA – 180 (180.102), MDMA – 194 (194.118), and MDEA – 208 (208.133).)

## **10.2 Unknown Sample Decision Criteria**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

### **10.2.1 Batch Acceptance**

No analytes of interest will be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the eight analytes in the Positive Control will be detected in either the GC/MS data, the LC/MS data, or both.

### **10.2.2 Unknown Sample Acceptance**

The d5-fentanyl will be detectable in the GC/MS data.

The d5-fentanyl, d5-alprazolam, d5-methamphetamine, d3-morphine, d3-oxycodone, d3-benzoyllecgonine and d3-ecgonine methyl ester will be detectable in the LC/MS data.

### **10.2.3 Unknown Sample Compound Identification**

In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control.

In situations where a reference material is not available for comparison, it may still be possible to identify a suspected compound in an extract<sup>5</sup>. In such circumstances, chromatographic fidelity and signal-to-noise requirements must be met, and the mass spectrum for the analyte of interest must compare favorably to a published reference library spectrum. Also, there must be strong additional supporting information that reasonably suggests the presence of the suspected compound. For example, a drug metabolite for which no reference standard is available might be considered identified on the basis of a good quality mass spectral library match combined with identification of the parent drug in the same sample through comparison to a reference standard.

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<sup>5</sup>See *General Approach to Report Writing for Toxicology* for details on how to report a positive finding when no reference standard is available.

### 10.2.3.1 Chromatography

The peak of interest will show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample will compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.2.3.1.1 GC Retention Time

The retention time of the peak will be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control or an appropriate deuterated analog.

#### 10.2.3.1.2 LC Retention Time

The retention time of the peak will be within 5% or  $\pm 0.5$  min (whichever is greater) of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

#### 10.2.3.1.3 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio will exceed 3. Further, the baseline signal for the peak of interest will be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

### 10.2.3.2 Mass Spectrometry

The mass spectrum of the analyte of interest will match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 10.3 Reporting EDDP

Methadone is known to break down to EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, a metabolite of methadone) in solution. To avoid false positive identification of EDDP in a sample, the following criteria must be met to report EDDP using this method: The ratio of the EDDP area (sum of the m/z 276 and m/z 277 ions in GC/MS data) to the methadone area (m/z 72 ion in GC/MS data) must be  $> 5\%$  to report EDDP in a sample.

## 10.4 Reporting Cocaine

To report cocaine qualitatively based upon this method, the area of the M+H peak for cocaine (from the LC/MS data) must be greater than or equal to 5% of the M+H peak for benzoylecgonine (from the same sample).

### **10.5 Screening LC/MS Data**

To screen high resolution LC/MS data for a wide number of analytes, M+1 ions for dozens of analytes may be traced at a mass tolerance of  $\pm 5$  mmu.

### **10.6 Cautionary Note**

Diphenhydramine, doxylamine, nortriptyline and other drugs may fragment in the ESI source.

### **10.7 Planned Action on QC Failure**

Refer to Quality Control for Toxicology Examinations (TOX101) for guidance on action steps in the event of a quality control failure.

### **10.8 Reporting Cut-offs for College of American Pathologists (CAP) T Series and FTC Series:**

See Quality Control for Toxicology Examinations (TOX101) for guidance on estimating the amount of an analyte in a specimen. When analyzing CAP T-Series or FTC specimens, if all decision criteria for an analyte of interest are met, but the concentration of 6-acetylmorphine, buprenorphine, fentanyl, norfentanyl, and/or norbuprenorphine is estimated to be below 5 ng/mL (or 15 ng/mL for all other alkaline drugs) in two independent analyses, the analyte will not be reported. Note: the second analysis may be a repeat of this procedure or via another validated procedure. A Positive Control at the Cut-off Level is recommended for the second analysis.

## **11 Calculations**

Not applicable.

## **12 Measurement Uncertainty**

Not applicable.

## **13 Limitations**

- a. Limit of Detection: While the limit of detection varies depending upon analyte and matrix, this assay readily detects a wide variety of alkaline drugs and metabolites in blood and urine specimens at concentrations of 5 ng/mL. See Appendix 3 and 4.

- b. Interferences: None known. Grossly decomposed or putrefied samples, as well as samples that have been embalmed, may affect detection limits.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

"Fentanyl and Analogues in Urine for GC or GC/MS Confirmations", *Solid Phase Extraction Applications Manual*, United Chemical Technologies, 2005.

Baselt, R.C. and Cravey, R.H. *J Anal Tox.* 1977, *1*, 81-103.

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*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Quality Control for Toxicology Examinations* (TOX101); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*General Approach to Report Writing for Toxicology* (Tox 106); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*ELISA Screening* (Tox 209); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
8	06/18/18	Section 2: updated scope language. Removed “subunit” from header and multiple locations. Section 3: defined EI and ESI. In Section 5-k specified several types of water that can be used (also removed DI from last location in procedure bench sheet). In section 5-l: added “Optima grade or better” (also removed ‘HPLC’ from procedure bench sheet). Corrected typos in Section 5 (s, t). Section 6-d: changed to “when available”. Section 8 (e, h): removed “HPLC”. Section 8-l: removed sentence describing combined GC/MS and LC/MS workflow, since LC/MS only workflow now also an option. Updated Section 8-l.2. Section 9.2: added LC autosampler parameters. Section 9.1.2: added quadrupole temperature of 150°C, updated instrument bench sheet and added “nominal” to mass spec solvent delay time. Throughout section 10: changed multipole instances of “should” to “will”. Section 10.1.1; clarified 14 analyte source. Section 10.3: defined EDDP. Removed “electron impact” from Section 10.2.3. Added Section 10.7 which references the updated TOX101 QC guidance document concerning planning action on QC failure. Added TOX101 to References. Renamed references to TOX106. Section 13: added reference to Appendix 3. In Appendix 3, updated dextromethorphan accurate mass from 272.200 to 272.201 m/z.
9	11/15/19	Revised 6i concentration and expiration date. Updated grammar 8l. Revised 9.2 mobile phase gradient hold and total run time and clarified 9.2.1 note section. Revised 10.1.1 and 10.2.3.1.2 retention time criteria. Added 10.8 reporting cut-offs for CAP proficiency tests. Revised Appendix 2 mobile phase gradient hold time. Appendix 3 - removed duplicate entries for olanzapine and phenazepam, updated LOD values for gabapentin, olanzapine, phenazepam, and ziprasidone.

### **Approval**

Redacted - Signatures on File

Acting Toxicology  
Technical Lead:

Date: 11/14/2019

Chemistry Unit Chief:

Date: 11/14/2019

**Appendix 1: Abbreviated version of the Alkaline SPE Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.**

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### Appendix 3: List of Analytes, approximate retention times and M+1, alphabetized

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
25B-NBOME	380.086	14.95	5	5	OK
25C-NBOME	336.136	14.78	5	5	OK
25H-NBOME	302.175	14.10	5	5	OK
25I-NBF	416.052	14.92	5	5	OK
25I-NBMD	442.051	15.00	5	5	OK
25I-NBOH	414.056	14.61	5	5	OK
25I-NBOME	428.072	15.15	5	5	OK
3-methoxyPCP	274.217	13.80	5	5	OK
6-AM	328.154	7.66	5	5	BP
7-aminoclonazepam	286.074	11.19	5	5	OK
7-aminoflunitrazepam	284.119	12.51	5	5	OK
acetylfentanyl	323.212	13.14	5	5	OK
alfentanyl	417.261	13.60	5	5	OK
alpha-PVP	232.170	12.29	5	5	OK
alprazolam	309.090	16.55	5	5	OK
amitriptyline	278.190	14.95	5	5	OK
amlodipine	409.152	14.72	25	25	OK
amoxapine	314.105	14.08	5	5	OK
amphetamine	136.112	6.05	25	25	BP
aripiprazole	448.155	14.84	25	25	OK
asenapine	286.099	14.34	5	10	I, MI
atenolol	267.170	3.44	5	10	SP
atomoxetine	256.170	14.50	5	5	OK
atropine	290.175	11.16	5	5	OK
baclofen	214.063	8.04	NS	NS	I
benzocaine	166.086	15.59	5	I	I
benzoylecgonine	290.139	11.70	5	5	OK
benztropine	308.201	15.04	5	5	OK
brexpiprazole	434.190	14.32	5	10	OK
bromazepam	316.008	14.70	5	10	OK
brompheniramine	319.080	12.83	5	5	OK
bupivacaine	289.227	13.28	5	5	I
buprenorphine	468.311	14.14	5	5	OK
bupropion	240.115	12.98	5	5	OK

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
buspirone	386.255	13.23	5	5	OK
butylone	222.112	9.85	5	5	BP
butyrylfentanyl	351.243	14.27	5	5	OK
BZP (benzylpiperazine)	177.139	2.25	5	10	SP
carbinoxamine	291.126	12.62	5	5	OK
carfentanil	395.233	14.19	5	5	OK
cetirizine	389.163	15.26	10	5	I
chlordiazepoxide	300.090	13.02	5	5	OK
chlorpheniramine	275.131	12.52	5	5	OK
chlorpromazine	319.103	15.26	5	5	OK
citalopram	325.171	14.15	5	5	OK
clobazam	301.075	18.36	NS	NS	NS
clomipramine	315.162	15.45	5	5	OK
clonazepam	316.048	16.77	NS	NS	NS
clonidine	230.025	5.20	5	5	BP
clozapine	327.137	12.94	5	5	OK
cocaethylene	318.170	13.34	5	5	OK
cocaine	304.154	12.62	5	5	OK
codeine	300.159	5.10	5	5	BP
cotinine	177.102	2.28	5	I	I
cyclobenzaprine	276.175	14.77	5	5	OK
cyproheptadine	288.175	14.74	5	5	OK
desalkylflurazepam	289.054	17.10	5	5	OK
desipramine	267.186	14.64	5	5	OK
desloratadine	311.131	12.11	25	25	I
desmethylcitalopram	311.155	14.04	5	5	OK
desmethyldesipramine	301.147	15.32	5	5	OK
desmethyldiazepam	313.121	12.44	10	10	OK
desmethyldoxepin	262.159	14.65	5	5	OK
desmethyldoxepin	266.154	14.10	5	5	OK
desmethyflunitrazepam	300.078	16.34	NS	NS	NS
desmethyldiazepam	208.170	12.20	5	5	OK
desmethyldiazepam	281.201	14.97	5	5	OK
desmethyldiazepam	264.196	11.38	5	5	OK
dextromethorphan	272.201	13.70	5	5	OK
dextrorphan	258.185	11.84	5	5	OK

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
diazepam	285.079	18.14	5	5	OK
dihydrocodeine	302.175	4.67	5	5	OK
diltiazem	415.169	14.22	5	5	OK
diphenhydramine	256.170	14.00	5	5	OK
donepezil	380.222	13.61	5	5	OK
doxepin	280.170	14.21	5	5	OK
doxylamine	271.180	6.00	5	5	BP
duloxetine	298.126	14.84	5	10	OK
EDDP	278.190	14.46	5	5	OK
EEE	214.144	2.26	25	25	BP
EME	200.128	2.10	5	5	BP
estazolam	295.075	16.30	5	5	OK
etizolam	343.077	17.11	5	5	OK
fentanyl	337.227	13.80	5	5	OK
fexofenadine	502.295	14.87	5	5	OK
flubromazepam	371.030	16.58	5	5	OK
flunitrazepam	314.094	17.35	NS	NS	NS
fluoxetine	310.141	15.18	5	5	OK
fluphenazine	438.182	15.15	5	10	OK
flurazepam	388.159	13.94	5	5	OK
fluvoxamine	319.163	14.77	5	5	OK
furanylfentanyl	375.207	14.00	5	5	OK
gabapentin	172.133	5.29	Unk*	Unk*	*Unk
guaifenesin	199.096	12.75	NS	NS	NS
haloperidol	376.149	14.33	5	5	OK
hydrocodone	300.159	8.17	5	5	BP
hydromorphone	286.144	3.55	5	5	SP
hydroxyzine	375.183	14.77	5	5	OK
iloperidone	427.203	14.36	5	5	OK
imipramine	281.201	14.76	5	5	OK
ketamine	238.099	11.16	5	5	OK
lacosamide	251.139	12.79	NS	NS	NS
lamotrigine	256.015	11.89	5	5	OK
lidocaine	235.180	10.37	5	5	OK
loperamide	477.230	15.77	5	5	OK
loratadine	383.152	15.93	5	5	I

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
lorazepam	321.019	16.58	NS	NS	NS
lormetazepam	335.035	17.75	NS	NS	NS
loxapine	328.121	14.31	5	10	I
LSD	324.207	12.97	5	5	SP
lurasidone	493.263	15.62	5	5	OK
maprotyline	278.190	14.86	5	5	OK
MBDB	208.133	11.50	5	5	OK
MDA	180.102	7.13	5	5	BP
MDEA	208.133	10.72	5	5	OK
MDMA	194.118	8.44	5	5	BP
MDPV	276.159	12.57	5	5	OK
meclizine	391.194	16.63	5	5	OK
medazepam	271.100	13.88	5	5	OK
meperidine	248.165	12.69	5	5	OK
mephedrone	178.123	10.30	5	5	OK
mescaline	212.128	6.12	5	5	BP
mesoridazine	387.156	13.79	5	5	OK
metaxolone	222.112	16.81	NS	NS	NS
methadone	310.217	15.02	5	5	OK
methamphetamine	150.128	7.35	5	5	BP
methocarbamol	242.102	13.37	NS	NS	NS
methoxetamine	248.165	11.92	5	5	OK
methyldone	208.099	5.63	5	5	BP
methylphenidate	234.149	12.10	5	5	OK
metoclopramide	300.147	11.60	5	5	OK
metoprolol	268.194	12.00	5	5	OK
midazolam	326.085	13.81	5	5	OK
mirtazapine	266.165	11.34	5	5	OK
molindone	277.191	11.95	NS	NS	NS
morphine	286.144	2.48	5	5	SP
MT45	349.264	14.90	5	5	BP
naloxone	328.154	4.89	5	5	BP
N-desmethyiltramadol	250.180	12.08	5	5	OK
nicotine	163.123	2.06	5	I	I
nifedipine	347.124	18.13	NS	NS	NS
nitrazepam	282.087	16.26	5	5	OK

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
norbuprenorphine	414.264	12.99	5	5	OK
norchlorcyclizine	287.131	14.76	25	25	I
norchlordiazepoxide	286.074	12.88	5	5	OK
norcodeine	286.144	4.69	5	5	BP
nordiazepam	271.063	16.60	5	5	OK
norfentanyl	233.165	11.37	5	5	OK
norfluoxetine	296.126	15.02	5	5	OK
norhydrocodone	286.144	7.62	5	10	BP
norketamine	224.084	10.83	5	5	OK
normeperidine	234.149	12.64	5	5	OK
normorphine	272.128	2.46	25	25	I
noroxycodone	302.139	6.43	5	5	BP
norpheniramine	227.154	5.63	5	5	BP
norpropoxyphene	326.211	14.79	5	5	OK
norquetiapine	296.122	13.12	5	5	OK
norsertraline	292.065	15.10	25	25	OK
nortriptyline	264.175	14.83	5	5	OK
norverapamil	441.275	14.75	5	5	OK
O-desmethyltramadol	250.180	7.65	5	5	BP
OH-alprazolam	325.085	15.86	5	5	OK
OH-bupropion	256.110	12.00	5	5	OK
OH-midazolam	342.080	13.76	5	5	I
OH-quetiapine	400.169	8.62	10	10	BP
OH-risperidone	427.214	12.71	5	5	OK
OH-triazolam	359.046	15.90	NS	NS	NS
olanzapine	313.148	5.13	100	NS	BP
orphenadrine	270.185	14.50	5	5	OK
oxycodone	316.154	6.79	5	5	BP
oxymorphone	302.139	2.48	5	5	SP
paroxetine	330.150	14.58	5	5	OK
pentylone	236.128	12.00	5	5	OK
perphenazine	404.156	14.66	NS	NS	NS
phenazepam	348.972	17.81	100	NS	NS
phenethylamine	122.096	4.23	5	5	BP
pheniramine	241.170	6.52	5	5	BP
phentermine	150.128	9.10	5	5	BP

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
phenacyclidine	244.206	13.48	5	5	OK
phenylephrine	168.102	2.41	5	5	SP
phenylpropanolamine	152.107	3.83	25	25	OK
PMA	166.123	7.94	5	5	BP
PMMA	180.138	9.54	5	5	BP
prazepam	325.111	20.23	5	5	OK
pregabalin	160.133	5.25	NS	NS	NS
procainamide	236.176	2.48	5	5	SP
prochlorperazine	374.145	14.90	25	25	BP
promethazine	285.142	14.40	5	5	OK
propoxyphene	340.227	14.94	5	5	OK
propranolol	260.165	13.60	5	10	OK
protriptyline	264.175	14.67	5	5	OK
pseudo/ephedrine	166.123	4.70	5	5	BP
psilocin	205.134	4.45	NS	NS	NS
psilocybin	285.100	3.24	NS	NS	NS
quetiapine	384.174	13.48	5	5	OK
quinine/quinidine	325.191	9.46	I	I	I
ranitidine	315.149	3.66	10	10	SP
risperidone	411.219	12.68	5	5	OK
ropinirole	261.196	11.08	5	5	OK
scopolamine	304.154	6.88	5	5	BP
sertraline	306.081	15.24	5	5	OK
strychnine	335.175	10.84	5	5	OK
suvorexant	451.164	20.74	5	5	OK
tapentadol	222.185	12.31	5	5	OK
temazepam	301.074	17.40	NS	NS	NS
tetrahydrozoline	201.139	9.87	5	5	BP
tetrazepam	289.111	16.37	5	5	OK
TFMPP	231.110	13.15	5	5	OK
thioridazine	371.161	15.80	100	100	OK
tizanidine	254.026	4.51	5	5	OK
tramadol	264.196	11.99	5	5	OK
trazodone	372.159	13.30	5	5	OK
triazolam	343.051	16.79	5	5	OK
triflupromazine	408.172	15.44	5	10	OK

Analytes Sorted Alphabetically			Blood	Urine	
Analyte	M+1	RT (min)	LOD	LOD	Notes
trimipramine	295.217	15.08	5	5	OK
triprolidine	279.186	12.91	5	5	BP
U47700	329.118	13.72	5	5	SP
venlafaxine	278.211	13.04	5	5	OK
verapamil	455.290	14.86	5	5	OK
W18	422.094	20.04	NS	NS	NS
zaleplon	306.135	16.08	NS	NS	NS
ziprasidone	413.120	13.82	25	100	OK
zolpidem	308.176	12.79	5	5	OK
zolpidem metabolite	338.150	11.19	5	5	OK
zopiclone	389.112	12.00	10	5	OK

Not suitable for analysis by this method	NS
LOD not thoroughly evaluated but consistently present in control spiked at 5 µg/mL	Unk*
Peak shape is broad; less than ideal	BP
Compound known to give split peaks	SP
Compound validated with no issues	OK
Matrix Interference	I



**Appendix 4: List of Analytes, approximate retention times and M+1, in retention time order**

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
nicotine	163.123	2.06	5	I	I
EME	200.128	2.10	5	5	BP
BZP (benzylpiperazine)	177.139	2.25	5	10	SP
EEE	214.144	2.26	25	25	BP
cotinine	177.102	2.28	5	I	I
phenylephrine	168.102	2.41	5	5	SP
normorphine	272.128	2.46	25	25	I
morphine	286.144	2.48	5	5	SP
oxymorphone	302.139	2.48	5	5	SP
procainamide	236.176	2.48	5	5	SP
psilocybin	285.100	3.24	NS	NS	NS
atenolol	267.170	3.44	5	10	SP
hydromorphone	286.144	3.55	5	5	SP
ranitidine	315.149	3.66	10	10	SP
phenylpropanolamine	152.107	3.83	25	25	OK
phenethylamine	122.096	4.23	5	5	BP
psilocin	205.134	4.45	NS	NS	NS
tizanidine	254.026	4.51	5	5	OK
dihydrocodeine	302.175	4.67	5	5	OK
norcodeine	286.144	4.69	5	5	BP
pseudo/ephedrine	166.123	4.70	5	5	BP
naloxone	328.154	4.89	5	5	BP
codeine	300.159	5.10	5	5	BP
olanzapine	313.148	5.13	100	NS	BP
clonidine	230.025	5.20	5	5	BP
pregabalin	160.133	5.25	NS	NS	NS
gabapentin	172.133	5.29	*Unk	*Unk	*Unk
methylone	208.099	5.63	5	5	BP
norpheniramine	227.154	5.63	5	5	BP
doxylamine	271.180	6.00	5	5	BP
amphetamine	136.112	6.05	25	25	BP
mescaline	212.128	6.12	5	5	BP
noroxycodone	302.139	6.43	5	5	BP

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
pheniramine	241.170	6.52	5	5	BP
oxycodone	316.154	6.79	5	5	BP
scopolamine	304.154	6.88	5	5	BP
MDA	180.102	7.13	5	5	BP
methamphetamine	150.128	7.35	5	5	BP
norhydrocodone	286.144	7.62	5	10	BP
O-desmethyltramadol	250.180	7.65	5	5	BP
6-AM	328.154	7.66	5	5	BP
PMA	166.123	7.94	5	5	BP
baclofen	214.063	8.04	NS	NS	I
hydrocodone	300.159	8.17	5	5	BP
MDMA	194.118	8.44	5	5	BP
OH-quetiapine	400.169	8.62	10	10	BP
phentermine	150.128	9.10	5	5	BP
quinine/quinidine	325.191	9.46	I	I	I
PMMA	180.138	9.54	5	5	BP
butylone	222.112	9.85	5	5	BP
tetrahydrozoline	201.139	9.87	5	5	BP
mephedrone	178.123	10.30	5	5	OK
lidocaine	235.180	10.37	5	5	OK
MDEA	208.133	10.72	5	5	OK
norketamine	224.084	10.83	5	5	OK
strychnine	335.175	10.84	5	5	OK
ropinirole	261.196	11.08	5	5	OK
atropine	290.175	11.16	5	5	OK
ketamine	238.099	11.16	5	5	OK
7-aminoclonazepam	286.074	11.19	5	5	OK
zolpidem metabolite	338.150	11.19	5	5	OK
mirtazapine	266.165	11.34	5	5	OK
norfentanyl	233.165	11.37	5	5	OK
desmethylvenlafaxine	264.196	11.38	5	5	OK
MBDB	208.133	11.50	5	5	OK
metoclopramide	300.147	11.60	5	5	OK
benzoylecgonine	290.139	11.70	5	5	OK
dextrophan	258.185	11.84	5	5	OK
lamotrigine	256.015	11.89	5	5	OK
methoxetamine	248.165	11.92	5	5	OK

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
molindone	277.191	11.95	NS	NS	NS
tramadol	264.196	11.99	5	5	OK
metoprolol	268.194	12.00	5	5	OK
OH-bupropion	256.110	12.00	5	5	OK
pentylone	236.128	12.00	5	5	OK
zopiclone	389.112	12.00	10	5	OK
N-desmethyiltramadol	250.180	12.08	5	5	OK
methylphenidate	234.149	12.10	5	5	OK
desloratadine	311.131	12.11	25	25	I
desmethyiltapentadol	208.170	12.20	5	5	OK
alpha-PVP	232.170	12.29	5	5	OK
tapentadol	222.185	12.31	5	5	OK
desmethyloclozapine	313.121	12.44	10	10	OK
7-aminoflunitrazepam	284.119	12.51	5	5	OK
chlorpheniramine	275.131	12.52	5	5	OK
MDPV	276.159	12.57	5	5	OK
carbinoxamine	291.126	12.62	5	5	OK
cocaine	304.154	12.62	5	5	OK
normeperidine	234.149	12.64	5	5	OK
risperidone	411.219	12.68	5	5	OK
meperidine	248.165	12.69	5	5	OK
OH-risperidone	427.214	12.71	5	5	OK
guaifenesin	199.096	12.75	NS	NS	NS
lacosamide	251.139	12.79	NS	NS	NS
zolpidem	308.176	12.79	5	5	OK
brompheniramine	319.080	12.83	5	5	OK
norchlordiazepoxide	286.074	12.88	5	5	OK
triprolidine	279.186	12.91	5	5	BP
clozapine	327.137	12.94	5	5	OK
LSD	324.207	12.97	5	5	SP
bupropion	240.115	12.98	5	5	OK
norbuprenorphine	414.264	12.99	5	5	OK
chlordiazepoxide	300.090	13.02	5	5	OK
venlafaxine	278.211	13.04	5	5	OK
norquetiapine	296.122	13.12	5	5	OK
acetylfentanyl	323.212	13.14	5	5	OK
TFMPP	231.110	13.15	5	5	OK

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
buspirone	386.255	13.23	5	5	OK
bupivacaine	289.227	13.28	5	5	I
trazodone	372.159	13.30	5	5	OK
cocaethylene	318.170	13.34	5	5	OK
methocarbamol	242.102	13.37	NS	NS	NS
phenycyclidine	244.206	13.48	5	5	OK
quetiapine	384.174	13.48	5	5	OK
alfentanyl	417.261	13.60	5	5	OK
propranolol	260.165	13.60	5	10	OK
donepezil	380.222	13.61	5	5	OK
dextromethorphan	272.200	13.70	5	5	OK
U47700	329.118	13.72	5	5	SP
OH-midazolam	342.080	13.76	5	5	I
mesoridazine	387.156	13.79	5	5	OK
3-methoxyPCP	274.217	13.80	5	5	OK
fentanyl	337.227	13.80	5	5	OK
midazolam	326.085	13.81	5	5	OK
ziprasidone	413.120	13.82	25	100	OK
medazepam	271.100	13.88	5	5	OK
flurazepam	388.159	13.94	5	5	OK
diphenhydramine	256.170	14.00	5	5	OK
furanylfentanyl	375.207	14.00	5	5	OK
desmethylocitalopram	311.155	14.04	5	5	OK
amoxapine	314.105	14.08	5	5	OK
25H-NBOME	302.175	14.10	5	5	OK
desmethyldoxepin	266.154	14.10	5	5	OK
buprenorphine	468.311	14.14	5	5	OK
citalopram	325.171	14.15	5	5	OK
carfentanil	395.233	14.19	5	5	OK
doxepin	280.170	14.21	5	5	OK
diltiazem	415.169	14.22	5	5	OK
butyrylfentanyl	351.243	14.27	5	5	OK
loxapine	328.121	14.31	5	10	I
brexpiprazole	434.190	14.32	5	10	OK
haloperidol	376.149	14.33	5	5	OK
asenapine	286.099	14.34	5	10	I, MI

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
iloperidone	427.203	14.36	5	5	OK
promethazine	285.142	14.40	5	5	OK
EDDP	278.190	14.46	5	5	OK
atomoxetine	256.170	14.50	5	5	OK
orphenadrine	270.185	14.50	5	5	OK
paroxetine	330.150	14.58	5	5	OK
25I-NBOH	414.056	14.61	5	5	OK
desipramine	267.186	14.64	5	5	OK
desmethylocyclobenzaprine	262.159	14.65	5	5	OK
perphenazine	404.156	14.66	NS	NS	NS
protriptyline	264.175	14.67	5	5	OK
bromazepam	316.008	14.70	5	10	OK
amlodipine	409.152	14.72	25	25	OK
cyproheptadine	288.175	14.74	5	5	OK
norverapamil	441.275	14.75	5	5	OK
imipramine	281.201	14.76	5	5	OK
norchlorcyclizine	287.131	14.76	25	25	I
cyclobenzaprine	276.175	14.77	5	5	OK
fluvoxamine	319.163	14.77	5	5	OK
hydroxyzine	375.183	14.77	5	5	OK
25C-NBOME	336.136	14.78	5	5	OK
norpropoxyphene	326.211	14.79	5	5	OK
nortriptyline	264.175	14.83	5	5	OK
aripiprazole	448.155	14.84	25	25	OK
duloxetine	298.126	14.84	5	10	OK
maprotyline	278.190	14.86	5	5	OK
verapamil	455.290	14.86	5	5	OK
fexofenadine	502.295	14.87	5	5	OK
MT45	349.264	14.90	5	5	BP
prochlorperazine	374.145	14.90	25	25	BP
25I-NBF	416.052	14.92	5	5	OK
propoxyphene	340.227	14.94	5	5	OK
25B-NBOME	380.086	14.95	5	5	OK
amitriptyline	278.190	14.95	5	5	OK
desmethyltrimipramine	281.201	14.97	5	5	OK
25I-NBMD	442.051	15.00	5	5	OK
methadone	310.217	15.02	5	5	OK

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
norfluoxetine	296.126	15.02	5	5	OK
benztropine	308.201	15.04	5	5	OK
trimipramine	295.217	15.08	5	5	OK
norsertaline	292.065	15.10	25	25	OK
25I-NBOME	428.072	15.15	5	5	OK
fluphenazine	438.182	15.15	5	10	OK
fluoxetine	310.141	15.18	5	5	OK
sertaline	306.081	15.24	5	5	OK
cetirizine	389.163	15.26	10	5	I
chlorpromazine	319.103	15.26	5	5	OK
desmethylclomipramine	301.147	15.32	5	5	OK
triflupromazine	408.172	15.44	5	10	OK
clomipramine	315.162	15.45	5	5	OK
benzocaine	166.086	15.59	5	I	I
lurasidone	493.263	15.62	5	5	OK
loperamide	477.230	15.77	5	5	OK
thioridazine	371.161	15.80	100	100	OK
OH-alprazolam	325.085	15.86	5	5	OK
OH-triazolam	359.046	15.90	NS	NS	NS
loratadine	383.152	15.93	5	5	I
zaleplon	306.135	16.08	NS	NS	NS
nitrazepam	282.087	16.26	5	5	OK
estazolam	295.075	16.30	5	5	OK
desmethylflunitrazepam	300.078	16.34	NS	NS	NS
tetrazepam	289.111	16.37	5	5	OK
alprazolam	309.090	16.55	5	5	OK
flubromazolam	371.030	16.58	5	5	OK
lorazepam	321.019	16.58	NS	NS	NS
nordiazepam	271.063	16.60	5	5	OK
meclizine	391.194	16.63	5	5	OK
clonazepam	316.048	16.77	NS	NS	NS
triazolam	343.051	16.79	5	5	OK
metaxolone	222.112	16.81	NS	NS	NS
desalkylflurazepam	289.054	17.10	5	5	OK
etizolam	343.077	17.11	5	5	OK
flunitrazepam	314.094	17.35	NS	NS	NS
temazepam	301.074	17.40	NS	NS	NS

Analytes Sorted by Retention Time			Blood LOD	Urine LOD	Notes
Analyte	M+1	RT (min)			
lormetazepam	335.035	17.75	NS	NS	NS
phenazepam	348.972	17.81	100	NS	NS
nifedipine	347.124	18.13	NS	NS	NS
diazepam	285.079	18.14	5	5	OK
clobazam	301.075	18.36	NS	NS	NS
W18	422.094	20.04	NS	NS	NS
prazepam	325.111	20.23	5	5	OK
suvorexant	451.164	20.74	5	5	OK

Not suitable for analysis by this method	NS
LOD not thoroughly evaluated but consistently present in control spiked at 5 µg/mL	Unk*
Peak shape is broad; less than ideal	BP
Compound known to give split peaks	SP
Compound validated with no issues	OK
Matrix Interference	I

## Color Tests

### 1 Introduction

Color tests are rapid toxicological qualitative methods used to establish the presence or absence of certain drugs based upon visible observation. The tests are primarily used to screen for the presence of drugs or metabolites in urine, but may also be used for other biological fluids (e.g., ~~urine~~, precipitated blood) and nonbiological samples (e.g., solids or liquids).

Positive results of a color test are only preliminary. A more specific analytical method must be used in order to obtain a confirmed analytical result. Gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS) are preferred confirmatory methods.

### 2 Scope

This procedure allows for screening of urine samples for acetaminophen, salicylic acid, trihalomethyl compounds (including chloral hydrate and its metabolites), and ethchlorvynol<sup>1</sup>. This procedure also allows for the screening of blood samples for acetaminophen and salicylic acid. Other drugs may be added to the routine screens as deemed necessary, provided the color tests are validated prior to use. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

Color tests involve the reaction of a specimen with a reagent or series of reagents to produce a change in color. The stronger the color change, the more drug or drug metabolite present in the sample.

- Acetaminophen and its metabolites are hydrolyzed in an acidic solution to p-aminophenol, which is coupled with o-cresol to give an indophenol blue color.
- Aspirin is hydrolyzed in an acidic solution to salicylic acid, which reacts with acidic ferric chloride solution to give a purple color.
- Trichlorinated compounds are polyhalogenated compounds. Compounds with at least two halogen atoms bound to the same carbon atom will react with pyridine to give a pink or deep yellow/orange color.
- Ethchlorvynol reacts with diphenylamine to form a purple color.

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<sup>1</sup> As of the date of this procedure, ethchlorvynol is not available on the legal drug market.



## 4 Specimens

This procedure has been validated for urine specimens. The acetaminophen and salicylate tests have been validated for blood specimens. Other matrices may be substituted provided appropriate controls are analyzed. A minimum of 1.7 mL of urine is needed to perform all four color tests. A minimum of 2.0 mL of blood is required to perform the salicylate and acetaminophen color tests.

## 5 Equipment/Materials/Reagents

- a. Deionized water
- b. Pyridine (certified A.C.S grade)
- c. Chloroform (HPLC grade)
- d. Hotplate – stirrer or Heating Block
- e. Pipettes (0.5 - 5 mL, 200-1000  $\mu$ L, 50-200  $\mu$ L) with disposable tips
- f. Test tubes (13 x 100 mm, 12 x 75 mm) with caps
- g. Vortexer
- h. 6 M Hydrochloric Acid (~ 50% v:v):  
To a 25-mL graduated cylinder containing 10 mL deionized water, add 12 mL concentrated hydrochloric acid and mix well. Bring to 24 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- i. 2 M Ammonium Hydroxide:  
Add 10 mL concentrated ammonium hydroxide to 50 mL deionized water in a 100-mL graduated cylinder. Fill to the 75-mL mark with deionized water and mix well. Store in glass at room temperature. Stable 1 month.
- j. 1% (by volume) o-Cresol:  
Place 1 mL o-cresol in a 100-mL volumetric flask and fill to the mark with deionized water. Mix well and allow to stand for at least 24 hours before use. Store refrigerated in brown glass. Stable 6 months.
- k. Trinder's Reagent:  
Add 400 mg mercuric chloride, 400 mg ferric nitrate nonahydrate, and 0.1 mL concentrated hydrochloric acid to 5 mL deionized water in a 10-mL volumetric flask. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.

- l. 5 M (20% w/v) Sodium Hydroxide:  
To a 100-mL Nalgene volumetric flask, add 60 mL water and 20 g sodium hydroxide. Mix well to dissolve and bring to volume with deionized water. Store in Nalgene containers at room temperature. Stable 1 year.
- m. Balance
- n. Volumetric flasks
- o. Diphenylamine Reagent
- p. Methanol (HPLC Grade)
- q. Acetonitrile (HPLC Grade)
- r. 0.45  $\mu$ m Millipore Filter Tubes
- s. Centrifuge
- t. Evaporator with nitrogen

## 6 Standards and Controls

- a. Negative Control Urine:  
Obtained in house<sup>2</sup>. Store refrigerated in plastic.  
A Negative Control Urine will be analyzed every time a urine color test is performed.
- b. Negative Control Blood:  
Obtained in house or from a suitable vendor. Store refrigerated in glass or plastic.  
Stability determined by manufacturer.  
A Negative Control Blood will be analyzed every time a blood color test is performed.
- c. Acetaminophen:  
Purchased from U.S.P., or another approved vendor. Stability and storage determined by manufacturer.
- d. Acetaminophen Working Stock Solution (1 mg/mL):  
Place 10 mg acetaminophen in 10-mL volumetric flask, dilute to mark with methanol.  
Store refrigerated in glass, stable at least 6 months.
- e. Acetaminophen Positive Control Urine (100  $\mu$ g/mL):

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<sup>2</sup> Note: Synthetic urine (Surine) is unsuitable for the salicylate color test.

Add 1 mL of the Acetaminophen Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.

The Acetaminophen Positive Control Urine will be analyzed with every urine acetaminophen color test.

- f. Acetaminophen Positive Control Blood (50 µg/mL):  
Add 0.5 mL of the Acetaminophen Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Blood. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Acetaminophen Positive Control Blood will be analyzed with every blood acetaminophen color test.
- g. Sodium Salicylate:  
Purchased from Sigma, or another approved vendor. Stability and storage determined by manufacturer.
- h. Sodium Salicylate Working Stock Solution (1 mg/mL):  
Place 10 mg sodium salicylate in 10-mL volumetric flask, dilute to mark with deionized water. Store refrigerated in glass. Stable at least 6 months.
- i. Sodium Salicylate Positive Control Urine (100 µg/mL):  
Add 1 mL of the Sodium Salicylate Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Sodium Salicylate Positive Control Urine will be analyzed with every salicylate urine color test.
- j. Sodium Salicylate Positive Control Blood (50 µg/mL):  
Add 0.5 mL of the Sodium Salicylate Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Blood. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Sodium Salicylate Positive Control Blood will be analyzed with every salicylate blood color test.
- k. Trichloroacetic Acid:  
Purchased from Sigma, or another approved vendor. Stability and storage determined by manufacturer.
- l. Trichloroacetic Acid Working Stock Solution (1 mg/mL):  
Place 10 mg trichloroacetic acid in 10-mL volumetric flask, dilute to mark with methanol. Store refrigerated in glass, stable at least 6 months.
- m. Trichloroacetic Acid Positive Control Urine (50 µg/mL):  
Add 0.5 mL of the Trichloroacetic Acid Working Stock Solution to a 10-mL volumetric

flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.

The Trichloroacetic Acid Positive Control will be analyzed with every Fujiwara Color Test (Trichlorinated Compound Color Test).

- n. Ethylchlorvynol:  
Purchased from U.S.P., or another approved vendor. Stability and storage determined by manufacturer.
- o. Ethylchlorvynol Working Stock Solution (100 µg/mL):  
Place 100 uL ethylchlorvynol in a 12 x 75 mm test tube, add 900 uL of methanol. Store refrigerated in glass, stable at least 6 months.
- p. Ethchlorvynol Positive Control Urine (30 µg/mL):  
Add 1 mL of the Ethchlorvynol Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Ethchlorvynol Positive Control will be analyzed with every Ethchlorvynol Color Test.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the Examiner or Chemist performing the procedure.

Results of the following tests should be photographed for documentation and review.

- a. Acetaminophen Color Test:
  - 1. Preparation of blood samples:
    - a. Add 1 mL of blood (sample or control) to a 13 x 100 test tube.
    - b. While vortexing, add 2 mL acetonitrile, dropwise.
    - c. Transfer supernatant to a 0.45 µm Millipore filter tube.
    - d. Centrifuge at approximately 4000 rpm for 15 minutes.

- e. Transfer filtrate to a new test tube, and evaporate to dryness under nitrogen at 35°C.
  - f. Reconstitute residue in 0.5 mL deionized water. Vortex well.
2. Place 0.5 mL of urine or reconstituted blood extract (sample or control) in a properly labeled test tube (13 x 100 mm). [Note: test also works at 1/5 volumes in a 10 x 75 mm test tube.]
  3. Add 0.5 mL of 6 M Hydrochloric Acid Solution to sample.
  4. Vortex samples for 10 - 15 seconds, and then place them in a boiling hot water bath or heating block at 98°C. Remove from water bath/block after 10 minutes.
  5. Add 3 mL of deionized water.
  6. Add 2 mL of 2 M Ammonium Hydroxide Solution.
  7. Add 0.5 mL of O-cresol Solution (1%).
  8. Wait 10 - 25 minutes. (Wait a minimum of 25 minutes if acetaminophen and salicylate are expected in a blood extract.)
  9. View color. A blue color (indicative of acetaminophen) will appear with a positive result.
- b. Salicylate Color Test - (Trinder's color test):
1. Preparation of blood samples:
    - a. Add 1 mL of blood (sample or control) to a 13 x 100 test tube.
    - b. While vortexing, add 2 mL acetonitrile, dropwise.
    - c. Transfer supernatant to a 0.45 mm Millipore filter tube.
    - d. Centrifuge at approximately 4000 rpm for 15 minutes.
    - e. Transfer filtrate to a new test tube, and evaporate to dryness under nitrogen at 35°C.
    - f. Reconstitute residue in 0.2 mL deionized water. Vortex well.
  2. Place 0.2 mL of urine or reconstituted blood extract (sample or control) in a

properly labeled test tube (12 x 75 mm).

3. Add 0.2 mL of Trinder's Reagent.
  4. Vortex for 10 - 15 seconds.
  5. Wait 5 minutes.
  6. View color. A tan/brown (indicative of methylsalicylate) or tan/purple (indicative of sodium salicylate) will appear with a positive result.
- c. Trichlorinated Compound Color Test - (Fujiwara color test):
1. Place 0.5 mL of urine (sample or control) in a properly labeled test tube (12 x 75 mm).
  2. Add 0.5 mL of 20% Sodium Hydroxide Solution.
  3. Add 0.5 mL of pyridine.
  4. Vortex samples for 10 - 15 seconds, and then place them in a boiling hot water bath or heating block at 98°C. Remove from water bath/block after 10 minutes.
  5. View color in the top (pyridine) layer. A pink (indicative of chloral hydrate or trichloroacetic acid) or deep yellow/orange (indicative of 2,2,2-trichloroethanol) will appear with a positive result.
- d. Ethchlorvynol Color Test:
1. Place 0.5 mL of urine (sample or control) in a properly labeled test tube (12 x 75 mm).
  2. Add 0.25 mL of diphenylamine reagent.
  3. Vortex for 10 - 15 seconds.
  4. Wait 5 minutes.
  5. Add 1 mL of chloroform.
  6. Vortex samples for 10 - 15 seconds.
  7. Wait approximately 1 minute.

8. View color. A purple color (indicative of ethchlorvynol) will appear in the lower (chloroform) layer with a positive result, or blue (indicative of negative specimen) will appear in the lower (chloroform) layer with a negative result.

## 9 Decision Criteria

Generally speaking, a specimen is considered to give a positive response for a particular drug class if the specimen compares favorably to the corresponding positive control.

- a. Acetaminophen Color Test: Blue color indicates positive result for acetaminophen.
- b. Salicylate Color Test: Tan/brown color indicates a positive result for methylsalicylate, tan/purple color indicates a positive result for sodium salicylate.
- c. Trichlorinated compounds Color Test: Pink (or very deep red-purple for strong positive) color in upper (pyridine) layer within minutes indicates a positive result for chloral hydrate or trichloroacetic acid. Deep yellow/orange color in top layer indicates a positive result for 2,2,2-trichloroethanol. Note: May not be a good indicator for 2,2,2-trichloroethanol since positive result (deep yellow/orange color) may be hard to differentiate from the color of urine specimen, even with analysis of a negative control.
- d. Ethchlorvynol Color Test: Purple color in the lower layer within 2-3 minutes indicates a positive result for ethchlorvynol.

It is the ultimate judgment of the Examiner to determine if the results suggest the need for additional testing. If a specimen is deemed positive for a particular drug class, a separate analysis utilizing a different principle must be performed in order to confirm the presumptive positive.

## 10 Calculations

Not applicable.

## 11 Measurement Uncertainty

Not applicable.

## 12 Limitations

### a. Limits of Detection (LOD):

1. Acetaminophen Color Test: 40 µg/mL for acetaminophen in urine  
10 µg/mL for acetaminophen in blood
2. Salicylate Color Test: 300 µg/mL for methylsalicylate in urine  
45 µg/mL for sodium salicylate in urine  
10 µg/mL for sodium salicylate in blood
3. Trichlorinated Compounds Color Test: 10 µg/mL chloral hydrate  
4 µg/mL trichloroacetic acid
4. Ethchlorvynol Color Test: 25 µg/mL ethchlorvynol

### b. Sensitivity:

1. Acetaminophen, Salicylate, and Ethchlorvynol Color Tests:  
Positive results can be obtained with therapeutic doses.
2. Trichlorinated Compounds Color Test:  
Positive results can be obtained with therapeutic doses in plasma and with overdoses in urine.

### c. Specificity:

1. Acetaminophen Color Test:  
Positive results may be obtained with phenacetin (acetaminophen and p-aminophenol are metabolites of phenacetin, which is not available within the US). False negative results may occur in patients with ketonuria. When both acetaminophen and salicylate are present in a blood sample, false negative results may be obtained if the wait time is not lengthened to 25 minutes.
2. Salicylate Color Test:  
False positive results may be obtained from the following compounds: aminosalicilic acid, diflunisal, labetalol. When both acetaminophen and salicylate are present in a blood sample, false negative results may be obtained for low levels of salicylate (less than 50 PPM).
3. Trichlorinated Compounds Color Test:  
Positive results indicate the presence of a polyhalogenated compounds. Polyhalogenated compounds that may also give positive results include: bromoform, carbon tetrachloride, chloramphenicol, chlorbutol, chlorobutanol,



chloroform, DDT, ethyl bromide, ethylene chloride, iodoform, methyl bromide, methylene chloride, pentachloroethylene, perchloroethylene, tetrachloroethane, tribromoethanol, and trichloroethylene.

4. Ethchlorvynol Color Test:  
False positive results may be obtained from phenothiazines.

### 13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for proper handling and disposal of all chemicals.

### 14 References

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*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
0	01/30/06	New document.
1	09/08/08	Updated title. Added note that ethchlorvynol no longer available on US drug market in section 2. Expanded sections 2, 4, 5, 6, 9 and 14 to cover newly validated analysis of blood samples for acetaminophen and salicylate testing. Clarified in-house urine in section 6a. Updated preparation of Trichloroacetic Acid Positive Control Urine in section 6. Extended wait time for Acetaminophen Color Test to 25 minutes.
2	02/09/18	Updated Scope language. Updated Sections 9a.4 and 9c.4 to allow for use of heating block. Updated Section 5d and Appendix 1 to include heating block. Updated approval lines. Removed footer. Removed Calibration section (formerly 7), and updated numbering. Changed to 'Measurement Uncertainty' for Section 12. Section 6a: removed expiration for urine control. Removed references to TOX103 in Section 5 and References, and updated 5: h.-l. Deleted Instrumental Conditions (formerly Section 9) and renumbered.

**Approval**

Redacted - Signatures on File

Toxicology  
Technical Lead:

Date: 02/08/2018

Chemistry Unit Chief:

Date: 02/08/2018

**QA Approval**

Quality Manager:

Date: 02/08/2018

**Appendix 1: Abbreviated version of the Color Test Procedure for bench use.**

Redacted - Form on File

## **Exclusionary Drug Screen by UPLC-ESI-FTMS**

### **1 Introduction**

A rapid ultra-performance liquid chromatography electrospray Fourier transform mass spectrometry (UPLC-ESI-FTMS) method can be used to quickly screen blood and urine specimens for common drugs of abuse and prescription medications. The amount of a detected analyte can be estimated by comparing the response (peak area) for the analyte to that for a corresponding internal standard. Positive findings are confirmed via a second technique.

### **2 Scope**

This procedure allows for the screening of blood and urine specimens for the presence of cocaine and metabolites, opioids, antihistamines, benzodiazepines and other hypnotics (see Section 13 for a list of target analytes and limits of detection). This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

Urine specimens are subjected to enzymatic hydrolysis. Blood and hydrolyzed urine specimens are diluted with buffer and made alkaline before purification via supported liquid extraction. Final extracts are analyzed by UPLC-ESI-FTMS in full scan mode.

### **4 Specimens**

0.3 mL of blood or urine is required for this assay.

### **5 Equipment/Materials/Reagents**

- a. 13 x 100 mm and 16 x 100 mm test tubes
- b. Ammonium Acetate (99.999% purity)
- c. Deionized Water
- d. Acetic Acid, glacial (17 M, ACS grade)
- e. pH meter

- f. Ammonium Acetate Buffer (0.5 M, pH5):  
Add 3.854 g ammonium acetate to a 100-mL volumetric flask containing about 75 mL deionized water. Mix well to dissolve. Add glacial acetic acid until pH registers between 4.5 and 5.5. Bring to volume with deionized water and mix well. Store refrigerated in glass or plastic. Stable at least 3 months.
- g. Vortex mixer
- h. Ammonium hydroxide, concentrated (15 M, ACS grade)
- i. Ammonium Hydroxide (4.5 M):  
Mix 1.4 mL deionized water and 0.6 mL concentrated ammonium hydroxide in a test tube. Prepare fresh daily.
- j.  $\beta$ -glucuronidase (>120,000 u/mL  $\beta$  glucuronidase activity; from Red Abalone, *H. Rufescena*; available from Kura Biotec)
- k. Supported Liquid Extraction (SLE) cartridges (Biotage Isolute SLE+; 2 mL sorbent mass; part number 820-0290-D)
- l. Dichloromethane (Optima grade)
- m. Isopropanol (HPLC grade)
- n. Elution Solvent (95/5 Dichloromethane/Isopropanol):  
Combine 95 mL dichloromethane and 5 mL isopropanol and mix well. Store at room temperature in brown glass. Stable for at least one month.
- o. Vacuum extraction box
- p. Heating block
- q. Heated evaporator
- r. Acetonitrile (Optima grade)
- s. Water/Acetonitrile (95/5):  
Combine 9.5 mL deionized water and 0.5 mL acetonitrile and mix well. Store at room temperature in glass. Prepare fresh weekly.
- t. 0.2  $\mu$ m centrifuge tubes
- u. Autosampler vials with caps
- v. Waters Cortecs<sup>®</sup> C18, 1.6  $\mu$ ; 2.1 x 50 mm UPLC Column with a Cortecs<sup>®</sup> C18 1.6  $\mu$

precolumn

- w. Seal Wash Solvent and Weak Wash Solvent (Water/Acetonitrile; 90/10):  
Combine 450 mL deionized water and 50 mL acetonitrile and mix well. Store at room temperature in glass. Stable for at least two weeks.
- x. Methanol (Optima grade)
- y. Strong Wash Solvent (Methanol/Acetonitrile/Water/Isopropanol; 45/40/10/5):  
Combine 90 mL methanol, 80 mL acetonitrile, 20 mL deionized water and 10 mL isopropanol and mix well. Store at room temperature in glass. Stable for at least six months.
- z. Liquid chromatograph capable of ultra-performance liquid chromatography coupled to a mass spectrometer capable of 70,000 resolution.
- aa. LC Mobile Phase B (Acetonitrile with 0.1% Formic Acid): Combine 500 mL acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least one month.
- bb. LC Mobile Phase A (5 mM Ammonium Formate with 0.1% formic acid): Add 0.158 g ammonium formate to a 500 mL volumetric flask. Add approximately 400 mL deionized water and mix well. Add 0.5 mL formic acid, and QS with deionized water. Store in glass at room temperature. Stable for one week.

## 6 Standards and Controls

- a. Negative Control Blood:  
Purchased from Cliniqa or another suitable commercial source. Stability and storage determined by manufacturer. A Negative Control Blood will be analyzed in every blood batch.
- b. Negative Control Urine:  
Obtained in-house. Store refrigerated or obtain fresh. Stable at least two years. Alternatively, synthetic urine may be purchased from Dynatek. Storage and stability determined by the manufacturer. A Negative Control Urine will be analyzed in every urine batch.
- c. Internal Standard Components:  
Purchased from Cerilliant, International or another suitable vendor as 0.1 mg/mL solutions. Storage and stability determined by manufacturer.
  - benzoylecgonine-d<sub>3</sub> or benzoylecgonine-d<sub>8</sub>
  - morphine-d<sub>3</sub>
  - hydrocodone-d<sub>3</sub>

- oxycodone-d<sub>6</sub>
- clonazepam-d<sub>4</sub>
- 7-aminoclonazepam-d<sub>4</sub>
- α-hydroxyalprazolam-d<sub>5</sub>
- alprazolam-d<sub>5</sub>
- oxazepam-d<sub>5</sub>
- diazepam-d<sub>5</sub>
- zolpidem-d<sub>6</sub>
- diphenhydramine-d<sub>3</sub>

- d. Internal Standard Working Solution:  
Combine the listed volumes of each Internal Standard Component in a 100-mL volumetric flask and bring to the mark with acetonitrile. Store refrigerated in glass. Stable for at least two years.

Component	Volume (μL)	Final Concentration (ng/mL)	Final Concentration in Specimen (ng/mL)
benzoylecgonine-d <sub>3</sub> or benzoylecgonine-d <sub>8</sub>	120	120	10
morphine-d <sub>3</sub>	120	120	10
hydrocodone-d <sub>3</sub>	60	60	5
oxycodone-d <sub>6</sub>	60	60	5
clonazepam-d <sub>4</sub>	60	60	5
7-aminoclonazepam-d <sub>4</sub>	36	36	3
α-hydroxyalprazolam-d <sub>5</sub>	36	36	3
alprazolam-d <sub>5</sub>	36	36	3
oxazepam-d <sub>5</sub>	36	36	3
diazepam-d <sub>5</sub>	36	36	3
zolpidem-d <sub>6</sub>	24	24	2
diphenhydramine-d <sub>3</sub>	36	36	3

- e. Positive Control Components  
Purchased from Cerilliant, International or another suitable vendor as 1.0 mg/mL solutions. Storage and stability determined by manufacturer.
- Benzoylecgonine
  - Diazepam
  - Diphenhydramine
  - Oxycodone
  - Morphine-3-β-glucuronide (or morphine-6-β-glucuronide)
- f. Positive Control Stock Solution (5.0 μg/mL):  
Combine 82 μL of the morphine glucuronide solution and 50 μL of the remaining



component solutions in a 10-mL volumetric flask and bring to the mark with acetonitrile. Store in glass at <0°C. Stable for at least two years.

- g. Positive Control Working Solution (0.15 µg/mL):  
Add 300 µL of the Positive Control Stock Solution to a 10-mL volumetric flask and bring to the mark with 1:1 methanol:water. Store in glass at <0°C. Stable for 6 months.
- h. Positive Control (10 ng/mL):  
Add 20 µL of the Positive Control Working Solution (0.15 µg/mL) to 0.3 mL Negative Control (urine or blood, as needed) on the day of analysis. Urine Positive Controls will be hydrolyzed enzymatically along with the Negative Control and case samples.  
  
A Positive Control will be analyzed with every batch.
- i. Performance Standard Mix:  
Mix 0.025 mL of the Internal Standard Working Solution with 0.375 mL of deionized water. Prepare fresh on day of use.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 8.1 Blood specimen preparation:

- a. Add 0.3 mL of each sample and control to a properly labeled 13 x 100 mm test tube.
- b. Add 25 µL of the Internal Standard Working Solution to each sample.
- c. Add 0.7 mL Ammonium Acetate Buffer (0.5 M, pH5).
- d. Add 0.6 mL Deionized Water and vortex.
- e. Add 80 µL Ammonium Hydroxide (4.5 M) and vortex.

### 8.2 Urine specimen preparation:

- a. Add 0.3 mL of each sample and control to a properly labeled 13 x 100 mm test tube.

b. Add 25  $\mu$ L of the Internal Standard Working Solution to each sample.

c. Enzymatic Hydrolysis:

- i. Add 0.6 mL Ammonium Acetate Buffer (0.5 M, pH5).
- ii. Add 100  $\mu$ L  $\beta$ -glucuronidase (H. Rufescena).
- iii. Vortex, cap and incubate for 30 minutes at approximately 68°C.
- iv. Cool to approximately room temperature.
- v. Add 0.6 mL Deionized Water and vortex.

d. Add 80  $\mu$ L Ammonium Hydroxide (4.5 M) and vortex.

### **8.3 Extraction (for all specimens):**

- a. Load samples onto SLE cartridges by gravity. (A brief application of vacuum will be necessary to start loading.)
- b. Allow to stand for 5 minutes.
- c. Apply 3 mL of Elution Solvent (95/5 Dichloromethane/Isopropanol) and allow to absorb.
- d. Allow to stand for 5 minutes. Do not apply vacuum.
- e. Elute by gravity into 16 x 100 mm test tubes with 2 x 4 mL Elution Solvent (95/5 Dichloromethane/Isopropanol). Briefly apply full vacuum to complete elution.
- f. Evaporate at approximately 45°C. When volume reaches 0.5 – 1 mL, briefly vortex before evaporating to dryness.
- g. Reconstitute with 0.1 mL Water/Acetonitrile (95/5).
- h. Filter with 0.2  $\mu$ m centrifuge tubes.
- i. Transfer each sample to properly labeled autosampler vials.
- j. Analyze 20  $\mu$ L by UPLC-ESI-FTMS.

## 9 Instrumental Parameters

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters				Column Parameters	
A: 5 mM ammonium formate in 0.1% formic acid	total flow	0.50 mL/min			type	Waters Cortecs™ C18
	time (min)	% A	% B	Curve	length	50 mm
B: 0.1% formic acid in acetonitrile	0	95	5	Initial	internal diameter	2.1 mm
	0.1	95	5	9	particle size	1.6 μ
Event table: at 6.25 min, cycle inject valve	0.9	95	5	9	temperature	30°C
	2.67	60	40	6	5 x 2.1 mm precolumn Autosampler Temp: 14°C Weak Wash Vol: 1000 μL Strong Wash Vol: 800 μL Sample Loop: 20 μL Needle Overfill Flush: 5 μL	
	4.67	60	40	6		
	5.56	0	100	6		
	7.0	0	100	6		
	7.25	95	5	6		
	10	95	5	6		
	total time		10 min			

### 9.2 Mass Spectrometer Parameters

Parameter	Value
Method duration	10 min
Mode	ESI, Full scan MS
Polarity	positive
Microscans	1
Resolution	70,000
AGC Target	1e6
Maximum IT	50 ms
Scan Ranges	1
Scan Range	180-525 m/z
Spectrum data type	profile

## 10 Decision Criteria

### 10.1 Performance Standard Decision Criteria

In addition to the performance checks specified in the instrument standard operating procedure, a performance standard mix is analyzed through the analytical column to monitor the performance of the column.

#### 10.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each analyte in the performance standard should have reasonable peak shape.

The retention times of the 12 analytes should be within  $\pm 0.05$  minutes of the previous run of the performance standard. If the retention times have shifted more than 0.05 minutes, the column or precolumn may need to be changed, or the column may not be at equilibrium.

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

#### 10.1.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the 12 analytes in the performance standard should be present. The following molecular ions should be present as the base peak for each analyte, with a tolerance of  $\pm 5$  mmu:

Internal Standard	Mass
d <sub>5</sub> -alprazolam	314.12150
d <sub>5</sub> -hydroxyalprazolam	330.11650
d <sub>4</sub> -clonazepam	320.07350
d <sub>4</sub> -7-aminoclonazepam	290.09927
d <sub>5</sub> -diazepam	290.11030
d <sub>5</sub> -oxazepam	292.08957
d <sub>6</sub> -zolpidem	314.21340
d <sub>6</sub> -oxycodone	322.19200
d <sub>3</sub> -morphine	289.16260
d <sub>3</sub> -hydrocodone	303.17830
d <sub>3</sub> -benzoylecgonine or d <sub>8</sub> -benzoylecgonine	293.1577 or 298.1889
d <sub>3</sub> -diphenhydramine	259.1887

### 10.2 Unknown Sample Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

### **10.2.1 Batch Acceptance**

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the 12 internal standards should be detected in each Control. If any of the internal standards are not detected in the Control, data for the drug class that the internal standard falls into should be interpreted with care for the batch. The analytes in the Positive Control should be detected; for hydrolyzed urine batches morphine should be detected. Positive control failures will be evaluated and reanalysis may be necessary.

### **10.2.2 Unknown Sample Acceptance**

All 12 internal standards should be detectable in the unknown sample. If any of the internal standards are not detected in a case sample, the data for that case should be interpreted with care.

### **10.2.3 Unknown Sample Compound Detection**

This procedure is used for screening purposes only and not for the identification of specific drugs.

#### **10.2.3.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution.

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

Unknown sample files are reviewed using M+1 layouts with a mass tolerance of  $\pm 2.5$  mmu. Any peak within  $\pm 0.05$  minutes of the target retention time should be explored as possible positive results.

#### **10.2.3.2 Mass Spectrometry**

Following is a list of validated analytes for this method and their corresponding molecular ions and retention times (Table 1). Detection of a peak at the proper retention time is an indication that the analyte may be present, and confirmation testing will be performed.

**Table 1: Analytes with retention times and molecular ions**

Analyte	Retention Time (RT; min) / RT of labeled Internal Standard, if applicable	Molecular Ion (M+1)
<b>Benzodiazepines and Metabolites</b>		
α-hydroxyalprazolam	2.9 / 2.9	325.0851
α-hydroxymidazolam	2.67	342.0804
α-hydroxytriazolam	2.9	359.0461
7-aminoclonazepam	2.07 / 2.07	286.0742
7-aminoflunitrazepam	2.27	284.1194
alprazolam	3.06 / 3.06	309.0902
bromazepam	2.66	316.0080
chlordiazepoxide	2.46	300.0898
clonazepam	3.07 / 3.07	316.0484
desalkylflurazepam	3.19	289.0539
desmethylflunitrazepam	2.95	300.0779
diazepam	3.7 / 3.67	285.0789
estazolam	3	295.0745
etizolam	3.25	343.0778
flunitrazepam	3.22	314.0936
flurazepam	2.71	388.1586
lorazepam	3.04	321.0192
lormetazepam	3.43	335.0348
medazepam	2.71	271.0996
midazolam	2.67	326.0855
nordiazepam	3.19	271.0633
oxazepam	2.98 / 2.98	287.0582
phenazepam	3.45	348.9738
prazepam	5.33	325.1102
temazepam	3.27	301.0738
tetrazepam	3.68	289.1102
triazolam	3.12	343.0511
<b>Opioids and Metabolites</b>		
6-acetylmorphine	1.77	328.1543
codeine	1.54	300.1594
dihydrocodeine	1.5	302.1751
dihydromorphone	0.49	288.1594

Analyte	Retention Time (RT; min) / RT of labeled Internal Standard, if applicable	Molecular Ion (M+1)
EDDP	2.87	278.1903
hydrocodone	1.8 / 1.79	300.1594
hydromorphone	0.86	286.1438
morphine	0.53 / 0.53	286.1438
norcodeine	1.46	286.1438
normorphine	0.44	272.1281
noroxycodone	1.69	302.1387
oxycodone	1.72 / 1.72	316.1543
oxymorphone	0.64	302.1387
<b>Cocaine and Metabolites</b>		
ecgonine methyl ester	0.26	200.1280
benzoylecgonine	1.96 / 1.94	290.1387
cocaethylene	2.5	318.1700
cocaine	2.3	304.1543
<b>Antihistamines and Related Compounds</b>		
brompheniramine	2.5	319.0804
chlorpheniramine	2.43	275.1309
dextromethorphan	2.65	272.2008
dextrophan	2.11	258.1852
diphenhydramine	2.73 / 2.71	256.1695
doxylamine	1.94	271.1804
hydroxyzine	2.98	375.1833
norchlorcyclizine	2.98	287.1309
pheniramine	1.93	241.1699
tetrahydrozoline	1.87	201.1386
<b>Hypnotics</b>		
zaleplon	2.86	306.1349
zolpidem	2.35 / 2.35	308.1757
zopiclone	2.13	389.1123
<b>Antidepressant</b>		
duloxetine	3.02	298.1260

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

a. Limit of Detection:

**Table 2: Limits of Detection**

Analyte	Blood LOD (ng/mL)	Urine LOD (ng/mL)
<b>Benzodiazepines and Metabolites</b>		
$\alpha$ -hydroxyalprazolam	1	1
$\alpha$ -hydroxymidazolam	1	1
$\alpha$ -hydroxytriazolam	1	1
7-aminoclonazepam	1	1
7-aminoflunitrazepam	1	1
alprazolam	1	1
bromazepam	1	1
chlordiazepoxide	1	1
clonazepam	1	1
desalkylflurazepam	1	1
desmethylflunitrazepam	1	1
diazepam	1	1
estazolam	1	1
etizolam	1	1
flunitrazepam	1	1
flurazepam	1	1
lorazepam	1	1
lormetazepam	1	1
medazepam	5	1
midazolam	1	1
nordiazepam	1	1
oxazepam	1	1
phenazepam	1	1
prazepam	5	1



Analyte	Blood LOD (ng/mL)	Urine LOD (ng/mL)
temazepam	1	1
tetrazepam	1	1
triazolam	1	1
<b>Opioids and Metabolites</b>		
6-acetylmorphine	1	3
codeine	1	1
dihydrocodeine	1	1
dihydromorphine	1	1
EDDP	1	1
hydrocodone	1	1
hydromorphone	1	3
morphine	1	3
norcodeine	3	3
normorphine	1	1
noroxycodone	1	1
oxycodone	1	1
oxymorphone	1	1
<b>Cocaine and Metabolites</b>		
ecgonine methyl ester	1	5
benzoylecgonine	1	1
cocaethylene	1	1
cocaine	1	1
<b>Antihistamines and Related Compounds</b>		
brompheniramine	1	1
chlorpheniramine	1	1
dextromethorphan	10	1
dextrorphan	5	1
diphenhydramine	5	1
doxylamine	5	1
hydroxyzine	50	1
norchlorcyclizine	5	1
pheniramine	1	1
tetrahydrozoline	1	1
<b>Hypnotics</b>		
zaleplon	1	1

Analyte	Blood LOD (ng/mL)	Urine LOD (ng/mL)
zolpidem	3	3
zopiclone	1	1
<b>Antidepressant</b>		
duloxetine	50	5

- b. Specificity: No known interferences. However, this procedure will be used as a screen only and all positive findings will be confirmed by a second procedure.
- c. This procedure is not suitable to screen specimens for buprenorphine, norbuprenorphine or the carboxylic metabolite of zolpidem.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

*FBI Laboratory Safety Manual*

*Rapid Screening for Drugs of Abuse in Biological Fluids by Ultra High Performance Liquid Chromatography/Orbitrap Mass Spectrometry.* Jagerdeo E, et al. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences.* 2016 Aug 1; 1027:11 8.

Rev. #	Issue Date	History
0	08/20/15	New document
1	04/29/16	Section 2, Table 1 and Table 2 were updated to add additional analytes and analyte classes. Section 5.v. was updated to add details on the precolumn. Section 5 bb. was updated to reflect lower number of significant figures on mass of buffer salt. Tolerances were tightened in sections 10.1.1, 10.1.2 and 10.2.3.1. Clarified acceptance criteria in 10.2.1 and 10.2.2. Added a note in 14.c concerning drugs and metabolites not suitable to screen for with this method.
2	08/25/16	In 5.aa, 5.bb, 9.1, and Appendix 2, switched Mobile Phase A to aqueous and Mobile Phase B to organic. In 5.bb, removed phrase "at least". For internal standards, removed d <sub>3</sub> -cocaine, added d <sub>3</sub> -diphenhydramine, and allowed for use of d <sub>3</sub> or d <sub>8</sub> -benzoylecgonine which caused updates to 6.c, 6.d and 10.1.2. In 6.d, added final concentration of internal standard in specimens. Updated wording in 10.2.3. Added internal standard retention times to Table 1. Added lot number slots for washes to instrument sheet in Appendix 2.
3	02/09/18	Rewrote Sections 6 (e-i), adding a Positive Control that adds four analytes (in addition to the existing morphine glucuronide). Changed Section 5t and 8.3h to read 0.2 micron filters instead of 0.45 micron filters. Updated scope language in Section 2. Specified that Negative Controls will be run in batches, updating Sections 6a and 6b. Updated 8.2c(iii and iv) to "approximately"; also renumbered this section as previous version used nested alphabetical scheme. Added "approximately" to 8.3f. Updated 10.2.1 to account for inclusion of Positive Controls. Added a published reference to Section 16. Updated approvers. Updated Section 1 "the" to "a corresponding internal standard".

### **Approval**

Redacted - Signatures on File

Toxicology  
 Technical Lead:

Date: 02/08/2018

Chemistry Unit Chief:

Date: 02/08/2018

### **QA Approval**

Quality Manager:

Date: 02/08/2018

**Appendix 1: Abbreviated version of the Procedure for bench use**

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**Appendix 2: Abbreviated version of the Instrumental Conditions for bench use**

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## **Volatile Chemicals by Headspace GC/MS(EI)**

### **1 Introduction**

The analysis of volatile chemicals is performed by headspace gas chromatography. This technique is based on various gas laws which state that when a volatile liquid in solution comes into contact with a closed air space, an equilibrium forms between the liquid phase and the headspace. At a given temperature, the partial pressure of the volatile in the "headspace" is directly proportional to its concentration in solution. This method affords a means of analyte separation from the biological matrix and produces a ready-made vapor for chromatographic analysis. It should be noted that this is a modification of a procedure used routinely in the analysis of biological specimens for ethanol.

### **2 Scope**

This procedure allows for the screening and confirmation of volatile chemicals that may be present in biological and non-biological samples.

### **3 Principle**

An aliquot of sample or control is combined with internal standard and sodium chloride in a headspace vial. The vial is heated for 30 minutes and then the headspace is analyzed by gas chromatography with mass spectral detection (GC/MS).

### **4 Specimens**

This procedure can be performed on a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or tissue homogenate. When available, a minimum of 0.25 mL of specimen is used in this assay. This procedure may also be performed on foods, beverages, or unknown solid or liquid samples, although dilution may be required for samples with high amounts of volatile chemicals present.

## 5 Equipment/Materials/Reagents

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Agilent Gas Chromatograph/Mass Spectrometer equipped with a headspace autosampler and a 30 m x 0.25 mm x 1.4  $\mu$ m DB-624 column, or equivalent
- b. 20-mL or 10-mL disposable headspace vials, magnetic caps, and crimper
- c. Vortex mixer
- d. Volumetric flasks (100-mL and 1000-mL)
- e. Pipette (Adjustable or 0.5 mL fixed)
- f. Sodium Chloride (NaCl) (ACS Reagent Grade)
- g. Deionized Water
- h. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
- i. Saturated sodium chloride solution (aka brine solution) :  
To a 500-mL volumetric flask, add 450 mL deionized water and 175 g sodium chloride. Gently heat with continuous stirring for at least one hour. Remove the stirbar, fill to volume with deionized water, and mix by inversion. A small amount of undissolved solid should remain in the bottom of the flask. Store in glass at room temperature. Stable for one year.

## 6 Standards and Controls

- a. Methanol (Reagent Grade)
- b. Ethanol (200 proof, pharmaceutical grade)
- c. Isopropanol (HPLC Grade)
- d. Acetone (HPLC Grade)
- e. Acetonitrile (HPLC Grade)

- f. Acetonitrile Internal Standard Solution (0.08% (w/v)):  
Add 100  $\mu$ L acetonitrile to about 90 mL deionized water in a 100-mL volumetric flask. Dilute to the mark with deionized water and mix thoroughly. Store at room temperature in a tightly sealed glass container. Stable for 6 months.
- g. Volatile Injection Solution / Positive Control (0.01 % v/v of each component): Prepare by adding 500 mL of deionized water into a 1000-mL volumetric flask. Add 0.1 mL each of methanol, ethanol, isopropanol, and acetone. Bring to the mark with deionized water. Store refrigerated in a tightly-sealed glass or plastic container. Stable for at least two years. This Injection Solution may be analyzed as the Positive Control for the assay. Another suitable positive control may be analyzed, as appropriate.
- h. Whole Blood Volatiles Control:  
Purchased from Cliniqua. Contains acetone, ethanol, isopropanol and methanol. Two levels are typically purchased for ethanol quantitations; either may be used. Concentrations of these analytes differ from lot to lot. Storage conditions and stability determined by manufacturer. This Volatiles Control may be analyzed as the Positive Control for the assay. Another suitable positive control may be analyzed, as appropriate.
- i. Negative Control:  
An appropriate negative matrix should be used as the Negative Control. In the absence of a more appropriate matrix, deionized water may serve as the negative control for this analysis. A Negative Control will be analyzed with every volatiles assay.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.



- a. Into properly-labeled 20-mL headspace vials add 0.5 g sodium chloride or 2.0 mL saturated sodium chloride solution. Add 0.5 mL (or 0.5 g) of specimen or control. Add 0.5 mL Acetonitrile Internal Standard Solution. Alternatively, half these amounts may be added to 10-mL headspace vials. Notes: Unknown liquids may have to be diluted before analysis. The acetonitrile internal standard solution may be omitted or substituted if acetonitrile is a suspected target analyte. All samples in a batch should be prepared the same way.
- b. Immediately cap.
- c. Vortex sample for 10 seconds.
- d. Analyze specimens by headspace GC/MS(EI) after confirming that the instrument is calibrated and in proper working condition.

## 10 Instrumental Conditions

Appendix 2 contains an abbreviated version of HS-GC/MS instrumental parameters used in this procedure that may be used at the bench to verify instrumental parameters.

### 10.1 Headspace Sampler Parameters

incubation temperature	80°C	syringe temperature	90°C
incubation time	30min	sample fill volume	1.0mL
agitator speed	300 RPM	sample fill rate	0.5 mL/sec
agitation timing	10 sec on 1 sec off	sample fill strokes	5
cycle time	48 min	sample injection speed	1.0 mL/sec
		syringe flush time	4.0min

## 10.2 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature 1	50°C	type	DB-624	inlet temp.	150°C
hold 1	3 min	length	30 m	injection mode	split
ramp 1	10°C/min	internal diameter	0.25 mm	carrier gas	ultrapure helium
temperature 2	250°C	film thickness	1.4 µm	carrier mode	constant pressure
hold 2	21.5 min			pressure	6.54 psi
total run time	44.5 min			split ratio	10:1

## 10.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	260°C
scan range	27 - 400 m/z	quadrupole temperature	150°C
multiplier offset	+106 V	solvent delay	1.6 min

## 11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or Positive Control.

### 11.1 Batch Acceptance Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

Each of the analytes in the Positive Control should be detected in the headspace GC/MS data. If a targeted run is being performed for a limited set of analytes, only those analytes need to be detected in the Positive Control.

## **11.2 Unknown Sample Acceptance Criteria**

### **11.2.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **11.2.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or Positive Control. The relative retention times of the components should agree with those listed in the enclosed table within  $\pm 2\%$ . If not, the shift in relative retention times should be noted and appropriate corrections made when analyzing the data generated from case specimens.

#### **11.2.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

### **11.2.2 Mass Spectrometry**

The mass spectrum of the analyte of interest should match that of a reference standard or Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure for further guidance.

Table 1: Approximate Relative Retention Times (RRT) to Acetonitrile on DB - 624 column

Chemical	RRT	Chemical	RRT	Chemical	RRT
Acetaldehyde	0.606	Diethylamine	1.203	1,4-Dioxane	2.241
Methanol	0.629	Hexane	1.250	Isoamyl Alcohol*	2.584
Pentane	0.783	1-Propanol	1.321	Toluene	2.635
Ethanol	0.800	Ethyl Acetate	1.530	Ethyl Benzene	3.305
Diethyl Ether	0.838	Chloroform	1.625	m/p-Xylene	3.359
Acetone	0.919	n-Butyl Chloride	1.739	o-Xylene	3.542
Isopropanol	0.951	Isobutyl Alcohol*	1.794	2,2,2-Trichloroethanol	3.894
Acetonitrile	1.000	Benzene	1.846	Octanol	4.688
Methylene Chloride	1.057	Isooctane	1.875	Cresol	5.067
t-Butanol	1.082	Butyl Alcohol*	2.062		

\*These alcohols were analyzed as nitrite standards.

## 12 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 13 Uncertainty of Measurement

Not applicable.

## 14 Limitations

### a. Limits of Detection:

Chemical(s)	LOD (%v/v)
methylene chloride, benzene	0.0001
toluene	0.0002
diethyl ether, t-butanol, ethyl acetate, chloroform, n-butyl chloride, octanol, isomayl alcohol, xylenes, ethyl benzene	0.0010
methanol, acetone, isopropanol, isobutyl alcohol, 2,2,2-TCE, butyl alcohol	0.0050
acetaldehyde, pentane, ethanol, hexane, isooctane, 1,4-dioxane, propanol, cresol	0.0100
diethylamine	0.1000

### b. Interferences: None known. Care should be taken when interpreting results from grossly decomposed or putrefied samples, as well as samples that have been embalmed. Severe or

extensive putrefaction will result in the generation of a wide variety of low molecular weight volatile compounds in biological specimens, including ethanol, higher weight n-alcohols, aldehydes, sulfides, mercaptans, and alkylamines.

- c. Processed Sample Stability: The following compounds are not stable when processed samples sit for one week at room temperature: methylene chloride, hexane, n-butyl chloride, toluene, octanol, xylenes, and ethyl benzene. Therefore, it is suggested that samples be processed the day of analysis, when possible.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

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Garriott, James, *Medicolegal Aspects of Alcohol*, 3<sup>rd</sup> ed., Lawyers and Judges Publishing: Tucson, AZ, 1996.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Ethanol in Biologicals by Automated Headspace GC-FID / GC-MS(EI)* Standard Operating Procedure (Tox 200); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
1	03/05/10	Removed "Automated" from title. Minimum sample size was updated to 0.25 mL in Section 4. Section 5 was updated to reflect Agilent GC/MS, direct reader to Tox 103, and include saturated sodium chloride solution. Section 6 was updated to reflect new provider of Whole Blood Volatiles Control. Section 7 was updated to direct reader to Tox 200 for quantitation of ethanol, methanol, acetone and isopropanol. Section 9 was updated to use the option of saturated sodium chloride solution, rather than NaCl. Updated MS scan range in Section 10.3. Added Appendix 2, instrument bench sheet.
2	05/17/12	Removed all quantitative references to the procedure which affected Sections 7, 13 and 16. Updated chromatography criteria in Section 11.1. Updated Table 1 with current validation data. Updated Section 14 with current validation data.
3	07/09/14	Added recipe for saturated sodium chloride solution in Section 5.i. Updated Positive Control analytes in 6.h. In Section 10.3, updated solvent delay to catch entire acetaldehyde peak. Added batch acceptance criteria in Section 11.1, and renumbered subsequent sections. Removed internal standard preparation instructions from Appendix 1. Reformatted Appendix 2 to include all pertinent instrumental parameters.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Volatile Procedure for bench use.**

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**Appendix 2: Abbreviated version of the instrumental parameters for bench use.**

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## Cyanide and Azide Analysis in Biologicals and/or Foodstuffs

### 1 Introduction

Cyanide, a rapidly acting poison, has a high affinity for iron in the ferric state. As such, when cyanide is biologically absorbed, it readily reacts with the trivalent iron of cytochrome oxidase. Reactions with mitochondrial enzymes cause an inhibition of cellular respiration that can lead to hypoxia and rapid death. The minimum adult lethal dose has been estimated as 100 mg for hydrocyanic acid and 200 mg for potassium cyanide.

Industrial uses of cyanide salts (fumigants, insecticides and electroplating solutions) make this toxic anion available for suicidal purposes and criminal mischief, such as food tampering.

Sodium azide is the most commonly encountered toxic form of the azide moiety. An inorganic compound, this colorless salt has a diverse set of uses. It is used as the gas-forming component of some automotive air-bag systems and airplane escape chutes. Industrially, it is used as a precursor for other azide compounds, although applications are limited due to its inherent explosion hazard. It can also be used as a biochemical research tool and as a preservative in laboratory environments.

Similar to cyanide, azide inhibits cytochrome oxidase by irreversible binding to the heme cofactor. Sodium azide is acutely toxic, with estimated adult lethal dose at 700 mg.

### 2 Scope

This procedure allows for the screening, identification, and quantitation of cyanide in biologicals and foodstuffs. This procedure also allows for the qualitative identification of azide in blood and aqueous samples. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

This procedure for cyanide and azide is predicated on the condition that the analytes will be released as hydrogen cyanide gas (HCN) and hydrozoic acid, respectively, upon acidification of the biological and/or food matrix.

For cyanide, screening is performed by headspace (HS) gas chromatography with selective nitrogen-phosphorus detection (GC/NPD). Qualitative confirmational analysis of cyanide and screening/confirmation of azide is performed by headspace gas chromatography-mass spectrometry. Quantitative confirmation of any cyanide positive sample may be achieved by headspace gas chromatography with nitrogen-phosphorus detection.

## 4 Specimens

For cyanide, sample matrices can be comprised of biologicals (blood, tissue homogenate, or stomach contents) as well as common foodstuffs such as cakes, candy and beverages. For azide, blood and aqueous matrices are currently validated. Typically, 0.5 mL or 1.0 mL (or gram) of sample is used for these analyses.

## 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Hewlett Packard Gas chromatograph equipped with an RT-QS-BOND (or equivalent) column, a nitrogen phosphorus detector and a headspace autosampler.
- b. Hewlett Packard Gas chromatograph equipped with a DB-624 (or equivalent) column, a mass spectrometric detector and a headspace autosampler.
- c. Volumetric flasks (100 and 200-mL)
- d. Glass reagent bottles (100 - 500 mL capacity)
- e. 10-mL and/or 20-mL disposable headspace vials and caps
- f. 1.0 and 2.5 cc syringes with needles
- g. Vortex mixer
- h. Homogenizer and/or mortar and pestle
- i. Potassium Cyanide (Reagent grade)
- j. Sodium Azide (Reagent grade)
- k. Acetonitrile (HPLC grade)
- l. Deionized Water
- m. 1-Butanol (ACS grade)

- n. **5 N Sulfuric Acid:**  
To a 100-mL graduated cylinder containing 70 mL deionized water, slowly add 12.5 mL concentrated sulfuric acid. Mix well and bring to 90 mL with deionized water. Store in glass at room temperature. Stable 1 year.
- o. **5 N Sodium Hydroxide:**  
To a 100-mL Nalgene volumetric flask, add 60 mL water and 20 g sodium hydroxide. Mix well to dissolve, and let cool. Bring to volume with deionized water. Store in Nalgene containers at room temperature. Stable 1 year.
- p. **0.4% Sodium Hydroxide**  
To a 25-mL volumetric flask, add approximately 15 mL of deionized water. Add 0.1 g of sodium hydroxide. Dilute to the mark with deionized water and mix well. Store in Nalgene containers at room temperature. Stable for 1 year.
- q. Routine laboratory supplies, including pH paper, disposable glass pipets, spatulas, test tube racks, graduated cylinders, etc.

## 6 Standards and Controls

- a. **Cyanide Stock Standard (0.2 mg/mL)<sup>1</sup>:**  
Prepared by adding 50 mg of potassium cyanide to a 100-mL volumetric flask containing 2 mL of 5 N NaOH. Dilute to volume with deionized water and mix thoroughly. Stable for at least one year.
- b. **Concentrated Cyanide Stock Standard (2 mg/mL; needed for quantitation only):**  
Prepared by adding 50 mg of potassium cyanide to a 10-mL volumetric flask containing 2 mL of 0.5 N NaOH. Dilute to volume with deionized water and mix thoroughly. Stable for at least one year.
- c. **Azide Stock Standard (1 mg/mL):**  
See the Safety Section prior to handling sodium azide. Using a plastic spatula, weigh out 10 mg of sodium azide into a tared plastic weigh boat. Add sufficient 0.4% sodium hydroxide solution to dissolve the powder. Pour the solution into a 10-mL volumetric flask, and dilute to the mark with 0.4% sodium hydroxide. Store in Nalgene containers at room temperature. Stable for 1 year.
- d. **Azide Working Standard (100 µg/mL)**  
See the Safety Section prior to handling sodium azide. Add approximately 5 mL of 0.4% Sodium Hydroxide to a 10-mL volumetric flask. Add 1 mL of the Azide Stock Standard (1 mg/mL). Dilute to the mark with 0.4% Sodium Hydroxide. Store in Nalgene containers at room temperature. Stable for 1 year.

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<sup>1</sup>For quantitative analyses, separate stocks will be prepared for controls and calibrators.

- e. Negative Control:  
Deionized water, blood or an appropriate matrix blank, is used as a Negative Control. A Negative Control will be extracted and analyzed with every assay. Note that some materials are preserved with sodium azide, and would therefore be not be appropriate for use as an azide negative control. Verify the negative control matrix does not contain sodium azide prior to use.
- f. 0.04% Acetonitrile (v/v) (Internal Standard):  
Add 40  $\mu$ L acetonitrile to about 90 mL deionized water in a 100-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store at room temperature in a tightly sealed glass or plastic container. Stable for 6 months.
- g. 0.1% 1-butanol (v/v) (Internal Standard):  
Prepared by dissolving 0.1 mL of 1-butanol in 50 mL of deionized water in a 100-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Stable for 6 months.
- h. Aqueous Positive Control (1  $\mu$ g/mL cyanide):  
Prepared by adding 0.05 mL of the Cyanide Stock Standard to a 10-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store in plastic or glass. Stable at least one year. A Positive Control will be analyzed with every assay. When possible, the Positive Control will be matrix matched.
- i. Aqueous Low Positive Control – Quantitative (3  $\mu$ g/mL cyanide):  
Prepared by adding 0.15 mL of the Cyanide Stock Standard to a 10-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store in plastic or glass. Stable at least one year. A Positive Control will be analyzed with every assay. When possible, the Positive Control will be matrix matched.
- j. Aqueous Positive Control (10  $\mu$ g/mL cyanide):  
Prepared by adding 0.5 mL of the Cyanide Stock Standard to a 10-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store in plastic or glass. Stable at least one year. A Positive Control will be analyzed with every assay. When possible, the Positive Control will be matrix matched.
- k. Aqueous High Positive Control - Quantitative (24  $\mu$ g/mL cyanide):  
Prepared by adding 1.2 mL of the Cyanide Stock Standard to a 10-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store in plastic or glass. Stable at least one year. A Positive Control will be analyzed with every assay. When possible, the Positive Control will be matrix matched.
- l. Positive Control Blood (1  $\mu$ g/mL cyanide):  
Prepared by first diluting the Cyanide Stock Standard to a concentration of 10  $\mu$ g/mL by

adding 5 mL of the Cyanide Stock Standard to a 100-mL volumetric flask and bringing to volume with deionized water. Add 0.1 mL of this 10 µg/mL solution to 0.9 mL of whole blood. Prepare fresh. A Positive Control will be extracted and analyzed with every assay. When possible, the Positive Control will be matrix matched. For quantitative assays, the Positive Control should be prepared in accordance with the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

- m. Positive Control Blood (10 µg/mL cyanide):  
Add 0.05 mL of the Cyanide Stock Standard to 0.95 mL of whole blood. Prepare fresh. A Positive Control will be extracted and analyzed with every assay. When possible, the Positive Control will be matrix matched. For quantitative assays, the Positive Control should be prepared in accordance with the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).
- n. Low Positive Control Blood Spiking Solution – Quantitative (30 µg/mL cyanide):  
Prepared by adding 1.5 mL of the Cyanide Stock Standard to a 10-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store in plastic or glass. Stable at least one year. On day of quantitative analysis, 0.05 mL of this Solution is added to 0.5 mL blood to represent a 3 µg/mL cyanide blood control.
- o. High Positive Control Blood Spiking Solution – Quantitative (240 µg/mL cyanide):  
Prepared by adding 1.2 mL of the Concentrated Cyanide Stock Standard to a 10-mL volumetric flask. Dilute to volume with deionized water and mix thoroughly. Store in plastic or glass. Stable at least one year. On day of quantitative analysis, 0.05 mL of this Solution is added to 0.5 mL blood to represent a 24 µg/mL cyanide blood control.
- p. Low Positive Control (2 µg/mL Azide)  
To 0.5mL of Negative Control Matrix, add 10µL of the Azide Working Standard, and vortex.
- q. High Positive Control (20 µg/mL Azide)  
To 0.5mL of Negative Control Matrix, add 100µL of the Azide Working Standard, and vortex.

This procedure may be used quantitatively for cyanide. A cyanide calibration graph is constructed from the aqueous cyanide calibrators per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Although the Acetonitrile Internal Standard Solution is stable for 6 months, slow evaporation means that any given calibration curve is only valid for about 24 hours. Cyanide concentrations greater than the highest calibrator require dilution and re-quantitation for accurate quantitative results. Table 1 provides direction on the preparation of recommended calibrators for the quantitation of cyanide.

Table 1: Preparation of Recommended Calibrators for the GC/NPD Quant

Level (ug/mL)	Amount of 0.2 mg/mL stock diluted to 10 mL DI water (mL)
1	0.050
2.5	0.125
5	0.250
10	0.500
20	1.000
30	1.500

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

Note: Sample preparation for all methods should be performed in a chemical fume hood.

### 8.1 Screening and Quantitation of Samples for Cyanide by GC/NPD

- Thoroughly homogenize solid or bulky specimens to ensure a representative aliquot is sampled. Tissue, stomach contents and food specimens are mixed (1:1) with deionized water prior to blending. Liquid samples (blood and beverages) can be directly assayed. Measure and record the pH of all food specimens and stomach contents. Large quantities of inorganic cyanide salts may be indicated by an alkaline pH (>10).
- Measure 0.5 mL of liquid sample or 1 gram of homogenate and 50 µL of 0.04% acetonitrile (Internal Standard) into a 10-mL headspace vial and cap. Process calibrators and controls similarly.
- Using a 1.0 cc syringe, inject 0.5 mL of 5 N H<sub>2</sub>SO<sub>4</sub> into the vial and thoroughly vortex the sample to uniformly distribute the acid.
- Allow the sample to equilibrate at room temperature for 30 minutes.
- Analyze headspace by GC/NPD using the instrumental parameters in Section 9.1.
- Upon completion of the analysis, check and record the pH of the sample matrix in the

vial to ensure acidity (pH<2).

## 8.2 Confirmation of Samples for Cyanide, and Screening/Confirmation of Samples for Azide by Headspace GC/MS

- Thoroughly homogenize solid or bulky specimens to ensure a representative aliquot is sampled. Tissue, stomach contents and food specimens are mixed (1:1) with deionized water prior to blending. Liquid samples (blood and beverages) can be directly assayed. Measure and record the pH of all food specimens and stomach contents. Large quantities of inorganic cyanide salts may be indicated by an alkaline pH (>10).
- Measure 0.5 mL of liquid sample or 1 gram of homogenate and 50 µL of 0.1% 1-butanol (Internal Standard) into a 10-mL headspace vial and cap. Process controls similarly. (Alternatively, measure 1.0 mL of a liquid sample or 2 grams of a homogenate and 100 µL of 0.1% 1-butanol into a 20-mL headspace vial and cap.)
- Using a plastic syringe, inject 0.5 mL of 5 N H<sub>2</sub>SO<sub>4</sub> into the vial and thoroughly vortex the sample to uniformly distribute the acid. (If using 20-mL headspace vials, add 1.0 mL of 5 N H<sub>2</sub>SO<sub>4</sub>.) Allow to equilibrate for 30 minutes at room temperature.
- For cyanide analysis, analyze the headspace by GC/MS using the instrumental parameters in Section 9.2.
- For azide analysis, analyze the headspace by GC/MS using the instrumental parameters in Section 9.3.
- Upon completion of the analysis, check and record the pH of the sample matrix in the vial to ensure acidity (pH<2).

## 9 Instrumental Conditions

Appendix 1 contains an abbreviated list of instrumental conditions. Following are the instrumental parameters for the instruments used in this procedure:

### 9.1 For GC/NPD Analysis of Hydrogen Cyanide

#### 9.1.1 Headspace Sampler Parameters

Syringe	2.5ml-HS	tray type	VT32-10/20
oven / syringe temp.	45°C / 55°C	Fill/injection volume	2500 / 250 µl
flush time	4.0 min	fill speed / strokes	500 µl/sec / 5
incubation time	5.0 min		
agitator speed	250 rpm	injection speed	1000 µl / sec
agitator on/off time	10 s / 1 s	Inj./Vial penetration	40 / 25 mm



### 9.1.2 Gas Chromatograph and NPD Parameters

Oven Parameters		Inlet and Carrier Parameters		NPD Parameters	
temperature	110°C	inlet temperature	150°C	temperature	225°C
ramp	4 °C/min	purged packed inlet pressure	10.894 psi	makeup flow	3.5 mL/min
final temperature	130 °C	carrier gas	N <sub>2</sub>	air flow	60 mL/min
final hold time	5 min	carrier mode	constant flow	hydrogen flow	3.0 mL/min
Column Parameters		carrier flow	1.5 mL/min	electrometer	on
type	RT-QS-BOND				
length	30 m				
internal diameter/film	320 µm /10 µm				

*Hydrogen Cyanide should elute within 2 minutes under these conditions*

## 9.2 For GC/MS Analysis of Hydrogen Cyanide

### 9.2.1 Headspace Sampler Parameters

Syringe	2.5ml-HS	tray type	VT32-10/20
oven / syringe temp.	45°C / 55°C	Fill/injection volume	2500 / 1000 µl
flush time	4.0 min	fill speed / strokes	500 µl/sec / 5
incubation time	5.0 min		
agitator speed	300 rpm	injection speed	1000 µl / sec
agitator on/off time	10 s / 1 s	Inj./Vial penetration	40 / 22 mm

### 9.2.2 Gas Chromatograph Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	60°C	inlet temperature	150°C	type	DB-624
hold 1	2 min	injection mode	split	length	30 m
ramp 1	50°C/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	120°C	carrier mode	constant pressure	film thickness	1.4 µm
hold 2	2.5 min	carrier pressure	6.54 psi		
total run time	5.7 min	split ratio	10:1		



### 9.2.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
solvent delay	1.5 min	transfer line temperature	260°C
scan mode	SIM	quadrupole temperature	150°C
group 1 (1.65 – 2.7 min)	m/z 12, 26, 27	multiplier offset	+200 V
group 2 (2.7 – 10.2 min)	m/z 31, 41, 56		

## 9.3 For GC/MS Analysis of Hydrozoic Acid

### 9.3.1 Headspace Sampler Parameters

Syringe	2.5 mL	Injection Volume	1000 µL
Syringe Temperature	55°C	Injection Speed	1000 µL / s
Flush Time	240 s	Pullup Delay	0 s
Incubation Temperature	45°C	Fill Volume	2500 µL
Incubation Time	10.00 min	Fill Strokes	5
Agitator On/Off Time	10 s / 1 s	Fill Speed	500 µL / s
Agitator Speed	300 rpm	Pre/Post Inj. Delay	0/0 s
Inj./Vial Penetration	40 / 22 mm	Sample Tray Type	VT32-10/20

### 9.3.2 Gas Chromatograph Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temp 1	40°C (1 min)	inlet temperature	150°C	type	DB-624
temp 2	110°C @ 15°C/min (0 min)	injection mode	split	length	30 m
temp 3	250 °C @ 35°C/min (4 min)	carrier gas	ultrapure helium	internal diameter	0.25 mm
Eq. Time	0.2 min	carrier mode	constant pressure	film thickness	1.4 µm
Run Time	14.167 min	carrier pressure	5.463 psi	split ratio	10:1

### 9.3.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
solvent delay	1.9 min	transfer line temperature	260°C
scan mode	SIM	quadrupole temperature	150°C
group 1	m/z 15, 29, 43, 45, 58	multiplier offset	+200 V

## 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed calibrator or extracted positive control. In most cases, all of the below should be met in order to identify cyanide or azide within a biological specimen.

### 10.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of a calibrator or extracted Positive Control of cyanide.

#### 10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank injected just prior to that sample.

### 10.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

### 10.3 Batch Acceptance

No analytes of interest should be detected in a Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected. High and Low Positive Controls should fall within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for further guidance.

## 11 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

### 13.1 Cyanide Analysis

- a. Linearity: 1 – 30 µg/mL with 1/x weighting
- b. Limit of Detection: GC/NPD: 0.65 µg/mL (water)  
 0.50 µg/mL (blood)  
 GC/MS: 1.0 µg/mL
- c. Lower Limit of Quantitation: 1.0 µg/mL in both blood and water
- d. Accuracy:

	3 µg/mL	8 µg/mL	24 µg/mL
% Bias (water)	+4.91%	-1.43%	+0.04%
%Bias (blood)	-2.53%	-8.24%	+1.83%

e. Precision:

	3 μg/mL	8 μg/mL	24 μg/mL
Repeatability (water)	2.35%	1.82%	4.53%
Repeatability (blood)	7.11%	4.91%	9.10%
Intermediate Precision (water)	6.51%	9.03%	4.53%
Intermediate Precision (blood)	9.96%	6.73%	9.10%

### 13.2 Azide Analysis

a. Limit of Detection: GC/MS: 2 μg/mL in blood and aqueous matrices

### 13.3 Interferences

Azide will interfere with the NPD quantitation of cyanide, but is chromatographically resolved from cyanide on the GC/MS, and so will not cause a false positive result. However, positive cyanide results must be confirmed by GC/MS analysis. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.

### 13.4 Reporting of Results

As this procedure generates hydrogen cyanide and hydrozoic acid which are then detected by GC/NPD or GC/MS, reported positive results will include language stating the limitations of this technique. Suggested language includes, but is not limited to:

*The identification of hydrogen cyanide in the Item 1 blood sample is indicative of exposure to a cyanide containing compound. Various cyanide compounds will produce hydrogen cyanide under acidic conditions, including sodium cyanide and potassium cyanide.*

*The identification of hydrozoic acid in the Item 1 blood sample is indicative of exposure to an azide containing compound. Various azide compounds will produce hydrozoic acid under acidic conditions, including sodium azide and lead azide.*

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Acid liberates hydrogen cyanide gas and care must be taken to isolate acid solutions from cyanide sources.

Similarly, acid also liberates the highly toxic hydrozoic acid, which then constitutes an inhalational hazard. Additionally, sodium azide in its powder form constitutes an explosion hazard. Handling of the powder is performed with plastic spatulas and plastic weigh boats. All

manipulations of the solid material must take place within a functioning fume hood. For storage of the powder and the solutions, keep azide materials segregated away from acids and metals. For disposal, do not discard sodium azide down the sink or mix with acidic waste. Collect all sodium azide waste as a separate hazardous waste stream. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 Precautionary Statement

Postmortem blood specimens may contain endogenous cyanogenic sources that render uncertain the toxicological significance of amounts less than 0.2 µg/mL. Also, it is not uncommon to find trace amounts of cyanide in the blood of smokers.

## 16 References

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*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit –

Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit - Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
2	09/19/12	Updated Sections 5 and 10.1 to reflect new chromatography column for NPD analysis. Added 1.0 cc syringes to Sections 5 and 9.2. Updated standard and control preparation in Section 6. Removed option to quantitate by GC/MS in Sections 7 and 9.2. Updated NPD curve in Section 9.2 and limitations in Section 14 based on new quantitative validation. Removed 20-mL vial option in Section 9.2. Updated Decision Criteria for chromatographic analysis in 11.1. Added a note about azide as a possible interference on the NPD in Section 14. Added Appendix 2 (instrumental parameters.)
3	02/09/18	Updated Scope language. Removed “reasonable degree of scientific certainty” language from Section 11.2. Updated approval lines. Updated Title, Introduction, and Scope to include azide description. Removed “necessitate” phrase from Introduction with regards to cyanide analysis. Removed all references to CYANTESMO, Merckoquant test kits, and Chloramine T derivatization in multiple sections. Updated Principle (3) section to better describe workflow. Updated Specimens (4) to remove references to the test strips and derivatization procedure. Removed materials and equipment related to deleted procedures in Section 5. Updated Section 6c to describe azide negative controls. *Updated Section 15 (Safety) to include cautionary statements about azide handling and disposal. Added 0.4% sodium hydroxide preparation to Section 5 (p) and renumbered. Added azide stock preparations in 6, c-d, and renumbered. Added azide blood controls to Section 6, p-q. Removed Calibration (formerly Section 7), and combined with Section 6, adding some information for azide analysis. Deleted former section 8.1 (Commercial test kits). Section 9: removed references to discontinued procedures and added azide procedure. Updated 9.1.1 to clarify method parameters. Removed footers. Section 12: renamed to Measurement Uncertainty. Broke Section 13 out into 13.1 Cyanide and 13.2 Azide sections, changed “ppm” to “µg/mL” for consistency, added statement requiring GC/MS analysis for confirmation of cyanide. Added 13.4 reporting language statements under Limitations section. Section 5: removed reference to TOX103, and updated items n and o. Also removed TOX103 reference in Section 16. Added 10.3: Batch Acceptance Criteria. Matched language in 8.1 and 8.2 for solid/bulky samples. Updated 10 to include azide.

**Approval**

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Toxicology  
Technical Lead:

Date: 02/08/2018

Chemistry Unit Chief:

Date: 02/08/2018

**QA Approval**

Quality Manager:

Date: 02/08/2018



**Appendix 1: Abbreviated version of the Cyanide Procedure for bench use.**

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## **Reinsch Analysis for Arsenic, Antimony, Bismuth, and Mercury in Foodstuffs and/or Stomach Contents**

### **1 Introduction**

While the human intake of sub-milligram amounts of metals such as arsenic, mercury, antimony and bismuth are common to a normal diet, suicidal as well as homicidal fatalities have occurred from the misuse of their toxic salts. The use of arsenic as a chemical murder weapon has historical origins in the odorless and nearly tasteless qualities of its trioxide salt. Human fatality from the ingestion of 'white arsenic' (arsenic trioxide) can occur from as little as 200-300 milligrams while the average lethal dose for an inorganic mercury salt is about one gram.

The following procedure describes the presumptive testing of common foodstuffs, as well as the viscous and liquid stomach contents arising from the ingestion of these foodstuffs, for the presence of these metals. It employs a well established analytical test, commonly known as the Reinsch test, which is simple and quick to perform.

### **2 Scope**

This procedure allows for the screening of several common heavy metals in foodstuffs and/or stomach contents. Its primary purpose is as a rapid exclusionary test for the presence of these toxic elements and/or their common salts. Positive findings are further analyzed by selective element analytical techniques.

### **3 Principle**

The analysis is based on the fact that metallic arsenic, antimony, bismuth and mercury will deposit on a copper foil placed within a sample matrix that is acidified and heated. This deposition is visually recognized as a black or silvery staining of the copper foil.

### **4 Specimens**

Sample matrices can be comprised of common foodstuffs (e.g., cakes, candy, and beverages) and/or liquid and viscous stomach contents. Typically, 5 mL or 5 grams of sample is used.

## 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Homogenizer and/or mortar and pestle
- b. 16 x 125 mm screw-top test tubes
- c. 5-mL Serological pipets
- d. Copper foil (~0.25 mm thick)
- e. 25-mL Graduated cylinder
- f. 33% (v/v) Nitric acid:  
Prepare fresh by mixing one part nitric acid (Reagent Grade) to two parts water
- g. Concentrated Hydrochloric acid (HCl) (Reagent Grade)
- h. 1 N Hydrochloric acid (Reagent Grade)
- i. Ethanol (Reagent Grade)
- j. Steam bath or equivalent
- k. Arsenic trioxide (SPEX Element Kit)
- l. Magnetic stirrer
- m. Ruler
- n. Aluminum foil
- o. Antimony trioxide (Reagent Grade, or better)
- p. Bismuth nitrate pentahydrate (Reagent Grade, or better)
- q. Mercuric chloride (Reagent Grade, or better)
- r. Glacial acetic acid (Reagent Grade, or better)
- s. Clear plastic tape

## 6 Standards and Controls

- a. **Arsenic Standard Solution (0.1 mg/mL):**  
A 0.1 mg/mL standard solution of arsenic is prepared by adding 13.2 mg of arsenic trioxide to a 100-mL volumetric flask. This is diluted with 1 N HCl to the 100 mL volume mark. Dissolution takes place with magnetic stirring (1-2 hours). Store at room temperature in glass. Stable as a qualitative standard for at least five years.
- b. **Antimony Standard Solution (0.1 mg/mL):**  
A 0.1 mg/mL standard solution of antimony is prepared by adding 12.0 mg of antimony trioxide to a 100-mL volumetric flask and dissolving in a few mLs of concentrated HCl. Following dissolution, the solution is brought to the mark with deionized water. Store at room temperature in glass. Stable as a qualitative standard for at least five years.
- c. **Bismuth Standard Solution (0.1 mg/mL):**  
A 0.1 mg/mL standard solution of bismuth is prepared by adding 23.2 mg of bismuth nitrate pentahydrate to a 100-mL volumetric flask and dissolving in a few mLs of glacial acetic acid. Following dissolution, the solution is brought to the mark with deionized water. Store at room temperature in glass. Stable as a qualitative standard for at least five years.
- d. **Mercury Standard Solution (0.1 mg/mL):**  
A 0.1 mg/mL standard solution of mercury is prepared by adding 13.6 mg of mercuric chloride to a 100-mL volumetric flask and dissolving in 5-10 mLs of concentrated HCl. Following dissolution, the solution is brought to the mark with deionized water. Store at room temperature in glass. Stable as a qualitative standard for at least five years.
- e. **Negative Control:**  
A deionized water blank or a matrix similar to the submitted specimen (if known and available) is used as the Negative Control. A Negative Control sample is analyzed every time the Reinsch test is performed.
- f. **Positive Control:**  
A portion (5 mL) of the arsenic standard solution (or any other heavy metal standard solution listed above) is used as the Positive Control. Alternatively, if the test sample amount permits, the Positive Control can be generated by mixing 5 mL of the arsenic standard solution (or other heavy metal standard solution) with 5 grams of the specimen. A Positive Control is analyzed every time the Reinsch test is performed.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Addendum 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Preparation of Specimen Material and Copper Foil Strip

- a. Thoroughly homogenize the submitted specimen to ensure a representative aliquot is sampled. This may be accomplished by grinding in a mortar and pestle (candy), grinding by hand (cakes, cookies), blending in a homogenizer (bulky stomach contents) or vigorously shaking the specimen (gastric lavages, beverages).
- b. Cut a 5 x 10 mm rectangular strip of copper foil. The copper foil strip should not be touched with bare hands.
- c. Immerse foil strip in a test tube of 33% nitric acid.
- d. Observe the effervescent copper foil strip for about 30 seconds and terminate the chemical reaction by discarding the acid solution and rinsing the foil strip three times with deionized water. Lastly, rinse the foil strip once with ethanol followed by a final deionized water rinse. The clean foil strip should have a shiny appearance.

### 9.2 Analysis

- a. Place a clean copper foil strip into a 16 x 125 mm screw cap test tube. Alternatively, for highly absorbant samples or samples that are difficult to homogenize, the copper foil strip can be placed into a 50-mL Erlenmeyer flask, or similar sized glass vessel.
- b. Add 5 grams or 5 mL of uniform specimen to the flask followed by 5 mL of deionized water. A Negative and Positive Control are similarly processed.

Note (1): If a specimen was initially homogenized with an equal volume of deionized water, use 10 grams of homogenate as the sample with no further addition of deionized water.

Note (2): A Positive Control that is generated in the specimen matrix also uses 10 grams of sample (5 grams specimen/5 mLs arsenic or other heavy metal standard solution) with no further addition of deionized water.



- c. Add 2 mL of concentrated hydrochloric acid.
- d. Cap the tube loosely with a screw cap or cap the flask with aluminum foil and swirl the contents to ensure a uniform distribution of the acid.
- e. Partially submerge the Erlenmeyer flask in a steam bath or equivalent boiling water bath for 45 minutes. Stir the contents by occasional swirling.
- f. After the elapsed time, remove the foil and rinse with deionized water. Visually inspect the foil for a deposited black, gray or silvery coating. Photographs may be used to document negative or positive results.

Note: Clear plastic tape may be used to secure the copper foil to a clean sheet of paper before photographing. This is highly recommended when bismuth or mercury are detected as they may be rapidly lost from the foil via sublimation.

## 10 Instrumental Conditions

Not applicable.

## 11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, all of the below should be met in order to consider this test a presumptive positive for metals of interest.

- a. The test is considered negative if the copper foil is not coated with a black, gray, or silvery coating.
- b. A deionized water Negative Control should yield no black, gray, or silvery deposit on the copper foil strip (see Figure 1).
- c. Arsenic trioxide used as the Positive Control will stain the copper foil black. Antimony yields a bluish-black stain. Bismuth yields a grey-black stain. Mercury coats the foil with a silvery deposit.

These stains are fixed and should not rinse off with deionized water, but mercury and bismuth may sublime and disappear from the copper foil over a matter of minutes (see Figure 1).

## 12 Calculations

Not applicable.

## 13 Uncertainty of Measurement

Not applicable.

## 14 Limitations

- a. Limits of Detection: Antimony = 1  $\mu\text{g/mL}$   
 Arsenic = 5  $\mu\text{g/mL}$   
 Bismuth and Mercury = 10  $\mu\text{g/mL}$

The primary value of this test is exclusionary and false negatives are unlikely. However, if a false negative is suspected and specimen amount permits, a Positive Control should be generated in the specimen matrix followed by a reassay of the sample to ensure staining of the copper foil.

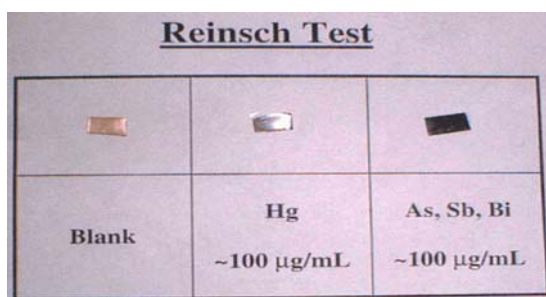


Figure 1: Examples of stains on copper foil

- b. Interferences: Food samples and gastric content specimens that are decomposing may affect detection limits.

## 15 Precautionary Statement

The deposit of a fixed (water insoluble) black or silvery stain on the copper foil only indicates and does not identify the presence of arsenic, antimony, bismuth or mercury. Tarnishing of the copper foil may occur from copious amounts of sulfur in decomposed biological material and/or the presence of other metals such as selenium or tellurium. If the Reinsch test is positive, a specific elemental analysis technique such as SEM/EDS of the deposited metal can be used to determine which metal is present. ICP/MS can be used to target the individual metal for qualitative as well as quantitative data.

## 16 Safety

Take standard precautions for the handling of chemicals and biological materials. Heating of specimens should take place in a hood or vented system due to the volatility of mercury. Metals are cumulative in the body and care should be taken to avoid skin contact with standard solutions. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 17 References

Alan S. Curry; *Poison Detection in Human Organs*; 4th edition; Charles C. Thomas; Springfield, Illinois; 1988, pp 108.

*Clarke's Isolation and Identification of Drugs*; Second Edition. The Pharmaceutical Press. London, 1986. Metals; pp 56-62.

Gettler, A.O. and Kaye, S., "A Simple and Rapid Analytical Method for Hg, Bi, Sb and As in Biologic Material", *Journal of Laboratory and Clinical Medicine* vol 35, no 1: 146-151 (1950).

*Methodology for Analytical Toxicology*. Heavy Metals; CRC Press; Cleveland, Ohio; 1975, pp 395-398.

Sidney Kaye; *Handbook of Emergency Toxicology*. Heavy Metals.; Charles C. Thomas; Springfield, Illinois; 5th edition; 1988, pp 58-64.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
0	06/21/06	New document that replaces a previous document also titled <i>"Reinsch Analysis for Arsenic, Antimony, Bismuth, and Mercury in Foodstuffs and/or Stomach Contents"</i> .
1	01/27/10	Updated thickness of copper foil in Section 5. Updated the stability of the arsenic trioxide standard solution in Section 6. Suggested SEM/EDS and ICP/MS for confirmation in Section 15. Added a reference in Section 17. Corrected a misspelling on the bench sheet.
2	09/19/12	Reduced sample size and updated Sections 4, 6f, and 9.2b and c. Allowed for use of 16 x 125 mm screw cap tubes and updated Sections 5b and 9.2a. Updated copper foil thickness in Section 5d. Updated preparation of Nitric Acid in Section 5f and noted new concentration in Section 9.1c. Added optional alternate positive controls in Sections 5o, p, q and r and 6a, b, c, d and f. Clarified number of rinses of foil required in Section 9.1d. Updated 9.2d to allow for use of test tube cap. Reduced water bath time in Section 9e. Noted in Section 9f that tape may be used to secure copper for photography and prevent sublimation of bismuth and mercury. Clarified colors viewed with various metals in Section 11. Updated LODs in Section 14. Added additional safety recommendation in Section 16. Updated bench sheet to coincide with updated procedure.

**Approval**

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**Appendix 1: Abbreviated version of the Reinsch Procedure for bench use.**

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## **Pesticide Analysis in Foodstuffs, in Stomach Contents, and/or Liver Tissue**

### **1 Introduction**

This procedure is used to identify pesticides that are insecticides or herbicides.

### **2 Scope**

This procedure allows for the screening and confirmation of common pesticides in a variety of foodstuffs, in stomach contents, or in liver tissue. This document applies to Chemistry Unit case working personnel who perform toxicology analyses and general chemistry analyses.

### **3 Principle**

Most of the pesticides analyzed by this procedure will be classified as an insecticide or herbicide. The presence of chlorine or phosphorus in many of the chemicals within these classes permits the use of a gas chromatograph equipped with selective detectors (electron capture and nitrogen phosphorus). To be detected by this procedure, the pesticide must have some degree of solubility in n-hexane (foodstuffs and stomach contents extraction). The identification of a pesticide is achieved by use of the orthogonal technique of gas chromatography with mass spectrometry operated in the full scan electron ionization mode.

### **4 Specimens**

Sample matrices typically comprise common foodstuffs, stomach contents, or liver tissue. Typically, 2.5 grams or 2.5 mL of sample are used for this analysis.

### **5 Equipment/Materials/Reagents**

- a. Gas Chromatograph capable of dual simultaneous injection and equipped with a 30-meter Rtx-CLPesticides column or equivalent connected to an electron capture detector (ECD) and a 30-meter Rtx-1701 column or equivalent connected to a nitrogen-phosphorous detector (NPD)
- b. Gas Chromatograph/Mass Spectrometer equipped with a 30-meter DB-5 capillary column or equivalent
- c. 5 N Sulfuric Acid (H<sub>2</sub>SO<sub>4</sub>)

- d. n-Hexane 95% (HPLC, spectrophotometry, or gas chromatography grade or better)
- e. Toluene (HPLC grade or better)
- f. Deionized Water
- g. Supported Liquid Extraction (SLE) cartridges (Biotage Isolute SLE+2; 2 mL sorbent mass; part number 820-0290-D)
- h. Dichloromethane (Optima grade or better)
- i. Acetone (HPLC grade or better)
- g. Routine laboratory supplies, including homogenizer and/or mortar and pestle, test tubes (16 x 125 mm screw-top, 16x100 mm culture, 12 x 75 mm culture), centrifuge, rotator, vortex mixer disposable glass pipets, pH paper, spatulas, scalpels, test tube racks, graduated cylinders, vacuum extraction box, heated evaporator, etc.

## 6 Standards and Controls

- a. Organochlorine (OC) Pesticides Stock Solution<sup>1</sup>:  
A hexane:toluene (1:1) solution approximately 1 mg/mL each of aldrin, 4,4'-DDT, endrin, endrin aldehyde, and lindane (gamma BHC). Purchased as a special order item from Chemservice, Inc. Store refrigerated in glass. Stable for at least two years, or as determined by manufacturer.
- b. Electron Capture Detector (ECD) Pesticides Testmix Solution<sup>2</sup>:  
Dilute 25 µL of the OC pesticides stock solution to 50 mL with hexane, yielding a solution approximately 0.5 µg/mL in each component. Store refrigerated in glass. Stable for at least two years. A portion of this testmix is analyzed prior to each batch of Gas Chromatography – Electron Capture Detection (GC-ECD) analyses.
- c. Organophosphate (OP) Pesticides Stock Solution<sup>1</sup>:  
A hexane solution approximately 1 mg/mL each in chlorpyrifos, diazinon, fenchlorphos, parathion (ethyl), and prophos. Purchased as a special order item from Chemservice, Inc. Store refrigerated in glass. Stable for at least two years, or as determined by manufacturer.

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<sup>1</sup> Standards can be individually purchased from Chemservice, Inc. or an equivalent supplier and prepared to an appropriate concentration in house.

<sup>2</sup> 100 µg/mL stock standards can be individually purchased from Chemservice, Inc. or an equivalent supplier and diluted to an appropriate concentration in house.

- d. Nitrogen Phosphorus Detector (NPD) Pesticides Testmix Solution<sup>2</sup>:  
Dilute 500 µL of the OP pesticides stock solution to 25 mL in hexane, yielding a solution approximately 20 µg/mL in each component. Store refrigerated in glass. Stable for at least two years. A portion of this testmix is analyzed prior to each batch of Gas Chromatography – Nitrogen Phosphorus Detection (GC-NPD) analyses.
- e. Carbamate Pesticides Stock Solution<sup>1,3</sup>:  
An acetonitrile solution approximately 1 mg/mL each in carbaryl, carbofuran, and propoxur. Purchased as a special order item from Chemservice, Inc. Store refrigerated in glass. Stable for at least six months, or as determined by manufacturer.
- f. Hexachlorobenzene (98% or better purity):  
Obtained as a solid from Sigma-Aldrich or an equivalent supplier. Storage and stability determined by manufacturer.
- g. Triphenylphosphate (98% or better purity):  
Obtained as a solid from Sigma-Aldrich or an equivalent supplier. Storage and stability determined by manufacturer.
- h. 4-Bromo-3,5-dimethylphenyl-N-methylcarbamate (BDMC) (98% or better purity):  
Obtained as a solid from Sigma-Aldrich or an equivalent supplier. Storage and stability determined by manufacturer.
- i. Internal Standards Working Solution:  
Weigh approximately 25 mg each of hexachlorobenzene, triphenylphosphate, and BDMC into a 25-mL volumetric flask and fill to the mark with toluene. Mix well and store refrigerated in glass. Stable for at least one year.
- j. Pesticides GC-MS Testmix Solution:  
Dilute 100 µL of the internal standards working solution to 10 mL in hexane, yielding a solution approximately 10 µg/mL in each component. Store refrigerated in glass. Stable for at least one year.
- k. OC Pesticides Mix AB#1:  
Obtained from Restek Corporation (Catalog #32291). This mixture contains twenty common organochlorine pesticides (aldrin, alpha-BHC, beta-BHC, delta-BHC, gamma-BHC, cis-chlordane, trans-chlordane, 4,4'-DDD, 4,4'-DDE, 4,4'-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide (isomer B), and methoxychlor. Store refrigerated in glass. Stability determined by manufacturer.
- l. OC Pesticides Retention Time Calibration Mix:  
Dilute 20 µL of the OC Pesticide Mix AB#1 to 10 mL in hexane. Store refrigerated in

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<sup>3</sup> Dilute stock solution prepared in house with acetone instead of acetonitrile.



glass. Stable for at least two years. A portion of this calibration mix should be analyzed by GC-ECD at least every six months or with every case batch, whichever is longer, to check retention time stability. Maintain a record of these samples with the instrument testmix records.

- m. **OP Pesticides Mix B:**  
Obtained from Restek Corporation (Catalog #32278). This mixture contains seven common organophosphate pesticides (tetraethylpyrophosphate (TEPP), sulfotepp, monocrotophos, dimethoate, malathion, parathion, and EPN). Store refrigerated in glass. Stability determined by manufacturer.
- n. **OP Pesticides Retention Time Calibration Mix:**  
Dilute 100  $\mu$ L of the OP Pesticides Mix B to 500  $\mu$ L with hexane. Store refrigerated in glass. Stable for at least two years. A portion of this calibration mix should be analyzed by GC-NPD at least every six months or with every case batch, whichever is longer, to check retention time stability. Maintain a record of these samples with the instrument testmix records.
- o. **Negative Control:**  
A deionized water blank or a matrix similar to the submitted specimen (if known and available) is used as the Negative Control. A negative control is extracted and analyzed with every assay.
- p. **Positive Control Foodstuff or Stomach Contents:**  
The suggested Positive Control is a spiked aliquot of the questioned sample. If sample volume is limited, a blank matrix similar to the submitted specimen may be used, or, if that is unobtainable, deionized water may be spiked to create a positive control. Alternatively, if a specific pesticide is being targeted it is acceptable to prepare a 10  $\mu$ g/mL solution of the targeted pesticide in a blank matrix or within deionized water. To prepare: add 25  $\mu$ L each of the OC Pesticides Stock Solution (1 mg/mL), the OP Pesticides Stock Solution (1mg/mL), and the Carbamate Pesticides Stock Solution (1mg/mL) to 2.5 mL or 2.5 g of the sample matrix. This will yield a spiked sample that is approximately 10  $\mu$ g/mL in each of the thirteen control analytes. A Positive Control is extracted and analyzed with every assay.
- q. **Positive Control Liver Tissue**  
To prepare: add 50  $\mu$ L each of the OC Pesticides Stock Solution (1 mg/mL), the OP Pesticides Stock Solution (1mg/mL), and the Carbamate Pesticides Stock Solution (1mg/mL) to 5 g of the liver homogenate (2.5 g liver tissue: 2.5 g deionized water). This will yield a spiked sample that is approximately 20  $\mu$ g/g in each of the thirteen control analytes. A Positive Control is extracted and analyzed with every assay.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 8.1 Foodstuff or Stomach Contents:

- a. Photograph the specimen if its appearance will be grossly altered by homogenizing.
- b. For liquid specimens, mix the specimen well, sample 2.5 mL into a screw-top tube, and mix this aliquot with 2.5 mL of deionized water.
- c. For solid or semi-solid specimens, homogenize a portion of the specimen 1:1 with deionized water (2.5 g specimen: 2.5 g deionized water) into a screw-top tube. Homogenization may be accomplished by grinding in a mortar and pestle (e.g., candy), grinding by hand (e.g., cakes, cookies), or blending in a homogenizer (e.g., bulky solids or stomach contents).
- d. Add 25  $\mu\text{L}$  of the internal standards working solution<sup>4</sup> and vortex for 30 seconds.
- e. Add 70  $\mu\text{L}$  of 5 N  $\text{H}_2\text{SO}_4$  and vortex for 30 seconds. Ensure the pH is less than 6 with indicator paper.
- f. Add 5 mL of n-hexane and extract by rotation for 20 minutes. Centrifuge for 15 minutes at high speed. If a severe emulsion forms, stir the emulsion with a clean wooden stick and re-centrifuge.
- g. Transfer the hexane supernatant to a 16x100 mm culture tube.
- h. Mix a 200  $\mu\text{L}$  portion of the extract with 800  $\mu\text{L}$  of hexane and analyze 1  $\mu\text{L}$  of this solution by GC-ECD.
- i. Concentrate a 1.5 mL portion of the extract to ca. 250-300  $\mu\text{L}$  under a stream of nitrogen at ca. 40°C. Analyze 1 - 2  $\mu\text{L}$  portions of this concentrate by GC-NPD and GC-MS.

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<sup>4</sup> Other internal standards may be substituted at relevant concentrations if deemed appropriate.

## 8.2 Liver Tissue

- a. Weigh a portion of liver tissue (2.5 g)<sup>5</sup>. Place specimen and an equal amount of deionized water (2.5 g specimen: 2.5 g deionized water) into a homogenizer. Blend for 2-3 minutes on high. Transfer homogenate to a 16 x 100 mm culture tube with a polypropylene snap-top.
- b. Add 50 µL of the internal standards working solution<sup>6</sup> and vortex for 30 seconds.
- c. Centrifuge homogenate at high speed for 15 minutes. Transfer supernatant to a clean 12 x 75 mm culture tube with a polypropylene snap-top. Re-centrifuge supernatant at high speed for 15 minutes (repeat above transfer and centrifuge if supernatant contains any homogenate),
- d. Load supernatant samples onto SLE cartridges by gravity. (A brief application of vacuum will be necessary to start loading.) DO NOT ELUTE.
- e. Allow to stand for 5 minutes.
- f. Apply 3 mL of Dichloromethane and allow to absorb.
- g. Allow to stand for 5 minutes. DO NOT APPLY VACUUM.
- h. Elute by gravity into 16 x 100 mm culture tubes with 2 x 4 mL Dichloromethane. Briefly apply full vacuum to complete elution.
- i. Evaporate at approximately 45°C. When eluent reaches 0.5 – 1 mL, briefly vortex before evaporating to dryness.
- j. Reconstitute with 0.1 mL Acetone. Transfer 25 µL to properly labeled autosampler vials, add 225 µL Acetone, analyze 1 µL of this solution by GC-ECD. Transfer remaining extract to properly labeled autosampler vials, analyze 1 – 2 µL of this solution by GC-MS(EI). Note: No GC/NPD analysis will be conducted on liver tissue samples.

## 9 Instrumental Conditions

Following are the suggested instrumental parameters for the instruments used in this procedure. Appendix 2 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

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<sup>5</sup> Other amounts of liver homogenate may be prepared as long as a 1:1 ratio of liver :water is maintained. If the test sample amounts permits, spike a second portion with positive control solution.

<sup>6</sup> Other internal standards may be substituted at relevant concentrations if deemed appropriate.

### 9.1 Gas Chromatograph/Nitrogen Phosphorus Detector / Electron Capture Detector Parameters (Simultaneous Dual Injection)

Inlet Parameters - ECD		Inlet Parameters - NPD		GC Oven Parameters	
inlet temperature	230°C	inlet temperature	250°C	temperature 1	125°C
injection mode	splitless	injection mode	split	hold 1	1
carrier gas	helium	carrier gas	helium	ramp 1	7°C/min
carrier mode	constant pressure	carrier mode	constant pressure	temperature 2	280°C
carrier pressure	16.85 psi	carrier pressure	13.39 psi	hold 2	22 min
splitless time	0.5 min	split ratio	15:1	Column Parameters ECD	
Detector Parameters - ECD		Detector Parameters - NPD		type	Rtx-ClPest
temperature	300°C	temperature	250°C	length	30 m
makeup gas	nitrogen	offset	10	inner diameter	0.32 mm
makeup flow	30 mL/min	makeup flow (nitrogen)	30 mL/min	film thickness	0.5 µm
		air flow	60 mL/min	Column Parameters - NPD	
		hydrogen flow	2 mL/min	type	Rtx-1701
				length	30 m
				inner diameter	0.32 mm
				film thickness	0.5 µm

### 9.2 Gas Chromatograph / Mass Spectrometer

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	60°C	inlet temperature	220°C	type	DB-5MS
hold 1	3.2 min	injection mode	split	length	30 m
ramp 1	35°C/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	280°C	carrier mode	constant flow	film thickness	0.25 µm
hold 2	31 min	carrier flow	1.2 mL/min		
total run time	40.5 min	split flow	12 mL/min		
		split ratio	10:1		
Mass Spectrometer Parameters					
ionization mode	electron impact (+)	source temperature	230°C		
scan mode	full scan	transfer line temperature	280°C		
scan range	35 500 AMU	solvent delay	5 min		

## **10 Decision Criteria**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, all of the below should be met in order to identify a pesticide within a foodstuff or gastric content sample.

### **10.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **10.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or an extracted positive control

#### **10.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank solvent injected just prior to the sample.

### **10.2 Mass Spectrometry**

The mass spectrum of the analyte of interest should compare favorably with that of a reference standard, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

## **11 Calculations**

Not applicable.

## **12 Measurement Uncertainty**

Not applicable.

### 13 Limitations

- a. **Limit of Detection:** The limit of detection varies depending on the pesticide of interest, and the matrix being analyzed. All thirteen target analytes contained in the pesticides stock solutions used in this procedure can be detected at levels of at least 10 µg/mL in a wide variety of food matrices. The limits of detection for pesticides in liver tissue are listed below in Table 1.

**Table 1; LOD Pesticides in Liver Tissue**

	GC-ECD	GC-MS
<b>Organochlorines</b>		
Lindane (g-BHC)	5 µg/g	5 µg/g
Aldrin	5 µg/g	5 µg/g
Endrin	5 µg/g	10 µg/g
Endrin Aldehyde	5 µg/g	10 µg/g
4,4'-DDT	5 µg/g	5 µg/g
<b>Carbamates</b>		
Carbaryl		5 µg/g
Carbofuran		5 µg/g
Propoxur		5 µg/g
<b>Organophosphates</b>		
Chlorpyrifos	10 µg/g	5 µg/g
Diazinon		5 µg/g
Fenchlorphos		5 µg/g
Parathion		5 µg/g
Profos		10 µg/g

- b. **Interferences:** None known. Grossly decomposed or putrefied samples may affect both detection.
- c. **Other Considerations:** Carbamate pesticides can undergo chemical breakdown to the corresponding phenolate compounds when subjected to GC injection. The extent of this breakdown depends upon sample matrix, analyte loading, and the age and condition of the GC injection port liner. For any carbamate pesticide, the presence of the phenolate breakdown product may be considered as evidence of the presence of the parent compound if the breakdown product is also observed in a contemporaneously analyzed matrix-matched positive control specimen. This procedure is not suitable for the detection of aldicarb and its metabolites.

### 14 Precautionary Statement

Care should be taken in the interpretation of pesticide levels in foodstuffs. Some pesticides have a legitimate use on food products and a qualitative identification in the absence of quantitative

data could produce confusion in interpreting results. The meaning of the toxicological significance of a negative pesticide finding should be considered in conjunction with its biodegradability. Exercise care in reporting and interpreting all pesticide results.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. It should be noted that many of the pesticides detected by this procedure may be extremely toxic and/or carcinogenic. The utmost caution should be taken in handling reference materials and case specimens containing such pesticides. Refer to the *FBI Laboratory Safety Manual* for guidance.

This procedure utilizes the following P-listed pesticides: aldrin, carbofuran, dimethoate, endosulfan, endrin & metabolites, heptachlor, parathion, and tetraethylpyrophosphate (TEPP). This SOP utilizes the following U-listed pesticides: carbaryl, DDD, DDT, hexachlorbenzene, methoxychlor, and propoxur.

## 16 References

*Clarke's Isolation and Identification of Drugs; Pesticides*. Second Edition. The Pharmaceutical Press. London, 1986. pp 70-86.

*Farm Chemicals Handbook*; Meister Publishing Company. Willoughby, Ohio. published annually.

*Mass Spectral Data Compilation of Pesticides and Industrial Chemicals*; Los Angeles District Laboratory; Mass Spectrometry Service Center; Los Angeles, California. 1987.

*Mass Spectrometry of Pesticides and Pollutants*; CRC Press. Cleveland, Ohio. 1973.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

FBI Laboratory - Instrument Operation and Support SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
3	08/05/19	Document was discontinued between 08/21/2018 – 08/04/2019. Updated Introduction language, Scope language and added General Chemistry. Updated wording Section 3, 4, 10, 11, 14. Section 5 updated reagent grades. Section 6 updated standards and controls. Section 7 Calibration – removed and renumbered Sections 7 – 16. Section 8 Procedure converted drops to microliters for sulfuric acid and increased injection volume. Section 9 updated typo in gc/ms scan range. Section 11 and 12 updated to not applicable. Section 15 Safety added P-listed and U-listed pesticides utilized in this procedure. Removed “reasonable degree of scientific certainty” language from Section 11.2. Updated approval lines. Removed references no longer needed.
4	09/11/19	Updated Title. Updated Section 2, 3 and 4 to include liver tissue. Added equipment, reagents and supplies needed to extract liver tissue in Section 5. Revised Positive Control scheme in Section 6 p and q. Modified procedure 8.1 for Foodstuff or Stomach Contents and 8.2 for Liver Tissue. Section 13 a added LOD for pesticides in liver tissue and updated 13c statement re: aldicarb.

### **Approval**

Redacted - Signatures on File

Acting Toxicology  
 Technical Leader: -

Date: 09/09/2019

General Chemistry  
 Technical Leader: -

Date: 09/09/2019

Chemistry Unit Chief: -

Date: 09/09/2019

### **QA Approval**

Quality Manager:

Date: 09/09/2019



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**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.**

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## **Carbon Monoxide Analysis in Biological Specimens**

### **1 Introduction**

Carbon monoxide (CO) is a colorless, odorless gas produced by the incomplete combustion of organic fuels. It is also a common trace pollutant in the atmosphere. CO is present in the body at low concentrations, where it is bound to hemoglobin (Hb), to form carboxyhemoglobin (COHb). Normal COHb concentrations in the body are typically under 5% in nonsmokers, and up to 9% in smokers. COHb values below 10% are usually considered normal in healthy human subjects. Serious toxicity is often associated with COHb levels above 25%, and the risk of fatality is high with levels over 70%.

When CO is inhaled, it competes with oxygen (O<sub>2</sub>) for the hemoglobin in red blood cells. The affinity of CO for hemoglobin is approximately 250 times that of O<sub>2</sub>. When COHb is formed, O<sub>2</sub> cannot be transported to the tissues that need it, putting those tissues in a state of anemic hypoxia. Overexposure to CO produces headache, tremor, nausea, weakness, confusion, stupor and coma. Carbon monoxide poisoning may occur in fire victims or as a result of inhalation of automobile exhaust, heating system/stove waste products or other combustion gases.

### **2 Scope**

This procedure quickly screens and quantitates biological specimens, typically whole blood, for elevated levels of COHb through a spectrophotometric method. Confirmation is completed by headspace gas chromatography with thermal conductivity detection.

### **3 Principle**

Blood samples are screened and quantitated by spectrophotometry using a CO-Oximeter.

Specimens that screen positive by the spectrophotometric method are confirmed by gas chromatography with thermal conductivity detection (GC/TCD). Sulfuric acid is added to liberate the CO from the hemoglobin. An automated headspace sampler then samples and injects a portion of the headspace onto the GC/TCD system. The %COHb can be estimated using the amount of Hb detected by the co-oximeter.

## 4 Specimens

The screening procedure method requires approximately 0.2 mL of whole blood. The GC/TCD confirmation procedure requires 0.33 mL of whole blood. Spleen or other blood-rich organs can be analyzed by these procedures.

## 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. AVOXimeter<sup>®</sup> 4000 Whole Blood CO-Oximeter
- b. Disposable Test Cuvettes for AVOXimeter<sup>®</sup> 4000 (Purchased from ITC, Edison, NJ)
- c. Disposable syringes (1 mL)
- d. Kimwipes and/or blood block pads
- e. Vortex mixer
- f. Saponin
- g. Concentrated sulfuric acid (Reagent Grade)
- h. Sulfuric Acid Solution (1M)
- i. 20-mL headspace vials (HSV) with appropriate crimper/decrimper, magnetic crimp caps and septa
- j. Calibrated pipettors (5 µL - 1000 µL volume capable)
- k. Gas chromatograph (Agilent 6890N or equivalent) equipped with thermal conductivity detector, analytical column (J&W HP-Molesieve 30 m x 0.32 mm x 12.00 µm or equivalent) and headspace autosampler (Gerstel MPS2 or equivalent)

## 6 Standards and Controls

- a. Formic acid (~89%, reagent grade)
- b. 0.05 M formic acid solution (GC/TCD performance check):

Dilute 215 µL of formic acid to 100 mL with deionized water in a graduated cylinder or flask. Mix well and store in glass at room temperature. Stable for at least one year.

c. AVOXimeter optical quality control filters (supplied with the instrument)

d. Blood COHb Controls:  
RNA Medical QC 253 Full Range CO-Oximeter Control, or equivalent.  
Purchased from RNA Medical, Division of Bionostics, Inc.,  
Available in three levels, as described in Table 1<sup>1</sup>:

Table 1: Three Levels of RNA Medical CO-Oximeter Controls

Level / Lot	total Hb, g/dL Average Value	total Hb, g/dL Range	COHb, % Average Value	COHb, % Range
1 / 34814	8.3	7.6-9.0	5.7	1.7-9.7
2 / 34913	13.7	12.6-14.7	16.1	11.6-20.6
3 / 35013	17.2	15.9-18.5	44.2	38.9-49.5

Since the Level 1 control contains COHb at a value less than 10%, it is considered to be a Negative Control. This Negative Control will be analyzed with each CO-Oximeter assay and with each TCD assay.

The Level 2 and Level 3 controls are considered Positive Controls, as they contain COHb at a level greater than 10%. Each Positive Control will be analyzed with each CO-Oximeter assay and with each TCD assay.

## 7 Calibration

Calibration is performed automatically in the AVOXimeter. The only variables that may be changed are the pathlength (which is supplied with each lot of cuvettes) and a value for Hüfner's number (1.39 is typically used). These values do need not to be changed unless a new lot of cuvettes is used. See the AVOXimeter Manual for guidance.

## 8 Sampling

Not applicable.

<sup>1</sup> Average and range values provided by RNA Medical package insert. Consult appropriate package insert for target values, lot numbers, stability and storage.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Screening and Quantitating %COHb by CO-Oximeter

#### 9.1.1 Daily CO-Oximeter Checks

- a. Turn the AVOXimeter on and wait for the “---READY--- Insert Cuvette” message to appear.
- b. Insert the yellow optical quality control filter.
- c. At the “Select Sample Type” screen, type “2/Enter” for QC.
- d. At the “Select QC Type” screen, type “2/Enter” for Optical.
- e. At the “Select Filter” screen, type “1/Enter” for Yellow. Hit “Enter” for OK if prompted.
- f. The results will appear within ten seconds. Press “Print” to print a copy. (Sometimes “Print” must be pressed twice.)
- g. Verify that the results are within the specifications on the sticker on the filter. Record whether the results Pass or Fail in the instrument logbook. (Target value ranges are printed on each filter.)
- h. Repeat steps b. through g. for the orange filter. (This time, type “2/Enter” in step e. for the orange filter.)

#### 9.1.2 Analyzing Samples and Controls by CO-Oximeter

- a. Verify that the “---READY--- Insert Cuvette” message appears.
- b. After mixing the blood sample via inversion, draw approximately 0.2 mL of a sample or control into a disposable syringe.
- c. Insert the syringe into the cuvette.
- d. Hold the syringe and cuvette at a 45 degree angle and gently press the plunger. Stop pressure when the sample reached the vent patch. (Do not allow vent patch to bulge.)
- e. Verify that the light path area is free of bubbles.

- f. Clean any drops of blood off of the exterior of the cuvette with a Kimwipe or blood block pad. If blood has broken through the vent patch, discard the cuvette and prepare a new one.
- g. Insert the cuvette (with syringe still attached) into the AVOXimeter.
- h. At the "Select Sample Type" screen, type "1/Enter" for Patient.
- i. The results will appear within ten seconds. Press "Print" to print a copy. (Sometimes "Print" must be pressed twice.) Label the printouts with the correct sample name or lot number. (Note: printouts may be photocopied for inclusion in case notes since printer tape is not easy to read after a few months.)
- j. After each sample is analyzed, the cuvette should be disposed of in biohazard waste.
- k. Analyze all case samples and control(s) in duplicate, using a fresh cuvette each time.

## **9.2 Confirmation of %COHb by GC/TCD**

The confirmation of elevated %COHb (defined as >10%) in a sample is analyzed using a headspace gas chromatographic method with detection by a thermal conductivity detector (GC/TCD). A liberating agent is added to affect the release of CO into the headspace. After an equilibration time, the samples are analyzed by GC/TCD.

### **9.2.1 Carbon Monoxide Testmix Analysis**

- a. To a 20 mL HSV, add 1 mL of concentrated sulfuric acid.
- b. Add 50  $\mu$ L of a 0.05 M formic acid solution.
- c. Immediately crimp-seal the HSV and vortex for 10 seconds.
- d. Incubate HSV at 100°C for 60 minutes in a laboratory heating block or a GC oven. (CO is produced quantitatively from the dehydration of formic acid in sulfuric acid.)
- e. Analyze the headspace as per the instrumental conditions provided in Section 10 of this procedure.
- f. Verify that the Decision Criteria for the Testmix defined in Section 11.1 of this procedure are met before continuing.



## 9.2.2 Analysis of Controls and Case Samples

- For each control and case sample to be analyzed, label a clean 20 mL HSV with the sample name.
- Using an adjustable pipettor, aliquot 0.33 mL portions of the appropriate blood sample or control into each HSV.
- Add 0.33 mL liberating agent (1 M sulfuric acid) to HSV, immediately sealing each vial with a crimp cap.
- Uniformly vortex each HSV for 30 seconds using a moderate setting. Avoid excessive splashing of sample onto crimp cap.
- Analyze each HSV using the GC/TCD using the instrumental parameters in Section 10 of this procedure.

## 10 Instrumental Conditions

### Confirmation of %COHb by GC/TCD

#### 10.1 Gerstel MPS2 Headspace Sampler Parameters

syringe size:	1.0 mL (HS)	shake time (on/off):	30 / 2 s
syringe temperature:	70°C	injection volume:	900 µL
flush time:	2 min	injection speed:	900 µL/s
incubation temperature:	50°C	number of fill strokes:	5
incubation time:	20 min	GC cycle time:	5.3 min
shake speed:	250 rpm	PrepAhead:	enabled

#### 10.2 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature	40°C	type	HP-Molesieve	inlet temp.	250°C
isothermal		length	30 m	injection mode	split
run time	5 min	internal diameter	0.32 mm	carrier gas	helium
equilibration time	0.2 min	film thickness	12 µm	carrier mode	constant flow

Thermal Conductivity Detector Parameters				carrier flow	5.0 mL/min
temperature	205°C	makeup gas	helium	split ratio	3:1
reference flow	20 mL/min	makeup flow	2.5 mL/min		

## 11 Decision Criteria

### 11.1 CO-Oximeter

#### 11.1.1 Daily Checks

Results from both optical filters should be within the manufacturer's specification ranges. If they are not, contact the instrument manufacturer for assistance.

#### 11.1.2 Blood Controls by CO-Oximeter

The Negative Controls and the Positive Controls should all give COHb values within the manufacturer's specifications.

#### 11.1.3 Unknown Samples by CO-Oximeter

Samples are considered "Negative" if the %COHb level is below 10%. Such samples will be reported as "None detected above a reporting limit of 10% COHb".

Samples are considered "Positive" if the %COHb level is above 10%. For these samples, the duplicate runs must be within 10% of each other. The average value for this test will be reported if the TCD confirmation results are also positive.

### 11.2 GC-TCD

#### 11.2.1 Testmix Decision Criteria

The CO peak should be well separated from the nitrogen and oxygen peaks (>0.5 min baseline separation), and have a peak area greater than 200 units.

The GC column used in this procedure is a molecular sieve column, which may retain water. The column may be reconditioned by heating the GC oven to 225°C for >4 hours or overnight. Insufficient column conditioning results in poor chromatographic separation between the CO and air peaks.

### 11.2.2 Blood Controls by TCD

A detectable CO peak will be obtained from each Positive Control as well as from the Negative Control. Using the calculations described in Section 12, the Level 1 and Level 3 controls should calculate to within 5% (absolute) of the target value for Level 1 and within 20% (relative) of the target value for Level 3.

### 11.2.3 Unknown Samples by TCD

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the retention time of the peak should be within  $\pm 2\%$  of the retention time obtained from injection of the testmix or positive control.

Using the calculations described in Section 12, the unknown sample results should agree within 20% (relative) of the average value obtained from the CO-Oximeter.

## 12 Calculations

%COHb is estimated from the TCD CO peak and the Hb amount measured by the CO-Oximeter as described in the example below:

1. Assume that the Level 2 Control Target Value (from the insert) is accurate. (For the example below, this value is 18.6%.)
2. Assume that the Hb average amount for each sample calculated by the AVOXimeter is correct.
3. Normalize the CO response for the unknown and the Level 2 Control.

$$\begin{aligned}
 \text{a.} \quad &= \frac{\text{Unknown sample CO response (TCD area counts)}}{\text{Unknown sample tHb (from AVOXimeter)}} \\
 &= \frac{2036623}{16.6} \\
 &= 122688 \\
 \text{b.} \quad &= \frac{\text{Level 2 CO response (TCD area counts)}}{\text{Level 2 tHb (from AVOXimeter)}}
 \end{aligned}$$

$$= \frac{654049}{13.6}$$

$$= 48091$$

4. Use the normalized response for the unknown, as the normalized response for the Level 2 Control, and the target value for the Level 2 control to solve for the %COHb in the unknown.

$$\frac{122688}{x} = \frac{48091}{18.6}$$

$$x = 47.45\% \text{ COHb}$$

### 13 Uncertainty of Measurement

Two control levels (purchased from RNA Medical) were analyzed in triplicate over five days during the validation period. The “true” value of the controls was taken from the package insert. Our testing showed an average 2.7% positive bias for the two levels, and gave an overall standard deviation value of 1.3%. Since there is no certified reference material that we can base our calibration on, we must account for both the bias and the precision of these measurements to estimate our uncertainty. Therefore, these values will be summed to achieve a value of 4%. This value will be used as the historical uncertainty for the method, rather than the 1.3% value. Any positive case specimens will therefore be reported with a +/-12% uncertainty (relative) at a 99.7% confidence level. (For example, COHb was identified at a concentration of 50% ± 6% COHb, 99.7% CL, k=3.396).

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

### 14 Limitations

- a. Bias of %COHb measurement (AVOXimeter; based on 15 measurements at each level on 5 days):
- +4.17% (Low)
  - +2.90% (Medium)
  - +2.57% (Low)

- b. Repeatability of %COHb measurement (AVOXimeter; based on 15 measurements at each level on 5 days):
  - 5.42% (Low)
  - 1.26% (Medium)
  - 0.96% (High)
- c. Intermediate Precision of %COHb measurement (AVOXimeter; based on 15 measurements at each level on 5 days):
  - 8.62% (Low)
  - 1.33% (Medium)
  - 1.16% (High)
- d. Reportable Range for %COHb (AVOXimeter): 10 - 75%
- e. Other considerations:
  - 1. Samples to be analyzed should be rich in red blood cells.
  - 2. Serum-separated or "spun-down" blood samples are not appropriate for CO analysis.
  - 3. Samples that do not give acceptable results by the AVOXimeter may be considered unsuitable for %COHb measurement.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

AVOXimeter<sup>®</sup> 4000 Whole Blood CO-Oximeter Operator's Manual.

Lewis, R.; Johnson, R.; Canfield, D. *J Anal Tox*, 2004, 28, 59-62.

Agilent Technologies, Publication A15836. "6890 Checkout Procedure TCD (Thermal Conductivity Detector)."

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Widdop, B. *Ann Clin Biochem* 2002, 39, 378-391.

Lee, C.W. et al. *For Sci Inter*, 2003, 23, 153-156.

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*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Instrument Subunit SOP Manual; FBI Laboratory Chemistry Unit.

Rev. #	Issue Date	History
0	06/21/06	New document that replaces a previous document also titled " <i>Carbon Monoxide Analysis in Whole Blood Samples</i> ".
1	10/15/07	Updated title and section 2 to include other types of biological samples. Section 1 updated to reflect current literature. Updated procedure for Gerstel autosampler including instrumental parameters. Sections 6 and 7 revised to eliminate the use of calibrators. In section 9.1, increased amount of sodium dithionite and clarified its use. In section 9.2.2, added suggestion to analyze controls and samples in duplicate. Deleted 9.2.2p and re-lettered section. Updated sections 11, 13 and 14 based on historical use of this procedure.
2	03/05/10	Updated section 5 to allow for the use of disposable UV cuvettes and updated UV-Vis instrument model. Updated section 6 to include prepared in-house GC-TCD controls and description of UV-Vis versus GC-TCD controls. Updated section 9 to include use of disposable UV cuvettes. Updated UV-Vis software, removed Microsoft Excel reference in Section 10. Updated criteria for evaluation of UV-Vis and GC-TCD controls in section 11. Updated the calculations to include directions for automatic calculations using the instrument software in section 12. In section 14 updated limitations to differentiate between UV-Vis and GC-TCD parameters.
3	04/11/13	Replaced spectrophotometry screen with CO-Oximeter analysis and updated all affected sections. Changed TCD analysis from quantitative to qualitative and updated all affected sections. In 9.2.1.d, added option to heat in a GC oven.

**Approval**

  
 Redacted - Signatures on File

**Appendix 1: Abbreviated version of the CO Procedure for bench use.**

Redacted - Form on File



# Analysis of Blood Specimens for Anticoagulant Rodenticides by LC-FTMSMS

## 1 Introduction

A series of compounds structurally related to 4-hydroxycoumarin have been used for many years as rodenticides. All of these compounds work by inhibiting the vitamin K epoxide reductase enzyme, leading to a decrease in circulating levels of vitamin K. This, in turn, causes a drop in blood levels of clotting factor VII and prothrombin, both of which are necessary for formation of blood clots. These compounds also tend to increase the permeability of the walls of blood vessels, leading to uncontrolled internal bleeding upon receipt of toxic doses of any of these compounds. In mid-2011 the U.S. Environmental Protection Agency moved to ban the use of the "second generation" anticoagulant rodenticides, including bromadiolone, brodifacoum, and difenacoum, in consumer products intended for residential application, although they remain in use for products intended for outdoor and commercial use. The "second generation" compounds have a higher toxicity, both acute and chronic, than older compounds such as warfarin, coumachlor, and coumatetralyl. Warfarin is also used, under the trade name Coumadin, as a therapeutic anticoagulant for prevention of thrombosis and embolisms.

## 2 Scope

This procedure qualitatively identifies six anticoagulant rodenticides in whole blood. It is derived from "The Determination of the Anticoagulant Rodenticide Brodifacoum in Blood Serum by Liquid Chromatography with Fluorescence Detection" and "Acute Bromadiolone Intoxication" published in the Journal of Analytical Toxicology in 1989 and 2006, respectively. The extraction procedure has been adapted from the *Analysis of Blood and Urine Specimens for THC and 11 COOH THC* standard operating procedure (Tox 405). Protein precipitation with acetonitrile is coupled with a solid phase cleanup and subsequent analysis of the isolate by LC-FTMSMS.

## 3 Principle

Blood and specimens may be screened and/or confirmed for brodifacoum, bromadiolone, coumachlor, coumatetralyl, difenacoum, and warfarin by this method. Blood samples are protein precipitated with acetonitrile followed by solid-phase extraction with Bond Elute Certify II cartridges. The resulting extract is taken to dryness and analyzed by LC-FTMSMS in the positive electrospray ionization mode.

#### 4 Specimens

This procedure uses two 1 mL portions of blood. It can also be adapted for analysis of rat bait pellets and other commercial products. When analyzing commercial samples, a 20-fold dilution or 20x homogenate in deionized water (DI) will typically be used.

#### 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Screw-top test tubes with caps
- b. Culture tubes with caps
- c. Volumetric flasks (10 mL and 100 mL)
- d. Quantitative pipetters covering the volume range 0.02-1.0 mL, with disposable tips
- e. Qualitative pipetter with disposable tips and/or glass serological pipets (0.5-5.0 mL range)
- f. Vortex mixer
- g. Centrifuge
- h. Evaporator with nitrogen
- i. SPE manifold (vacuum or positive pressure)
- j. Bond Elut Certify II solid-phase extraction cartridges
- k. Liquid chromatography-high resolution (30000 FWHM) mass spectrometry system equipped with a 15 cm x 2.1 mm x 5  $\mu$ m  $d_p$  Grace Altima C18 (or equivalent) column
- l. Routine laboratory supplies, including: Pasteur pipets, pH paper, graduated cylinders, etc
- m. Acetonitrile (HPLC grade)
- n. Toluene (HPLC grade)
- o. Methanol (HPLC grade)

- p. Deionized water (DI)
- q. Sodium Acetate Buffer (0.1 M, pH 7)
- r. Sodium Acetate Buffer (0.1 M, pH 7) with 5% Methanol
- s. Rodenticides Wash Solvent (95:5 hexane:ethyl acetate)
- t. Rodenticides Elution Solvent (75:25:1 hexane:ethyl acetate:acetic acid)
- u. Methanol:water (1:1)
- v. Rodenticides LC Mobile Phase #1 (0.06% acetic acid in water). Stable for a maximum of 2 weeks; do not extend expiration date.
- w. Rodenticides LC Mobile Phase #2 (0.06% acetic acid in methanol). Stable for a maximum of 2 weeks; do not extend expiration date.

## 6 Standards and Controls

- a. Brodifacoum:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- b. Bromadiolone:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- c. Coumachlor:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- d. Coumatetralyl:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- e. Difenacoum:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- f. Warfarin Stock Solution (1 mg/mL in methanol):  
Obtained from Cerilliant or another approved vendor. Stability and storage conditions

determined by manufacturer.

- g. Brodifacoum Stock Solution (1 mg/mL):  
Weigh 10 mg of brodifacoum into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- h. Bromadiolone Stock Solution (1 mg/mL):  
Weigh 10 mg of bromadiolone into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- i. Coumachlor Stock Solution (1 mg/mL):  
Weigh 10 mg of coumachlor into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- j. Coumatetralyl Stock Solution (1 mg/mL):  
Weigh 10 mg of coumatetralyl into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- k. Difenacoum Stock Solution (1 mg/mL):  
Weigh 10 mg of difenacoum into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- l. Rodenticides Working Solution (1 µg/mL of each component)  
Combine 100 µL each of the warfarin, brodifacoum, bromadiolone, coumachlor, coumatetralyl, and difenacoum stock solutions in a 100 mL volumetric flask, fill to the mark with methanol, and mix well. Store refrigerated in glass. Stable for at least 3 months.
- m. Rodenticide LC/MSMS Performance Mix (0.05 µg/mL):  
Mix 50 µL of the rodenticides working solution with 950 µL of the rodenticides LC mobile phase #2. Prepare fresh daily.
- n. Negative Control Blood:  
Blood is purchased from Cliniqa or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

- o. Positive Control Blood:  
Prepared at 25 ng/mL by spiking 1 mL of Negative Control Blood with 25 µL of the Rodenticides Working Solution and at 100 ng/mL by spiking 1 mL of Negative Control Blood with 100 L of the Rodenticides Working Solution. Positive Control Blood samples will be extracted and analyzed with every blood assay. Additionally, when sample volume permits, a 1 mL portion of the case specimen to be analyzed will be fortified with 25 µL of the rodenticides working solution to demonstrate recovery from that specific matrix.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Into properly-labeled test tubes, add 1 mL of control or case samples and enough DI water to bring the volume to 1.2 mL. Prepare in duplicate if specimen volume allows in order to prepare a Positive Control Blood sample as directed in Section 6 above.
- b. Add 4 mL of acetonitrile drop wise while vortexing sample. Vortex thoroughly for a minimum of 3 minutes.
- c. Centrifuge samples for 15 minutes.
- d. Transfer the supernatant to a clean test tube and evaporate sample to about 2 mL under a slow stream of nitrogen at approximately 50°C.
- e. Add 6 mL of 0.1 M sodium acetate buffer (pH 7).
- f. Prepare the Bond Elut Certify II columns by sequentially passing 2 mL methanol and 2 mL 0.1 M sodium acetate buffer (pH 7) with 5% methanol. Do not allow sorbent bed to dry.
- g. Pour the sample into the appropriately labeled column reservoir. Draw the sample through

the column at a flow rate of approximately 1 - 2 mL/minute.

- h. Rinse column with 1 mL of 0.1 M sodium acetate buffer (pH 7).
- i. Dry column under full vacuum for 1 minute.
- j. Sequentially rinse column with 2 mL of Rodenticide Wash Solvent followed by 5 mL of methanol:deionized water (1:1).
- k. Dry column under full vacuum for 1 minute.
- l. Elute rodenticide fraction: Place rack with labeled tubes in the SPE manifold and wipe tips of needles. Slowly elute with 2 mL of the Rodenticide Elution Solvent at about 1 mL/minute. Evaporate under nitrogen at approximately 50°C.
- m. Reconstitute dried extract in 100 µL of Rodenticide Mobile Phase #2.
- n. Analyze 10 µL of the LC/MS Performance Mix to determine that the LC/MS is in proper working condition.
- o. Analyze 10 µL of each extract by LC-FTMSMS.

## 10 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

### 10.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
1: Water with 0.06% Acetic Acid	flow rate	0.3 mL/min		type	C-18
	time (min)	%1	%2	length	15 cm
2: Methanol with 0.06% Acetic Acid	0.0	22	78	internal diameter	2.1 mm
	3.0	22	78	particle size	5 µm
	8.0	5	95	temperature	40°C
	20	5	95		
	21	22	78		
	28	22	78		

## 10.2 Mass Spectral Parameters

3 Segments		
Segment 1 – 0-5 minutes – 2 scan events		
Event #1	full scan m/z 240-400 profile at 30000 resolution	
Event #2	MS/MS at 7500 resolution	collision energy: 30 (rel) for 343.073 and 309.112; 40 (rel) for 293.117
	precursor from cyclic scan table: 2-5 min for m/z 293.117 and m/z 343.073; 1.3-4.3 min for m/z 309.112	
	isolation width: 3.0 AMU	scan range: software control
Segment 2 – 5-13 minutes – 2 scan events		
Event #1	full scan m/z 390-580 profile at 30000 resolution	
Event #2	MS/MS at 7500 resolution	collision energy: 30 (rel)
	precursor from cyclic scan table: 8-11.5 min for m/z 445.180; 5.5-9 min for m/z 509.075; 9-12.5 min for m/z 523.090	
	isolation width: 3.0 AMU	scan range: software control
Segment 3 – 13-28 minutes – 1 scan event		
Event #1	Full scan m/z 240-580 profile at 30000 resolution	

NOTE: The precursor ion for bromadiolone (509.08) is the protonated dehydrated pseudomolecular ion. In validation it proved impossible to produce reasonable source yield of the unfragmented pseudomolecular ion.

## 11 Decision Criteria

### 11.1 Performance Mix Suitability

Proper calibration and sensitivity of the LC/MS (ESI) are demonstrated each day samples are analyzed. The Rodenticide LC/MS Performance Mix (0.05 µg/mL) is used to verify system suitability. Retention times for each analyte should compare favorably with the last performance mix analysis and each analyte should yield correct exact masses ( $\pm 0.005$  m/z for full MS,  $\pm 0.01$  m/z for MSMS, base peak only) for the ions as shown in Table 1. Commercially available standards of bromadiolone are a mixture of orientational isomers, and it is normal for the chromatographic peak for this compound to be asymmetric and exhibit a “shoulder”.

Table 1: Exact MS and MSMS fragment masses for anticoagulant rodenticides

Compound	Full MS Mass(es)	MSMS masses (base peak in bold)
Coumatetralyl	293.117	131.085, 163.039, <b>175.039</b>
Warfarin	309.112	147.081, <b>163.039</b> , 251.071
Coumachlor	343.073, 345.070	<b>163.039</b> , 181.042, 285.032
Difenacoum	445.180	189.054, <b>257.133</b> , 291.102
Bromadiolone	509.075, 511.073	<b>251.071</b> , 277.086, 321.027
Brodifacoum	523.090, 525.088	189.054, 291.102, <b>335.043</b>

## 11.2 Analyte Suitability

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control. In most cases, all of the below should be met in order to identify a target analyte within a biological specimen:

### 11.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 11.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time obtained from injection of a reference standard or extracted Positive Control of the analyte of interest.

#### 11.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank sample injected just prior to that sample. Signal to noise will normally be evaluated based upon extracted ion profiles for the ion(s) of interest, with a  $\pm 0.01$  m/z extraction window.



### 11.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of the appropriate reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See Table 1 for a list of the ions to be evaluated for each target analyte.

### 12 Calculations

Not applicable.

### 13 Uncertainty of Measurement

Not applicable.

### 14 Limitations

a. Figures of Merit:

Compound	Warfarin	Coumachlor	Coumatetraly 1	Difenacoum	Bromadiolone	Brodifacoum
LOD (note 1)	10 ng/mL	5 ng/mL	2 ng/mL	2 ng/mL	5 ng/mL	2 ng/mL
Recovery (at 10 ng/mL)	9%	34%	23%	69%	58%	59%
Recovery (at 100 ng/mL)	25%	74%	45%	94%	78%	71%
MS Supression (at 10 ng/mL)	75%	90%	76%	73%	79%	63%
MS Supression (at 100 ng/mL)	22%	36%	17%	47%	38%	31%
MSMS Supression (at 10 ng/mL)	37%	56%	46%	52%	17%	60%
MSMS Supression (at 100 ng/mL)	12%	15%	-11%	33%	5%	25%
Stability (note 2)	-33.3%	-5.6%	-15.8%	+24.7%	+6.3%	+7.2%

Note 1: Limits of detection were determined solely from MSMS data.

Note 2: Change in normalized signal for 100 ng/mL positive controls 11 days after analysis vs. day of original analysis. Samples were refrigerated in darkness for the interim.

- b. Interferences: High levels of alprazolam may lead to false negative results for warfarin. Grossly decomposed or putrefied samples may affect limits of detection.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. See the *FBI Laboratory Safety Manual* for further guidance.

## 16 References

Felice, L and Murphy, M.J., "The Determination of the Anticoagulant Rodenticide Brodifacoum in Blood Serum by Liquid Chromatography with Fluorescence Detection", *Journal of Analytical Toxicology* 13: 229-231 (1989).

Grobosch, T, et al., "Acute Bromadiolone Intoxication", *Journal of Analytical Toxicology* 30: 281-286 (2006).

"Extraction of THC and THC Metabolite from Blood Using Certify II", Publication from Varian Sample Preparation Products, Harbor City, California.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Analysis of Blood and Urine Specimens for THC and 11 COOH THC* (Tox 405); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
0	10/27/06	New document.
1	01/19/12	Converted to high resolution MS, and added MSMS analysis, changing: title and sections 2, 3, 5, 10, and 11. Rewrote section 1 to include information about history and pharmacological action of target analytes. Added coumachlor, coumatetraly, difenacoum, and warfarin to targetanalyte list, changing: title and sections 2, 3, 6, 10, 11, and 14. Added additional validation data to comply with current Laboratory Division guidelines, changing: sections 11 and 14. Reduced sample volume used to 1 mL, changing: sections 4, 6, and 9. Updated procedural terminology to match current Chemistry Unit usage, changing: sections 2, 3, 5, 9, and 11. Revised "bench sheet" (appendix 1) for procedural revisions, and added an appendix 2 with instrument parameters checklist.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Rodenticide Procedure for bench use.**

**Redacted - Form on File**

**Appendix 2: Abbreviated version of the Rodenticide instrumental conditions for bench use.**

Redacted - Form on File

## **Analysis of Poisonous Glycols from Blood and Aqueous Samples**

### **1 Introduction**

Ethylene glycol (EG) and diethylene glycol (DEG) are two toxic glycols used in coolants and antifreezes. Triethylene glycol (TEG) is less toxic than EG and DEG, and can be used in plastics or air disinfectants. Propylene glycol (PG) or 1,2-propanediol is generally recognized as safe for use in foods, cosmetics and medicines. It can cause skin irritation and may be toxic in high doses in children.

### **2 Scope**

This procedure allows for the screening and confirmation of blood samples for EG, DEG, TEG and PG. It also allows for the analysis of aqueous samples for EG.

### **3 Principle**

For screening or confirmation of EG in blood samples, specimens are crashed out with acetonitrile, taken to dryness, and derivatized with heptafluorobutyric anhydride (HFBA) before analysis by gas chromatography with mass spectrometry (electron impact) [GC/MS(EI)]. For screening of multiple glycols in blood samples, or for a second test for confirming EG, samples are extracted in acetonitrile and converted to their trimethylsilyl derivatives for improved retention on a typical capillary column. Analysis of derivatized extracts is by GC/MS(EI) or GC/MS (chemical ionization) [GC/MS(CI)].

Aqueous samples are screened for EG via direct analysis in real time (DART) time of flight mass spectrometry (TOFMS). Positive findings will be confirmed via GC/MS.

### **4 Specimens**

This procedure is validated for multiple glycols in blood. It is also validated for EG in aqueous samples.

### **5 Equipment/Materials/Reagents**

- a. Gas Chromatograph / Mass Spectrometer (GC/MS) capable of EI and CI ionization and equipped with a 30 m x 0.25 mm x 0.25 µm film thickness DB-5 (or equivalent) column (dedicated to silyl derivatives)

- b. Gas Chromatograph / Mass Spectrometer (GC/MS) equipped with a 30 m x 0.25 mm x 0.25 µm film thickness DB-5 (or equivalent) column
- c. Vortex mixer
- d. Centrifuge
- e. Evaporator with nitrogen
- f. Heating block
- g. Adjustable volume pipettes (0.025 mL to 1 mL) with appropriate tips
- h. Routine laboratory supplies, including 12 x 75 mm test tubes, autosampler vials with crimp caps, disposable glass pipettes, test tube racks, graduated cylinders, parafilm etc.
- i. Acetonitrile (HPLC grade)
- j. Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/TMCS) (obtained from Sigma-Aldrich Chemical Company, or an equivalent supplier)
- k. Ethyl acetate (HPLC grade)
- l. Direct Analysis in Real Time Time-of-Flight Mass Spectrometer (DART TOFMS)<sup>1</sup>
- m. Heptafluorobutyric anhydride (HFBA), ≥99%, for GC derivatization
- n. Hexane (UV grade)
- o. Sodium sulfate (Reagent grade)

## 6 Standards and Controls

- a. Ethylene glycol (EG) Stock Standard (10 mg/mL):  
Ethylene glycol traceable to United States Pharmacopoeia (USP) can be purchased from USP or another approved vendor. Storage and stability are determined by the manufacturer. Add 100 mg of EG to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- b. EG Working Stock (500 µg/mL):  
Add 0.5 mL of the EG Stock Standard (10 mg/mL) to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass; stable for at

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<sup>1</sup> As of the time of the issuance of this procedure, only DART-1 has been validated for this application.

least 1 year.

- c. Diethylene glycol (DEG) Stock Standard (1 mg/mL):  
Diethylene glycol can be purchased from Sigma-Aldrich or another approved vendor. Storage and stability are determined by the manufacturer. Add 100 mg of DEG to a 100-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- d. Triethylene glycol (TEG) Stock Standard (1 mg/mL):  
Triethylene glycol can be purchased from Sigma-Aldrich or another approved vendor. Storage and stability are determined by the manufacturer. Add 100 mg of TEG to a 100-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- e. Propylene glycol (PG) or 1-2 Propanediol Stock Standard (1 mg/mL):  
Propylene glycol can be purchased from Sigma-Aldrich or another approved vendor. Storage and stability are determined by the manufacturer. Add 100 mg of PG to a 100-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- f. Negative Control Blood:  
Purchased from Clinica or another approved vendor. Storage and stability are determined by the manufacturer. A Negative Control Blood sample is analyzed with every blood assay.
- g. Positive Control Blood for BSTFA Derivatization (100 µg/mL):  
Positive Controls will be prepared fresh. When 25 µL of the appropriate 1 mg/mL stock solution, or 50 µL of the EG Working Stock (500 µg/mL) is added to 0.25 mL Negative Control Blood, the resulting control is 100 µg/mL. A Positive Control will be prepared for each analyte of interest. TEG and DEG are routinely combined into one Positive Control, while EG and PG are typically analyzed individually.
- h. d<sub>4</sub>-Ethylene glycol (d<sub>4</sub>-EG) Internal Standard Stock Standard (2.5 mg/mL):  
d<sub>4</sub>-Ethylene glycol can be purchased from Isotec or another approved vendor. Storage and stability are determined by the manufacturer. Add 25 mg of d<sub>4</sub>-EG to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- i. d<sub>4</sub>-Ethylene glycol (d<sub>4</sub>-EG) Internal Standard Solution (500 µg/mL):  
Add 2.0 mL of d<sub>4</sub>-EG Internal Standard Stock Standard (2.5 mg/mL) to a 10-mL volumetric flask. Bring to the mark with deionized water and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.



- j. **Positive Control Blood for HFBA Derivatization (EG at 72 µg/mL and 1200 µg/mL):**  
 Positive Control Blood will be prepared on the day of analysis as described in Table 1.

Table 1: Blood Control Preparation

Ctl Level (µg/mL)	Blood Volume (mL)	µL EG Working Stock (500 µg/mL)
72	0.25	36
1200	0.25	600

- k. **Negative Control Water:**  
 Obtained from an appropriate commercial source or from the in-house tap. A Negative Control Water sample is analyzed with every water assay.
- l. **Positive Control Water (EG at 100 µg/mL):**  
 Add 0.01 mL of the EG Control Stock Standard (10 mg/mL) to 0.99 mL of Negative Control Water. Prepare fresh. When sample size permits, an unknown sample can also be spiked with the EG Stock Standard as an additional Positive Control sample. A Positive Control Water sample is analyzed with every water assay.

## 7 Sampling

Not applicable.

## 8 Procedure

### 8.1 Screening for EG in Aqueous Matrices

- a. Control and unknown samples are analyzed directly in duplicate on the DART-TOF MS using the instrumental parameters in Section 9.1 of this procedure. (No sample preparation is necessary.)

### 8.2 Screening or Confirmation of EG in Blood Specimens (HFBA Derivative)

Appendix 1 contains an abbreviated version of this part of the procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Label centrifuge tubes for each sample and control.
- b. Aliquot 0.25 mL of negative control blood.
- c. Aliquot 0.25 mL of each case sample(s) in duplicate.

- d. Prepare the positive controls in duplicate as directed in 6.j.
- e. Add 50  $\mu$ L of the d<sub>4</sub>-EG Internal Standard Solution (500  $\mu$ g/mL) to one replicate of each sample and control. Note: The replicate without internal standard will be used for ion ratio comparison.
- f. Bring the total volume in the centrifuge tube to approximately 1.5 mL with acetonitrile.
- g. Vortex well. Centrifuge at approximately 10,000 rpm for 5 minutes.
- h. Remove the acetonitrile layer to a labeled 12 x 75 test tube. Evaporate to dryness under nitrogen at 50°C.
- i. Reconstitute extracts in 0.1 mL acetonitrile and vortex well.
- j. Add 50  $\mu$ L HFBA. Cap with a snap cap and parafilm and vortex well.
- k. Heat at 60°C for 30 minutes. Cool to room temperature.
- l. Vortex with 0.5 mL hexane and 0.5 mL deionized water.
- m. Centrifuge for 1 minute at approximately 3000 rpm. Remove hexane layer to a labeled 12 x 75 test tube. Add a small scoop of sodium sulfate (approximately 0.2 g) and vortex.
- n. Remove 0.05 mL of the hexane layer to a labeled autosampler vial. Add 0.1 mL hexane to each autosampler vial.
- o. Analyze 1  $\mu$ L by GC/MS(EI) using the parameters in Section 9.2 after ensuring that hexane is in the autosampler rinse vials.

### **8.3 Confirmation for EG, DEG, TEG and PG in Blood and Confirmation of EG in Aqueous Samples**

- a. Add 0.25 mL of specimen or control to an appropriately labeled 12 x 75 mm test tube.
- b. Spike positive controls, as appropriate.
- c. Add 0.5 mL acetonitrile to each sample.
- d. Cap and vortex for approximately 20 seconds.
- e. Centrifuge at approximately 2500 rpm for 2 minutes.
- f. Remove supernatant to a new 12 x 75 mm test tube.

- g. Evaporate the organic layer to dryness with nitrogen at approximately 40°C.
- h. Reconstitute the residue with 50 µL BSTFA/TMCS.
- i. Cap tubes and incubate all samples at approximately 60°C in a heating block for at least 30 minutes.
- j. Allow extracts to cool down to room temperature. Transfer extracts to autosampler vials. Analyze 1 µL by GC/MS(EI) or (CI) using the instrumental parameters in Section 9.3 of this procedure. It is important to analyze the extracts on a GC column that is dedicated to silyl derivatives. To compensate for known carryover within this procedure, ethyl acetate blanks and BSTFA/TMCS blanks should precede every unknown sample.

## 9 Instrumental Conditions

### 9.1 DART-TOF MS Analysis

#### 9.1.1 DART Ionization Source Parameters:

Anode Polarity:	Positive (+)
Needle Voltage:	3999 V
Electrode #1 Voltage:	75 V
Electrode #2 Voltage:	150 V
Gas Control:	~ 2.4 LPM
Temperature Control:	set 410°C (actual ~ 400°C)

#### 9.1.2 TOF-MS Parameters:

Tune File: DART +	
Needle Voltage:	0 V
Ring Lens Voltage:	5 V
Orifice 1 Voltage:	30 V
Orifice 2 Voltage:	5 V
Peaks Voltage:	300 V
Mass Range:	43-500 <i>m/z</i>

## 9.2 GC/MS Parameters for HFBA Derivative

Appendix 2 contains an abbreviated version of the instrumental conditions in Section 9.2 and 9.3 of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.2.1 GC Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	40°C	inlet temperature	300°C	type	DB-5
hold 1	1 min	injection mode	split	length	30 m
ramp 1	10°C/min	split	10:1	internal diameter	0.25 mm
temperature 2	130°C	carrier gas	ultrapure helium	film thickness	0.25 µm
ramp 2	30°C/min	carrier mode	constant flow		
temperature 3	325°C	flow	1.2 mL/min		
hold 2	1.5 min				

### 9.2.2 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	23 0°C
scan mode	full scan	transfer line temperature	280°C
scan range	35 - 500 m/z	quad temperature	150°C
		solvent delay	5.0 min

## 9.3 GC/MS Parameters for BSTFA Derivatives

### 9.3.1 GC Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	60°C	inlet temperature	250°C	type	DB-5
hold 1	2 min	injection mode	splitless	length	30 m
ramp 1	10°C/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	180°C	carrier mode	constant flow	film thickness	0.25 µm
ramp 2	35°C/min				
temperature 3	250°C				
hold 2	10 min				

### 9.3.2 Mass Spectrometer Parameters (EI Analysis)

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	270 °C
scan range	70 - 500 m/z	solvent delay	5.0 min
		quad temperature	150°C

### 9.3.3 Mass Spectrometer Parameters (CI Analysis)

ionization mode	methane chemical ionization (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	270 °C
scan range	70 - 500 m/z	solvent delay	5.2 min
		quad temperature	150°C

## 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this procedure. In general, compound identification will be based on comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or Positive Control. In most cases, all of the below should be met in order to identify one of the target analytes within a biological specimen.

### 10.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. It is noted, however, that derivatized glycols often produce wide chromatographic peaks on the analytical column used in this procedure. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of an extracted Positive Control.

#### 10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10-fold



greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank injected just prior to that sample.

## 10.2 Mass Spectrometry of HFBA derivative of EG

The following ions may be traced for ion ratio comparison of an unknown to a positive control: 169, 197, 213, 241. Only the 241 and 213 ions are from the EG; the other ions are from the HFBA. Therefore, the 169 and 197 will be present in the EI spectrum of the d<sub>4</sub>-EG-HFBA derivative as well. For this reason, it is best to use samples with no internal standard added for ion ratio comparisons.

Detectable 255 ion in a peak eluting near the internal standard may indicate the presence of PG. If the 255 ion is detected in an unknown near the retention time of the internal standard (within a few scans), this sample should be analyzed by a different method to verify that PG is not present, as it will interfere with the quantitation of EG.

## 10.3 Mass Spectrometry of BSTFA derivatives (EI data)

The following ions may be traced for each analyte:

- EG: 191, 133, 103, 147
- PG: 133, 147, 117
- DEG: 103, 147, 117
- TEG: 103, 147, 161

The mass spectrum of the analyte of interest should match that of an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 10.4 DART-TOF MS Data

The following two ions are used to screen for EG in aqueous samples: 63.0446 and 45.0340 (EG – water). Unknown samples should be spiked with EG at a concentration of 100 µg/mL to rule out the possibility of false negative results if the sample may not be pure water and if sample size permits.

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

### 13 Limitations

- a. There are only two ions in the HFBA derivative of EG that are unique to EG. The other ions that are found in the MS of the EG-HFBA derivative are HFBA ions.

- b. Limit of Detection:

The limit of detection has been administratively set to 100 µg/mL for DEG, TEG and PG in blood samples.

The limit of detection has been administratively set to 25 µg/mL for EG in blood samples. |

The limit of detection for EG in aqueous samples is 100 µg/mL.

- c. Interferences: EG cannot be accurately identified using the HFBA derivative method in the presence of PG. Grossly decomposed or putrefied samples may affect detection limits. |

### 14 Safety

The derivatizing reagents used in this procedure have noxious odors. They should be used in the fume hood to prevent excess exposure to their odor.

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

### 15 References

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Gembus, V.; Goulle, J.P.; Lacroix, C.; *J Anal Tox.* 2002, 26, 280-285.

Pan, Y.M., et al.; *J Anal Tox.* 2001, 25, 328-332.

Wurita, A., et al.; *Forensic Toxicol.* 2013, 31, 272-280.

Rev. #	Issue Date	History
3	08/23/12	Specified use of DART-1 in Section 5k. Updated chromatography decision criteria in Section 11.1.
4	03/21/14	A new method to quantitate ethylene glycol in blood specimens was added, leading to updates in Sections 3, 5b, 5h, 5m, 5n, 5o, 6a-6d, 6i, 6k-l, 6n, 7, 9.2, 10.2, 11.2, 12, 14 and 16. In Section 6a-6g and 6j-6k, specified that standard solutions should be stored in glass and updated provider of Negative Control Blood in section 6h. Updated wording for measurement uncertainty in Section 13. Added Appendix 1.
5	06/03/16	Removed all quantitative aspects of the procedure resulting in changes to the following Sections: 2, 3, 6, 8.2, 8.3, 11, 12 and 13 as well as Appendices 1 and 2. Removed Calibration Section (Section 7) and renumbered subsequent sections. Retitled Section 12 (Measurement Uncertainty).

**Approval**

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**Appendix 1: Abbreviated version of the EG Screening and Confirmation procedure for bench use.**

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**Appendix 2: Abbreviated Version of the EG Screening and Confirmation Instrumental  
Parameters for bench use.**

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## **Helium Analysis by Gas Chromatography with Thermal Conductivity Detection**

### **1 Introduction**

In recent years there has been an increase in reported instances of death by asphyxiation with helium, both as a means of suicide and accidentally in practitioners of autoerotic asphyxia. The use of helium in these activities has been promoted by various groups as a less unpleasant alternative to other means of asphyxiation. Breathing pure helium produces almost no secondary physiological responses when compared to other common asphyxiants such as nitrogen (narcosis) and carbon monoxide (severe nausea), and compressed helium is readily available from party supply stores. An exclusionary screening method for the presence of helium in biological specimens provides a tool for alerting investigators to cases where the cause of death may be more complicated than evidence at the scene might otherwise suggest.

### **2 Scope**

This procedure allows for exclusionary screening of helium that may be present in biological and non-biological samples.

### **3 Principle**

Thermal conductivity detectors (TCD) function by measuring the change in temperature of a heated wire placed at the exit of a GC column that occurs when an analyte with a thermal conductivity different than that of the carrier gas exits the GC column. Normally either hydrogen or helium is used as a carrier gas with a TCD, since these gases have much higher thermal conductivity (at least 3-fold greater at 500 K) than any other gases, providing strong response. For this method, the normal practice is reversed, and nitrogen, with a moderate thermal conductivity (38 W/K at 500 K) is used as carrier gas, providing high sensitivity for helium (222 W/K at 500 K). Specificity is enhanced by the fact that relatively few gases have higher thermal conductivities than nitrogen, meaning that only a few compounds are capable of producing interfering signals. At 500 K, the only common room-temperature gases with thermal conductivity differences greater than 5% of that for helium are: ammonia (7%), ethane (8%), ethylene (6%), hydrogen (>100%), methane (15%), and neon (17%).

## **4 Specimens**

This procedure can be performed on a variety of biological fluids and tissue samples. Specimens for this exam should be collected as soon as possible after death, and must be kept under a gas-tight seal until they are analyzed. For fluid specimens, the preferred container is a Vacutainer blood collection tube approximately 2/3 to 3/4 full. A 20 cc crimp-top headspace vial is suitable for small tissue samples. For large tissue samples, the best container is a new metal paint can of the smallest size necessary to contain the sample.

## **5 Equipment/Materials/Reagents**

- a. Gas chromatograph (Agilent 6890N or equivalent) equipped with thermal conductivity detector, analytical column (J&W HP-Molesieve 30 m x 0.32 mm x 12.00  $\mu$ m or equivalent)
- b. 16 x 100 mm disposable glass culture tubes
- c. deionized water
- d. fold-over rubber septa
- e. drug-free blood (Clinical Controls, or equivalent)
- f. vacuum source
- g. high-purity helium (GC-grade)
- h. compressed air source
- i. plastic syringe, 3 cc
- j. syringe needles (various sizes)
- k. syringe filters (Restek 25 mm 0.22 $\mu$ m PTFE, or equivalent)
- l. centrifuge
- m. heating block with thermometer

- n. electrical tape (or other well-sealing adhesive tape)
- o. standard GC syringe, 10  $\mu$ L
- p. hammer and metal probe/punch
- q. volumetric flasks (100-mL)
- r. basin or other container suitable for filling with water and inversion of flasks
- s. routine laboratory supplies, including disposable pipettes, test tube racks, etc.

## 6 Standards and Controls

- a. Air Standard:  
An air standard serves to demonstrate the absence of helium or target analyte in the source of air used for the procedure. The air standard is made by filling a 100 mL volumetric flask completely with deionized water. The flask is then inverted in a deionized water bath and the water is displaced by air taken from the laboratory compressed air supply. The flask is then capped with a fold-over rubber septum while still inverted in the water bath. Alternatively, an air standard may be obtained by simply sampling the ambient atmosphere with a standard 10  $\mu$ L GC syringe.
- b. High Purity Helium Standard:  
The high purity helium standard is analyzed to demonstrate that the target analyte source material is free of interferences and as a source for creating the mixed helium/air control. It is prepared in the same manner as the Air Standard, substituting a high purity helium source for the laboratory compressed air supply.
- c. Mixed Helium/Air Control (1% helium in air):  
Prepare an air standard as in (a) above. Then use a 3 cc syringe with a fine-gauge needle to transfer 1.0 mL of the high purity helium standard into this flask. A sample of this control is analyzed prior to each batch of samples to demonstrate that the instrument is performing properly.
- d. Negative Control:  
Measure 9 mL of drug-free blood into a 16 x 100 mm culture tube and cap with a fold-over rubber septum. Centrifuge at low speed ( $\leq 1000$  rpm) for 5 min and, using a fine-gauge needle, vent the tube headspace to the laboratory vacuum system for about 5 s. A negative

control will be analyzed with every batch of samples.

- e. **Positive Control:**  
Measure 9 mL of drug-free blood into a 16 x 100 mm culture tube and cap with a fold-over rubber septum. Run a long large-gauge needle through the septum into the blood sample and use a short fine-gauge needle, with an attached syringe filter, to vent the tube headspace. Gently bubble high-purity helium through the blood sample for about 30 min. The blood sample will generate copious quantities of foam, and the vent needle must be equipped with a syringe filter to prevent the sample from foaming out of the tube. After this sparge, centrifuge the sample at low speed ( $\leq 1000$  rpm) for 5 min and, using a fine-gauge needle, vent the tube headspace to the laboratory vacuum system for about 5 s. A positive control will be analyzed with every batch of samples.

## **7 Calibration**

Not applicable.

## **8 Sampling**

Not applicable.

## **9 Procedure**

- a. Where possible, centrifuge samples at low speed ( $\leq 1000$  rpm) for 5 min prior to equilibration and analysis. This will help prevent contamination of the sampling syringe with biological material.
- b. Place all controls and unknowns into a laboratory heating block set at approximately 36°C to equilibrate the headspace. The container type for the unknown samples may preclude placement in a heating block in which case another suitable equilibration method should be substituted. Equilibrate for at least 30 minutes.
- c. While the samples are equilibrating, perform the necessary QC checks for the GC-TCD instrument. The Air Standard, the High Purity Helium Standard, and the Mixed Helium/Air Control will be analyzed at this time to verify proper instrument performance.
- d. Using a 10  $\mu$ L standard GC syringe sample the headspace of each control or unknown and

analyze using the conditions given below. For headspace vials and Vacutainer (or similar) tubes, the headspace is sampled directly through the container septum. For specimens in paint cans (or similar containers) use a hammer and a metal punch or probe to make a pinhole in the lid of the container. Immediately cover this hole with a double layer of electrical tape, and then sample the headspace through the electrical tape.

## 10 Instrumental Conditions

### 10.1 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature 1	35°C, isothermal	type	HP-Molesieve	inlet temp.	200°C
hold 1	10 min	length	30 m	injection mode	manual, 1-10 µL
		internal diameter	0.32 mm	carrier gas	nitrogen
		film thickness	12 µm	carrier mode	constant flow
				flow	1.0 mL/min
				split ratio	2:1

### 10.2 Thermal Conductivity Detector

temperature	250°C	makeup gas	nitrogen
reference flow	20 mL/min	makeup gas flow rate	5.0 mL/min
polarity	negative	reference gas	nitrogen
data sampling rate	5 Hz	reference gas flow	20.0 mL/min

## 11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. Evaluation of results should be based upon comparison of analytical data for an unknown sample to data from analysis of positive and negative control samples.

## **11.1 GC-TCD Performance Criteria**

The Air Standard should be free of helium or target analyte. The Helium Standard (or target standard) should be free from other interferences. The air peak and the helium (or target) peak should be well-resolved.

## **11.2 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### **11.2.1 Retention Time**

The retention time of the presumptive helium peak should be within  $\pm 2\%$  of the retention time obtained from injection of a positive control sample.

### **11.2.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a negative control injected just prior to the sample.

## **12 Calculations**

Not applicable.

## **13 Uncertainty of Measurement**

Not applicable.

## **14 Limitations**

- a. Limit of Detection: This method will detect helium at a level of 0.5% v/v in air standards. The response for a 1% v/v standard of helium in air is less than 5% of that observed for the



positive control blood specimen.

- b. Interferences: None known.
- c. Results of this analysis are exclusionary in nature. At present, there is no method available to confirm presumptive positive helium results. Appropriate caution should be used in reporting presumptive positive analytical results.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance. When preparing the positive control blood sample, ensure that a syringe filter is attached to the vent needle in order to prevent aerosol formation from the blood sample.

## 16 References

CRC Handbook of Chemistry and Physics, 89<sup>th</sup> Ed., section 6, pp. 206 – 207.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

FBI Laboratory Safety Manual.

Rev. #	Issue Date	History
0	05/22/09	Original issue.
1	01/03/13	Updated Chromatography Decision Criteria in Section 11.2.

**Approval**

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## Cardiac Glycosides from Food Products

### 1 Introduction

The cardiac glycosides digoxin, digitoxin and oleandrin are naturally occurring chemicals that are found in various plants. Digitoxin and digoxin are found in *Digitalis lanata* and *Digitalis purpurea* (commonly known as foxglove); they are also used in the treatment of congestive heart failure and atrial fibrillation. Oleandrin is found in *Nerium oleander* (bay laurel). Toxic effects of cardiac glycosides include nausea, vomiting, visual disturbances and cardiac arrhythmias.

### 2 Scope

This procedure is designed to detect digoxin, digitoxin and/or oleandrin in food products, beverages and plant materials.

### 3 Principle

Samples are alkalized with a buffer and extracted into a mixture of chloroform and isopropanol. Resulting extracts are taken to dryness, reconstituted, and analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) in the Fourier Transform (FT) mode.

### 4 Specimens

This procedure has been validated for a variety of food products, beverages and plant materials. It can easily be validated for new matrices by determining the limit of detection of the analyte(s) of interest in a specific matrix. Typically, 0.5 mL of a 1:1 aqueous homogenate will be extracted and analyzed.

### 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Screw-top test tubes (16 x 100 mm) with caps and Teflon inserts
- b. Disposable glass pipettes with rubber bulbs
- c. Volumetric flasks

- d. Pipetters with disposable tips
- e. pH paper
- f. Vortex mixer
- g. Centrifuge
- h. Evaporator with nitrogen
- i. Blender
- j. Chloroform (HPLC grade)
- k. Isopropanol (HPLC grade)
- l. Cardiac Glycoside Extraction Solvent (95:5 chloroform:isopropanol):  
Combine 5 mL isopropanol and 95 mL chloroform and mix well. Store at room temperature in brown glass. Stable 1 month.
- m. Deionized water
- n. Ammonium chloride (Reagent grade)
- o. Ammonium hydroxide (ACS grade)
- p. Ammonium Chloride Buffer (1.2 M, pH 9.5):  
Add 6.25 g ammonium chloride to a 100-mL volumetric flask. Add approximately 50 mL deionized water and mix. Add 6.0 mL of ammonium hydroxide. Bring to the mark with deionized water and mix well. Verify that pH is between 9 and 10. Store in plastic at room temperature. Stable for six months.
- q. Methanol (Optima grade)
- r. Acetonitrile (Optima grade)
- s. Ammonium formate (99%+)
- t. Formic acid (99.8%+)
- u. Water (Optima grade)

- v. Ammonium Formate (2 mM, pH 3):  
Add 0.126 g ammonium formate to a 1-L graduated cylinder, bring to the 1-L mark with Optima grade water, and mix well. Add 0.2 mL formic acid. Verify pH is between 2.5 and 3.5. Store in glass at room temperature. Stable for one month.
- w. Liquid chromatograph/mass spectrometer capable of high mass resolution equipped with a 15 cm x 2.1 mm x 5  $\mu$ m Altima-C18 (or equivalent) column.

## 6 Standards and Controls

- a. Digoxin: Purchased as a 1.0 mg/mL solution from Cerilliant Corporation in Round Rock, Texas. Storage and stability determined by manufacturer.
- b. Digitoxin: Purchased from ChromaDex in Irvine, California. Storage and stability determined by manufacturer.
- c. Oleandrin: Purchased from ChromaDex in Irvine, California. Storage and stability determined by manufacturer.
- d. Digoxin Working Solution (5 PPM):  
Add 0.050 mL of the digoxin standard to a 10 mL volumetric flask. Bring to the mark with methanol. Store refrigerated in glass or plastic. Stable at least one year.
- e. Digitoxin Stock Solution (1 mg/mL):  
Weigh 10 mg digitoxin. Dilute to 10 mL with methanol in a 10 mL volumetric flask. Store refrigerated in glass or plastic. Stable at least one year.
- f. Oleandrin Stock Solution (1 mg/mL):  
Weigh 10 mg oleandrin. Dilute to 10 mL with methanol in a 10 mL volumetric flask. Store refrigerated in glass or plastic. Stable at least one year.
- g. Digitoxin Working Solution (5 PPM):  
Add 0.050 mL of the digitoxin standard to a 10 mL volumetric flask. Bring to the mark with methanol. Store refrigerated in glass or plastic. Stable at least one year.
- h. Oleandrin Working Solution (5 PPM):  
Add 0.050 mL of the oleandrin standard to a 10 mL volumetric flask. Bring to the mark with methanol. Store refrigerated in glass or plastic. Stable at least one year.

- i. **Negative Control:**  
Typically, deionized water will be used as a negative control. A 1:1 deionized water homogenate of a blank matrix similar to the questioned item may also be used as a negative control. A negative control will be extracted and analyzed with every assay.
- j. **Positive Control:**  
When sample size permits, a portion of the questioned sample will be spiked with the analyte of interest to prepare the positive control. The concentration of the positive control will be based on limit of detection experiments in that matrix, but 1.0 PPM is a good starting point for most matrices. A positive control will be extracted and analyzed with every assay.
- k. **Cardiac Glycoside Column Check Mix (1 PPM each component):**  
Combine 0.1 mL of each appropriate Working Solution above. (Only analytes of interest for the day's batch must be in the column check mix.) Bring to 0.5 mL with methanol. Prepare fresh.

## 7 Calibration

If quantitative results are required, consult the *Guidelines for Toxicological Quantitations* procedure (Tox 101) for guidance.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. For food and plant material specimens, homogenize the sample 1:1 with deionized water. Beverages may be analyzed directly.
- b. Into properly-labeled test tubes (16 x 100 mm), add 0.5 mL homogenate, or 0.25 mL undiluted liquid sample. For confirmatory, targeted analyses, a cardiac glycoside not present in the sample may be added as an internal standard at a concentration of 1 PPM by adding 50 µL of the Working Solution of the compound. For a screen, the unknown may be analyzed in duplicate; one sample will be run without an internal standard and one will be spiked with one of the three analytes.

- c. Add 1.5 mL deionized water, 0.5 mL ammonium chloride buffer, and vortex.
- d. Add 5 mL Cardiac Glycoside Extraction Solvent and mix via gentle inversion for 30 minutes.
- e. Centrifuge at approximately 3000 rpm for 5 minutes.
- f. Remove organic layer (bottom) to a clean test tube (12 x 75 mm) and evaporate to dryness under nitrogen at approximately 45 °C.
- g. Reconstitute extracts in 0.12 mL methanol and analyze by LC/MS/MS using the conditions in Section 10.

## 10 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

### 10.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
B: Acetonitrile	flow rate	0.3 mL/min		type	C-18
	time (min)	%B	%C	length	15 cm
C: Ammonium Formate	0	20	80	internal diameter	2.1 mm
	5	38	62	particle size	5 µm
	6	65	35	temperature	30 °C
	9	70	30		
	12	70	30	autosampler T	15 °C
	13	20	80		
	25	20	80		

## 10.2 Mass Spectral Parameters

<b>Ionization mode</b>	electrospray (+)
<b>Scan Events</b>	2
<b>Scan Event #1</b>	FTMS; res=30000; 450-900 amu
<b>Scan Event #2</b>	ms/ms of the most intense of the following: 594.3637 (oleandrin), 798.4634 (digoxin) and 782.4685 (digitoxin) amu; res = 7500; CID CE = 60%; isolation width = 8.0

## 11 Decision Criteria

### 11.1 Column Check Mix Suitability

Proper calibration and sensitivity of the LC/MS/MS are demonstrated each day samples are analyzed. The Cardiac Glycoside Column Check Mix effectively evaluates system suitability. By analyzing this standard mix the analytes can be evaluated for proper mass assignments, elution times and signal to noise responses. Table 1 shows the parameters evaluated by use of this Mix.

Table 1: Parameters used in evaluating system suitability

Analyte	Retention Time	Full Scan Ammoniated Adduct Ion	Product Ions
Digoxin	7.64 min	798.4634	651.374, 391.248, 521.311
Digitoxin	9.11 min	782.4685	375.253, 505.316, 635.379
Oleandrin	9.26 min	594.3637	433.258, 373.238, 517.316

Retention times may shift as an analytical column ages or with minor differences in mobile phase composition. From run to run, retention times should vary less than 0.5 minute.

High resolution mass spectrometric data is used in this procedure. Therefore, the full scan ammoniated adduct ion for each analyte in full scan mode should be within 5 mnu of the expected value. Additionally, product ions should be within 5 mnu of the expected value.

### 11.2 Analyte Suitability

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control. In most cases, all of the below should be met in order to identify a cardiac glycoside within a biological specimen:



### **11.2.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **11.2.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control of the analyte of interest.

#### **11.2.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank sample injected just prior to that sample. Note: Visual evaluation of S/N is acceptable as reconstructed ion chromatograms in FTMS may have mathematically nonsensical S/N values.

### **11.2.2 Mass Spectrometry**

The mass spectrum of the analyte of interest should match that of the appropriate reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See Table 1 for expected ions. Full scan ammoniated adduct ions and product ions should agree with the expected values within 5 mmu.

## **12 Calculations**

Not applicable.

## **13 Uncertainty of Measurement**

Not applicable.

## 14 Limitations

- a. Limits of Detection: Limits of detection for the cardiac glycosides will vary according to matrix, and will be determined with each new matrix analyzed. The following detection limits have been determined in the following matrices:

Oleandrin	- 1 PPM in coffee - 2 PPM in pancakes and salad
Digoxin	- 0.2 PPM in pancakes - 0.25 PPM in salad
Digitoxin	- 0.25 PPM in salad - 25 PPM in dried leaf material (based on 5 mg sample size)

- b. Interferences: None known.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. See the *FBI Laboratory Safety Manual* for further guidance.

## 16 References

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*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit –  
Toxicology Subunit SOP Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology  
Subunit SOP Manual.

*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
0	05/19/10	New document.
1	08/23/12	Updated chromatography decision criteria in Section 11.2.1.

**Approval**

  
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**Appendix 1: Abbreviated version of the Cardiac Glycoside Procedure for bench use.**

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## **Arsenic in Urine and Hair by ICP/MS**

### **1 Introduction**

This procedure is used to detect and quantitate arsenic (As) in urine and hair specimens. This procedure does not discriminate between the nontoxic organic forms of arsenic and the toxic inorganic form of arsenic.

### **2 Scope**

This procedure is used to selectively assay arsenic in urine and hair specimens by Inductively Coupled Plasma Mass Spectrometry (ICP/MS) with a collision cell. Urine samples are diluted, spiked with an internal standard and analyzed. Hair samples are first chemically digested then diluted, spiked with an internal standard and analyzed.

### **3 Principle**

Urine specimens are mixed with the Indium Internal Standard Working Solution, diluted up to 10 mL in 2% Nitric Acid in Water Solution and analyzed contemporaneously with a complete matrix matched calibration curve and duplicate sets of matrix matched controls. Hair specimens are massed, completely digested in Tetramethylammonium hydroxide (TMAH), mixed with the Indium Internal Standard Working Solution, diluted up to 10 mL in 2% Nitric Acid in Water Solution and analyzed contemporaneously with a complete urine calibration curve, duplicate sets of controls in urine, a Negative Control Hair sample and duplicate sets of Positive Control Hair samples.

### **4 Specimens**

This procedure is validated for urine and hair. Typically for urine, two 100  $\mu$ L samples are analyzed. For hair, two 5.5 mg samples are analyzed. However, if it is suspected that the arsenic concentration is above the procedure's linear range, smaller sample sizes or further dilutions of the samples may be analyzed.

### **5 Equipment/Materials/Reagents**

- a. 10 mL and 25 mL volumetric flasks, class A, PMP (VITLAB<sup>®</sup> or equivalent)
- b. 15 mL and 50 mL conical tubes with screw tops, PP (Falcon<sup>®</sup> or equivalent)

- c. 100 mL volumetric flask, class B, PP (Nalgene<sup>®</sup> or equivalent)
- d. 1 L volumetric flask, class B, PP (Nalgene<sup>®</sup> or equivalent)
- e. 25 mL plastic graduated cylinder (Nalgene<sup>®</sup> or equivalent)
- f. Concentrated nitric acid (Optima grade)
- g. Water (Deionized, 18 MΩ)
- h. 2% Nitric Acid in Water Solution (v:v):  
To a 1 L Nalgene<sup>®</sup> volumetric flask, add approximately 800 mL of deionized water. Add 20 mL of Optima grade concentrated nitric acid, fill to the mark with deionized water and mix well. Store at room temperature in plastic. Stable for at least one year.
- i. Tetramethylammonium hydroxide (TMAH), 25% w/w aqueous solution (Electronic grade)
- j. Methanol (HPLC grade or better)
- k. Balance capable of measuring  $\pm 0.1$  mg
- l. Routine laboratory supplies including but not limited to: pipettes, disposable pipettes, forceps, hand shears, etc.
- m. Vortexer
- n. Inductively Coupled Plasma-Mass Spectrometer with a collision cell installed (Thermo-Fisher iCAP Q or equivalent)
- o. Autosampler (CETAC ASX-260 or equivalent)
- p. Cryogrinder (optional)

## 6 Standards and Controls

- a. Indium Internal Standard Stock Solution (1 mg/L in 2% nitric acid solution):  
Purchased from SPEX CertiPrep or an equivalent supplier. Stability and storage determined by manufacturer.
- b. Indium Internal Standard Working Solution (10 µg/L in 2% Nitric Acid in Water Solution):  
To a 100 mL Nalgene<sup>®</sup> volumetric flask that has been washed with 2% Nitric Acid in

Water Solution, add about 80 mL of 2% Nitric Acid in Water Solution. Add 1 mL of Indium Internal Standard Stock Solution, fill to the mark with 2% Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.

- c. Arsenic Calibrator Stock Solution (1 mg/L in 2% Nitric Acid in Water Solution): Purchased from SPEX CertiPrep or an equivalent supplier. Stability and storage determined by manufacturer.
- d. Arsenic Calibrator Working Solutions for Urine (10 µg/L – 1000 µg/L):  
The following table shows the preparation of the individual Calibrator Working Solutions for Urine. The Calibrator Working Solutions are prepared in individual 25 mL class A volumetric flasks that have been washed with 2% Nitric Acid in Water Solution. Store at room temperature in plastic. Fill to the mark with 2% Nitric Acid in Water Solution and mix well. Stable for at least one year.

Calibrator Working Solutions for Urine (µg/L)	Volume of Arsenic Calibrator Stock Solution (mL)
10	0.250
25	0.625
50	1.25
100	2.5
250	6.25
500	12.5
800	20.0
1000	25.0

- e. Arsenic Control Stock Solution (100 mg/L in 2% nitric acid solution)  
Purchased from High Purity Standards or an equivalent supplier. Stability and storage determined by manufacturer.
- f. Arsenic Intermediate Control Working Solution (10 mg/L in 2% Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been washed with 2% Nitric Acid in Water Solution, add about 15 mL of 2% Nitric Acid in Water Solution. Add 2.5 mL of the Arsenic Control Stock Solution (100 mg/L), fill to the mark with 2% Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- g. Arsenic High Control Working Solution for Urine (800 µg/L in 2% Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been washed with 2% Nitric Acid in Water Solution, add about 15 mL of 2% Nitric Acid in Water Solution. Add 2.0 mL of the Arsenic Intermediate Control Working Solution (10 mg/L), fill to the mark with 2% Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.



- h. Arsenic Low Control Working Solution for Urine (30 µg/L in 2% Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been washed with 2% Nitric Acid in Water Solution, add about 15 mL of 2% Nitric Acid in Water Solution. Add 75 µL of the Arsenic Intermediate Control Working Solution (10 mg/L), fill to the mark with 2% Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- i. Arsenic High Control Working Solution for Hair (500 µg/L in 2% Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been washed with 2% Nitric Acid in Water Solution, add about 15 mL of 2% Nitric Acid in Water Solution. Add 1.25 mL of the Arsenic Intermediate Control Working Solution (10 mg/L), fill to the mark with 2% Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- j. Arsenic Low Control Working Solution for Hair (50 µg/L in 2% Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been washed with 2% Nitric Acid in Water Solution, add about 15 mL of 2% Nitric Acid in Water Solution. Add 125 µL of the Arsenic Intermediate Control Working Solution (10 mg/L), fill to the mark with 2% Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- k. Negative Control Urine:  
Prepared from in-house anonymous donations that are pooled. Collected negative specimens are screened for arsenic. If arsenic is present in an individual specimen, the level must be below 10 µg/L. Combine and stored refrigerated in plastic. Stable for at least one year.
- l. Negative Control Hair:  
Prepared from in-house anonymous donations. Collected negative specimens are screened for arsenic. If arsenic is present, the level must be below 1 ng/mg (50 µg/L). Store at room temperature in paper. Stable indefinitely.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

1. Prepare the calibration curve:

To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with 2% Nitric Acid in Water Solution add:

- Approximately 5 mL 2% Nitric Acid in Water Solution;
- 100  $\mu$ L of pooled in-house Negative Control Urine;
- 100  $\mu$ L of the appropriate Calibrator Solution (10 – 1000  $\mu$ g/L);
- 100  $\mu$ L of the Indium Internal Standard Working Solution;
- 2% Nitric Acid in Water Solution to the mark, mix well;

Transfer calibrators to labeled 15 mL Falcon<sup>®</sup> tubes.

A zero calibrator/blank is prepared in the same manner except 100  $\mu$ L of deionized water is used instead of 100  $\mu$ L of a calibrator solution.

2. Have a second chemist prepare urine controls:

To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with 2% Nitric Acid in Water Solution add:

- Approximately 5 mL 2% Nitric Acid in Water Solution;
- 100  $\mu$ L of pooled in-house Negative Control Urine;
- 100  $\mu$ L of the appropriate Control Solution (30 or 800  $\mu$ g/L);
- 100  $\mu$ L of the Indium Internal Standard Working Solution;
- 2% Nitric Acid in Water Solution to the mark, mix well;

Transfer controls to labeled 15 mL Falcon<sup>®</sup> tubes.

Positive controls are prepared in duplicate.

3. Prepare unknown urine samples:

To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with 2% Nitric Acid in Water Solution add:

- Approximately 5 mL 2% Nitric Acid in Water Solution;
- 100  $\mu$ L of unknown sample urine;
- 100  $\mu$ L of deionized water;
- 100  $\mu$ L of the Indium Internal Standard Working Solution;
- 2% Nitric Acid in Water Solution to the mark, mix well;

Transfer calibrators to labeled 15 mL Falcon<sup>®</sup> tubes.

Samples are typically prepared in duplicate.

4. Prepare hair controls (if necessary):

a. To individually labeled 15 mL Falcon<sup>®</sup> tubes:

- Add a minimum of 27.5 mg of negative hair. Accurately record the mass to the nearest 0.1 mg.
- Based upon the recorded mass, add enough TMAH to establish a solution of 5 mg of hair per 100  $\mu$ L of TMAH. (For example, 550  $\mu$ L TMAH is needed for 27.5 mg of hair.)

Allow the hair to completely digest, vortexing occasionally. (This process typically takes at least 8 hours, and the process may be left to proceed overnight.)

b. To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with 2% Nitric Acid in water solution add:

- Approximately 5 mL 2% Nitric Acid in Water Solution;
- 100  $\mu$ L of negative hair digest (prepared in 8.4.a above);
- 100  $\mu$ L of deionized water or appropriate Control Working Solution for hair;
- 100  $\mu$ L of the Indium Internal Standard Working Solution;
- 2% Nitric Acid in Water Solution to the mark, mix well;

Transfer controls to labeled 15 mL Falcon<sup>®</sup> tubes.

A minimum of one Negative Control Hair is to be analyzed and the Low and High Hair Controls are to be analyzed in duplicate.

- Note: The low hair control corresponds to 1 ng As per mg hair.
- Note: The high hair control corresponds to 10 ng As per mg hair.

5. Prepare unknown hair samples (if necessary):

a. To individually labeled 15 mL Falcon<sup>®</sup> tubes:

- Add a minimum of 5.5 mg of specimen hair in duplicate. Accurately record the mass to the nearest 0.1 mg.
  - Note: 5.5 mg is a small amount of hair. In order to ensure that a representative hair sample is analyzed, a larger amount of hair may be cut into small snippets and mixed before removing the 5.5 mg sample. Alternatively, a larger hair sample may be cryoground to mix it well.
- Based upon the recorded mass, add enough TMAH to establish a solution of 5 mg of hair per 100  $\mu$ L of TMAH. (110  $\mu$ L TMAH is added to 5.5 mg hair.)

Allow the hair to completely digest, vortexing occasionally. (This process typically takes at least 8 hours, and the process may be left to proceed overnight.)

b. To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with 2% Nitric Acid in Water Solution add:

- Approximately 5 mL 2% Nitric Acid in Water Solution;
- 100  $\mu$ L of specimen hair digest prepared in 8.5.a above;
- 100  $\mu$ L of deionized water;
- 100  $\mu$ L of the Indium Internal Standard Working Solution;

- 2% Nitric Acid in Water Solution to the mark, mix well;  
 Transfer controls to labeled 15 mL Falcon® tubes.

6. Analyze calibration samples, control samples and unknown samples by ICP/MS using the instrumental conditions in Section 9 below.

Note: A Negative Control Urine (BLK) should be first in the sequence, followed by the calibrators (STDs), and unknown samples (UNKNOWN) bracketed by Positive Control Urines (QC – LCS). The Negative Control Urine may be reanalyzed as an UNKNOWN between specimens. When hair samples are analyzed, the Negative Control Hair should be analyzed after the urine specimens (as an UNKNOWN) followed by unknown samples (UNKNOWN) bracketed by Positive Control Hair samples (QC – LCS).

## 9 Instrumental Conditions

The following conditions are written to follow Thermo-Fisher's LabBooks software package.

Analytes: As (arsenic) and In (indium)

Acquisition parameters:

Identifier	Dwell time (s)	Channels	Spacing (u)	Measurement mode	Resolution
75As (KED)	0.05	1	0.1	KED	Normal
115In (KED)	0.05	1	0.1	KED	Normal
				# sweeps = 10	

Monitor analytes:

	Uptake	Wash
Minimum	30	30
Maximum	300	300

Survey scan settings:

Start mass (u)	End mass (u)	Dwell Time (s)	Spacing (u)	Resolution	Measurement mode
4.60	245.00	0.01	0.2	Normal	KED

Interference correction: not applicable

Standards: covered in cal/ctrl section

Quantification:

Analyte	Measurement mode	Quantify	Internal Standard	Fit type	Weighting	Forcing	Use for Semi-Quant
75As (KED)	KED	Yes	115In (KED)	Linear	None	Blank	Yes
115In (KED)	KED	No	Use as Internal Standard	Linear	None	Blank	Yes
IS Recovery		Low warning limit: 80%		Low failure limit: 75%			
		High warning limit: 120%		High failure limit: 125%			

Ratios: not applicable

Quality Control:

Calibration Tests / Laboratory Control Standard (LCS)

Analyte	Low failure limit	Low warning limit	High warning limit	High failure limit
75As (KED)	80%	81%	119%	120%

Continuous Tests / Relative Stability Verification (RSV)

Analyte	Verify	Ignore concentration below	unit	Concentration warning limit	Concentration failure limit
75As (KED)	concentration	11	ppb	10%	15%

Autosampler:

Time Settings:	Wash Time (s):	120	Take up Time (s):	45
Rack Settings:	Rack 1 Type:	60-vials (12x5)	Rack 2 Type:	60-vials (12x5)
Autotune Settings:	Autotune rack:	Standard	Autotune vial:	1
Rinse settings	Rinse Rack:	Rinse Station		

## 10 Decision Criteria

### 10.1 Batch Acceptance Criteria

Arsenic should not be detected in the Negative Control Urine specimen above a level of 10 µg/L.

Arsenic should not be detected in the Negative Control Hair specimen above a level of 1 ng/mg.

Arsenic in the Positive Control Urine and Positive Control Hair specimens should quantitate within ±20% of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for more information.

### 10.2 Sample Acceptance Criteria

The Indium (Internal Standard) response in the unknown specimen(s) should fall within 80% and 120% of the Internal Standard response for the initial blank in the run.

## 11 Calculations

Quantitation is performed by constructing a multi-point calibration curve based on the ratio of the intensity for each calibrator level and the internal standard. The curve is forced through a blank urine specimen with no weighting. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

For hair specimens, 5 mg of hair is used in the place of 0.1 mL of urine. Therefore, results as received from the instrument for hair should be divided by 50. For example, if a result of 100 µg/L of As is obtained for a hair specimen, that corresponds to 2 ng As per mg of hair.

When a hair specimen contains arsenic above the method's lower limit of quantitation, it may be analyzed again using a method of standard addition to verify the As concentration.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

This method does not discriminate between nontoxic organic forms of arsenic and the toxic inorganic form of arsenic.

- a. Urine - Limit of Detection/Limit of Quantitation (administratively set): 10 µg/L

Urine:	@30 µg/L	@400 µg/L	@800 µg/L
% Bias	2.23	3.20	3.06
% Repeatability	3.06	1.63	2.06
%Intermediate Precision	5.44	2.66	3.07

- b. Hair - Limit of Detection/Limit of Quantitation (administratively set): 1 ng/mg

Hair:	@ 1 ng/mg (50 µg/L)	@ 10 ng/mg (500 µg/L)
% Bias	9.29	11.92
% Repeatability	1.47	2.73
%Intermediate Precision	2.71	3.01

- c. Interferences for urine and hair: no endogenous material/matrices interfered with the analysis of arsenic. For urine, a mixture of nickel, beryllium, cerium, indium, lithium, barium, bismuth, cobalt, lead and uranium at a concentration of 1000 µg/L for each element was analyzed and found not to interfere with the analysis of arsenic.
- d. Carryover: No carryover was observed when a negative control urine specimen was analyzed immediately following a 1000 µg/L calibrator. No carryover was observed when a negative control hair specimen was analyzed immediately following a 10 ng/mg control.
- e. Processed sample stability: For urine, sample stability was evaluated up to seven days after

the initial analysis and remained within  $\pm 20\%$  with refrigerated storage. For hair, sample stability was evaluated up to 24 hours after the initial analysis and remained within  $\pm 25\%$  with refrigerated storage.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual*.



Rev. #	Issue Date	History
0	01/08/2016	New document.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Procedure for bench use.**

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Redacted - Form on File

Redacted - Form on File

## **Thallium in Hair by ICP/MS**

### **1 Introduction**

Thallium is a toxic metal that may cause gastrointestinal, neurological or dermatological symptoms. Thallium can be detected in hair following chronic exposure or following survival of an acute exposure. This procedure is used to detect and quantitate thallium (Tl) in hair specimens.

### **2 Scope**

This procedure is used to selectively assay thallium in hair specimens by Inductively Coupled Plasma Mass Spectrometry (ICP/MS) with a collision cell. Hair samples are first chemically digested then diluted, spiked with an internal standard and analyzed.

### **3 Principle**

Hair specimens are weighed, completely digested in Tetramethylammonium hydroxide (TMAH), mixed with the Iridium Internal Standard Working Solution, diluted up to 10 mL in Dilute Nitric Acid in Water Solution and analyzed contemporaneously with a complete matrix matched calibration curve, a Negative Control Hair sample and duplicate sets of Positive Control Hair samples.

### **4 Specimens**

This procedure is validated for hair. Two 5.5 mg samples are analyzed. However, if it is suspected that the thallium concentration is above the procedure's linear range, smaller sample sizes or further dilutions of the samples may be analyzed.

### **5 Equipment/Materials/Reagents**

- a. 10 mL and 25 mL volumetric flasks, class A, PMP (VITLAB<sup>®</sup> or equivalent)
- b. 5 mL 12 x 75 mm round bottom tubes with caps, PP (Falcon<sup>®</sup> or equivalent)
- c. 15 mL and 50 mL conical tubes with screw tops, PP (Falcon<sup>®</sup> or equivalent)
- d. 100 mL volumetric flask, class B, PP (Nalgene<sup>®</sup> or equivalent)
- e. 1 L volumetric flask, class B, PP (Nalgene<sup>®</sup> or equivalent)

- f. 25 mL plastic graduated cylinder (Nalgene<sup>®</sup> or equivalent)
- g. Concentrated nitric acid (Optima grade)
- h. Water (Deionized, 18 MΩ)
- i. Dilute Nitric Acid in Water Solution (v:v):  
To a 1 L Nalgene<sup>®</sup> volumetric flask, add approximately 800 mL of deionized water. Add 40 mL of Optima grade concentrated nitric acid, fill to the mark with deionized water and mix well. Store at room temperature in plastic. Stable for at least one year.
- j. Tetramethylammonium hydroxide (TMAH), 25% w/w aqueous solution (Electronic grade)
- k. Methanol (HPLC grade or better)
- l. Balance capable of measuring  $\pm 0.1$  mg
- m. Routine laboratory supplies including but not limited to: pipettes, disposable pipettes, forceps, hand shears, etc.
- n. Vortexer
- o. Inductively Coupled Plasma-Mass Spectrometer with a collision cell installed (Thermo-Fisher iCAP Q or equivalent)
- p. Autosampler (CETAC ASX-260 or equivalent)
- q. Cryogrinder (optional)
- r. Acetone (Reagent grade or better)
- s. Heating block

## 6 Standards and Controls

- a. Iridium Internal Standard Stock Solution (1  $\mu\text{g/mL}$ ):  
Purchased from SPEX CertiPrep or an equivalent supplier. Stability and storage determined by manufacturer.
- b. Iridium Internal Standard Working Solution (10  $\mu\text{g/L}$ ):  
To a 100 mL Nalgene<sup>®</sup> volumetric flask that has been rinsed with Dilute Nitric Acid in

Water Solution, add about 80 mL of Dilute Nitric Acid in Water Solution. Add 1 mL of Iridium Internal Standard Stock Solution, fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.

- c. Thallium Calibrator Stock Solution (1000 mg/L in Dilute Nitric Acid in Water Solution): Purchased from SPEX CertiPrep or an equivalent supplier. Stability and storage determined by manufacturer.
- d. Thallium Calibrator Working Stock Solution - High (5 mg/L in Dilute Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been rinsed with Dilute Nitric Acid in Water Solution, add about 15 mL of Dilute Nitric Acid in Water Solution. Add 0.125 mL of the Thallium Calibrator Stock Solution (1000 mg/L), fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- e. Thallium Calibrator Working Stock Solution - Low (0.5 mg/L in Dilute Nitric Acid in Water Solution):  
To a 10 mL class A volumetric flask that has been rinsed with Dilute Nitric Acid in Water Solution, add about 7 mL of Dilute Nitric Acid in Water Solution. Add 1.0 mL of the Thallium Calibrator Working Stock Solution High (5 mg/L), fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- f. Thallium Calibrator Working Solutions (1 µg/L – 250 µg/L):  
The following table shows the preparation of the individual Calibrator Working Solutions. The Calibrator Working Solutions are prepared in individual 25 mL class A volumetric flasks that have been washed with Dilute Nitric Acid in Water Solution. Store at room temperature in plastic. Fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Stable for at least one year.

Calibrator Working Solutions (µg/L) (Prepared in 25-mL volumetric flasks)	Volume of Thallium Calibrator Working Stock Solution - High (5 mg/L) (µL)	Volume of Thallium Calibrator Working Stock Solution - Low (0.5 mg/L) (µL)	Corresponds to xx ng Thallium per mg of hair as prepared
1		50	0.02
2.5		125	0.05
5		250	0.10
10	50		0.20
25	125		0.50
50	250		1.00
75	375		1.50
100	500		2.00
175	875		3.50
250	1250		5.00

- g. Thallium Control Stock Solution (10,000 mg/L in Dilute Nitric acid solution)  
Purchased from High Purity Standards or an equivalent supplier. Stability and storage determined by manufacturer.
- h. Thallium Intermediate Control Solution High (10 mg/L in Dilute Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been rinsed with Dilute Nitric Acid in Water Solution, add about 15 mL of Dilute Nitric Acid in Water Solution. Add 0.025 mL of the Thallium Control Stock Solution (10,000 mg/L), fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- i. Thallium Intermediate Control Solution Low (0.5 mg/L in Dilute Nitric Acid in Water Solution):  
To a 10 mL class A volumetric flask that has been rinsed with Dilute Nitric Acid in Water Solution, add about 15 mL of Dilute Nitric Acid in Water Solution. Add 0.5 mL of the Thallium Intermediate Control Solution High (10 mg/L), fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- j. Thallium High Control Working Solution (200 µg/L in Dilute Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been rinsed with Dilute Nitric Acid in Water Solution, add about 15 mL of Dilute Nitric Acid in Water Solution. Add 0.5 mL of the Thallium Intermediate Control Solution High (10 mg/L), fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- k. Thallium Low Control Working Solution (7.5 µg/L in Dilute Nitric Acid in Water Solution):  
To a 25 mL class A volumetric flask that has been rinsed with Dilute Nitric Acid in Water Solution, add about 15 mL of Dilute Nitric Acid in Water Solution. Add 0.375 mL of the Thallium Intermediate Control Solution Low (0.5 mg/L), fill to the mark with Dilute Nitric Acid in Water Solution and mix well. Store at room temperature in plastic. Stable for at least one year.
- l. Negative Control Hair:  
Prepared from in-house anonymous donations. Collected negative specimens are screened for thallium. If thallium is present, the level must be below 0.02 ng/mg (1 µg/L). Store at room temperature in paper. Stable indefinitely.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

1. Prepare negative control hair digest for the calibration curve and controls:
  - a. To a labeled 15 mL Falcon<sup>®</sup> tube:
    1. Add a minimum of 100 mg of negative hair. Accurately record the mass to the nearest 0.1 mg. Alternatively, a larger hair sample may be cryoground to mix it well. (The negative control hair digest will be prepared in the same manner as the unknown sample.)
    2. Based upon the recorded weight, add enough TMAH to establish a solution of 5 mg of hair per 100  $\mu$ L of TMAH. (For example, 2000  $\mu$ L TMAH is needed for 100 mg of hair.)
  - b. Allow the hair to completely digest, vortexing occasionally. (This process typically takes at least 8 hours, and the process may be left to proceed overnight.)
2. Prepare the calibration curve:
  - a. To individually labeled 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with Dilute Nitric Acid in Water Solution add:
    1. Approximately 5 mL Dilute Nitric Acid in Water Solution;
    2. 100  $\mu$ L of negative hair digest (prepared in 8.1 above);
    3. 100  $\mu$ L of each Calibrator Working Solutions (prepared in 6.f above);
    4. 100  $\mu$ L of the Iridium Internal Standard Working Solution (prepared in 6.b above);
    5. Dilute Nitric Acid in Water Solution to the mark, mix well;
  - b. Transfer controls to labeled 15 mL Falcon<sup>®</sup> tubes.
  - c. Prepare a zero calibrator and a Negative Control in the same manner but add 100  $\mu$ L of deionized water instead of a Calibrator Working Solution. Additionally, prepare a Blank (no matrix added) with Internal Standard by adding 100  $\mu$ L Iridium Internal Standard Working Solution to 10 mL with Dilute Nitric Acid in Water Solution.
  - d. Prepare Negative Control in duplicate.
3. Have a second chemist prepare the Positive Controls:
  - a. To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with Dilute



Nitric Acid in Water Solution add:

1. Approximately 5 mL Dilute Nitric Acid in Water Solution;
  2. 100  $\mu$ L of negative hair digest (prepared in 8.1 above);
  3. 100  $\mu$ L of the appropriate Control Solution (7.5 or 200  $\mu$ g/L);
  4. 100  $\mu$ L of the Iridium Internal Standard Working Solution;
  5. Dilute Nitric Acid in Water Solution to the mark, mix well;
- b. Transfer controls to labeled 15 mL Falcon<sup>®</sup> tubes.
  - c. Prepare positive controls in duplicate.

Note: As prepared, the Low Control corresponds to 0.15 ng thallium / mg hair and the High Control corresponds to 4 ng thallium / mg hair.

4. Prepare the unknown samples:

- a. Optional: If specimen size allows, positive specimens are repeated following an external wash. Submerge the hair specimen in acetone, and mix for 10 minutes. Repeat the process three times with deionized water for ten minutes each, and finally for 10 more minutes with acetone. When the unknown hair specimen is washed in this manner, a negative control should also be contemporaneously washed and analyzed alongside the unknown. Allow the specimen to dry in a fume hood or in a heating block at 50°C.
- b. To individually labeled 5 mL (12 x 75) Falcon<sup>®</sup> tubes:
  1. Add a minimum of 5.5 mg of specimen hair in duplicate. Accurately record the mass to the nearest 0.1 mg.  
Note: 5.5 mg is a small amount of hair. In order to ensure that a representative hair sample is analyzed, a larger amount of hair may be cut into small snippets and mixed before removing the 5.5 mg sample. Alternatively, a larger hair sample may be cryoground to mix it well. (The negative control hair digest will be prepared in the same manner as the unknown sample.)
  2. Based upon the recorded weight, add enough TMAH to establish a solution of 5 mg of hair per 100  $\mu$ L of TMAH. (110  $\mu$ L TMAH is added to 5.5 mg hair.)
- c. Allow the hair to completely digest, vortexing occasionally. (This process typically takes at least 8 hours, and the process may be left to proceed overnight.)
- d. To individual 10 mL Vitlab<sup>®</sup> volumetric flasks that have been washed with Dilute Nitric Acid in Water Solution add:
  1. Approximately 5 mL Dilute Nitric Acid in Water Solution;
  2. 100  $\mu$ L of unknown hair digest (prepared above);
  3. 100  $\mu$ L of water;
  4. 100  $\mu$ L of the Iridium Internal Standard Working Solution;
  5. Dilute Nitric Acid in Water Solution to the mark, mix well;
- e. Transfer to labeled 15 mL Falcon<sup>®</sup> tubes.

5. Centrifuge all samples at 3500 rpm for 15 minutes and transfer to new labeled 15 mL Falcon<sup>®</sup> tubes before analysis by ICP/MS.

6. When setting up the sequence, run a nitric acid blank four times followed by a Blank with Internal Standard, followed by a Negative Control. Then run the calibrators, followed by the unknowns bracketed by the Positive Controls. A Blank with Internal Standard followed by a Negative Control may be analyzed between specimens and before the Positive Controls.

## 9 Instrumental Conditions

The following conditions are written to follow Thermo-Fisher's LabBooks software package.

Analytes: Tl (Thallium) and Ir (Iridium)

Acquisition parameters:

Identifier	Dwell time (s)	Channels	Spacing (u)	Measurement mode	Resolution
205Tl (KED)	0.05	1	0.1	KED	Normal
191Ir (KED)	0.05	1	0.1	KED	Normal
				# sweeps = 20	

Monitor analytes:

	Uptake (s)	Wash (s)
Minimum	30	30
Maximum	300	300

Survey scan settings:

Start mass (u)	End mass (u)	Dwell Time (s)	Spacing (u)	Resolution	Measurement mode
4.60	245.00	0.01	0.2	Normal	KED

Interference correction: not applicable

Standards: covered in cal/ctrl section

Quantification:

Analyte	Measurement mode	Quantify	Internal Standard	Fit type	Weighting	Forcing	Use for Semi-Quant
205Tl (KED)	KED	Yes	193 Ir (KED)	Linear	None	Blank	Yes
191Ir (KED)	KED	No	Use as Internal Standard	Linear	None	Blank	Yes

IS Recovery		Low warning limit: 80%	Low failure limit: 75%	
		High warning limit: 120%	High failure limit: 125%	

Ratios: not applicable

Quality Control:

Calibration Tests / Laboratory Control Standard (LCS)

Analyte	Low failure limit	Low warning limit	High warning limit	High failure limit
205Tl (KED)	75%	80%	120%	125%

Continuous Tests / Relative Stability Verification (RSV)

Analyte	Verify	Ignore concentration below	unit	Concentration warning limit	Concentration failure limit
205Tl (KED)	concentration	10	ppb	5%	10%

Autosampler:

Time Settings:	Wash Time (s):	200	Take up Time (s):	45
Rack Settings:	Rack 1 Type:	60-vials (12x5)	Rack 2 Type:	60-vials (12x5)
Autotune Settings:	Autotune rack:	Standard	Autotune vial:	1
Rinse settings	Rinse Rack:	Rinse Station		

## 10 Decision Criteria

Normal hair contains thallium levels less than 0.02 ng/mg. Chronic thallium hair levels in occupational workers have ranged from 0.020 to 0.57 ng/mg. During acute thallium toxicity, thallium hair levels can be greater than 1 ng/mg.

### 10.1 Batch Acceptance Criteria

Thallium should not be detected in the Negative Control Hair specimen above a level of 0.02 ng/mg.

Thallium in the Positive Control Hair specimens should quantitate within  $\pm 20\%$  of the target value.

See the Guidelines for Toxicological Quantitations standard operating procedure (Tox 101) for more information.

## 10.2 Sample Acceptance Criteria

The Iridium (Internal Standard) response in the unknown specimen(s) should fall within 80% and 120% of the Internal Standard response for the initial blank in the run.

## 11 Calculations

Quantitation is performed by constructing a multi-point calibration curve based on the ratio of the intensity for each calibrator level and the internal standard. The curve is forced through a Negative Control / Zero Calibrator specimen with no weighting. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

For hair specimens, 5 mg of hair is used in the place of 0.1 mL of liquid. Therefore, results as received from the instrument for hair should be divided by 50. For example, if a result of 100 µg/L of Tl is obtained for a hair specimen, that corresponds to 2 ng Tl per mg of hair.

When a hair specimen contains thallium above the method's lower limit of quantitation, it may be analyzed again using a method of standard addition to verify the Tl concentration.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- a. Limit of Detection = 0.02 ng/mg (1 µg/L)

b. Limit of Quantitation = 0.02 ng/mg (1 µg/L)

c.

Hair:	@ 0.15 ng/mg (7.5 µg/L)	@ 2.4 ng/mg (1200 µg/L)	@ 4 ng/mg (200 µg/L)
% Bias	-3.63%	-3.68%	-2.91%
% Repeatability	1.83%	1.63%	1.16%
% Intermediate Precision	2.49%	2.79%	2.63%

d. Interferences: no endogenous material/matrices interfered with the analysis of thallium. Additionally, elemental standards of gallium, arsenic, indium, rhodium, cadmium, lead, mercury, bismuth, holmium, lithium, scandium, terbium, yttrium, barium, cerium, cobalt and uranium did not interfere with the thallium signal.

e. Carryover: No carryover was observed when a negative control hair specimen was analyzed immediately following a 5 ng/mg (250 ug/L) calibrator.

f. Processed sample stability: Sample stability was evaluated up to four days after the initial analysis and remained within ± 20% with refrigerated storage.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Kempson I.M., et al. (2012) A Comparison of Washing Methods for Hair Mineral Analysis: Internal Versus External Effects. *Biol Trace Elem Res.* 150 (1-3).

Hirata, et al. (1998) A Probable Case of Chronic Occupational Thallium Poisoning in a Glass Factory. *Industrial Health*. 36, 300-303.

Rusniak (2002) Thallium and Arsenic Poisoning in a Small Midwestern Town. *Annals of Emergency Medicine* 39(3):307-311

Rev. #	Issue Date	History
0	06/03/2016	New document.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Procedure for bench use.**

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## **Analysis of Ethylene Glycol from Blood**

### **1 Introduction**

Ethylene glycol (EG) is a toxic glycol used in coolants and antifreezes. It causes central nervous system depression similar to that of ethanol. It is metabolized in the body to oxalic acid, which is damaging to the kidney.

### **2 Scope**

This procedure allows for the qualitative and quantitative analysis of blood samples for EG. This procedure applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

After addition of an internal standard, blood specimens are mixed with phenylboronic acid in acetone. A small amount of the acetone layer is added to a headspace vial which is crimped. The vial is thermostatted to cause total volatilization of the acetone and ethylene glycol derivative. The headspace is analyzed by HS-GC/MS(EI) (headspace gas chromatography mass spectrometry in electron impact mode).

### **4 Specimens**

0.1 mL of blood is required for each replicate; quantitative analyses are performed in duplicate.

### **5 Equipment/Materials/Reagents**

- a. Gas Chromatograph / Mass Spectrometer (GC/MS) with a headspace autosampler capable of EI ionization and equipped with a 30 m x 0.25 mm x 0.25  $\mu$ m film thickness DB-5 (or equivalent) column
- b. Vortex mixer
- c. Centrifuge
- d. Volumetric pipets with appropriate tips
- e. Routine laboratory supplies, including centrifuge tubes with caps, 10 cc headspace vials with caps, etc.
- f. Phenylboronic acid (HPLC grade)

- g. Acetone (HPLC grade)
- h. Methanol (HPLC grade)
- i. Phenylboronic Acid Derivatizing Reagent (5 mg/mL):  
Add 125 mg of phenylboronic acid to a 25-mL volumetric flask. Dilute to the mark with acetone and mix well. Store at room temperature in glass. Stable for at least one month.

## 6 Standards and Controls

- a. Ethylene glycol (EG) Stock Standard (10 mg/mL):  
For quantitation, two sources of ethylene glycol should be obtained. Ethylene glycol traceable to United States Pharmacopoeia (USP) can be purchased from USP or another approved vendor. Storage and stability are determined by the manufacturer. Add 100 mg of EG to a 10-mL volumetric flask. Bring to the mark with methanol and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- b. EG Calibrator Working Stock - High (2000 µg/mL):  
Add 1.0 mL of the EG Stock Standard (10 mg/mL) to a 5-mL volumetric flask. Bring to the mark with methanol and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- c. EG Calibrator Working Stock - Low (200 µg/mL):  
Add 0.2 mL of the EG Stock Standard (10 mg/mL) to a 10-mL volumetric flask. Bring to the mark with methanol and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- d. EG Control Working Stock - High (2000 µg/mL):  
Add 1.0 mL of the EG Stock Standard (10 mg/mL; different source than that used to make the Calibrator Working Stock) to a 5-mL volumetric flask. Bring to the mark with methanol and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- e. EG Control Working Stock - Low (200 µg/mL):  
Add 0.2 mL of the EG Calibrator Stock Standard (10 mg/mL; different source than that used to make the Calibrator Working Stock) to 10-mL volumetric flask. Bring to the mark with methanol and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.
- f. Negative Control Blood:  
Purchased from Cliniq or another approved vendor. Storage and stability are determined by the manufacturer.  
  
A Negative Control Blood sample is analyzed with every blood assay.
- g. d<sub>4</sub>-Ethylene glycol (d<sub>4</sub>-EG) Internal Standard Working Standard (400 µg/mL):  
d<sub>4</sub>-Ethylene glycol (98% or better) can be purchased from Isotech or another approved vendor.

Storage and stability are determined by the manufacturer. Add 9  $\mu\text{L}$  of  $\text{d}_4\text{-EG}$  to a 25-mL volumetric flask. Bring to the mark with methanol and mix well to dissolve. Store refrigerated in glass; stable for at least 1 year.

h. **Calibrator Sample Preparation:**

On the day of analysis, add EG and makeup methanol to 0.1 mL blood as described in the table below in order to generate a 6 point calibration curve:

Calibrator Level	EG Conc ( $\mu\text{g/mL}$ )	EG Calibrator Working Stock Conc ( $\mu\text{g/mL}$ )	Volume EG Calibrator Working Stock ( $\mu\text{L}$ )	Volume Makeup Methanol ( $\mu\text{L}$ )
1	40	200 (low)	20	70
2	300	2000 (high)	15	75
3	600		30	60
4	1000		50	40
5	1400		70	20
6	1800		90	0

i. **Positive Control Sample Preparation:**

On the day of analysis, have a second chemist add EG and makeup methanol to 0.1 mL blood as described in the table below in order to create low and high positive controls:

Control Level	EG Conc ( $\mu\text{g/mL}$ )	EG Control Working Stock Conc ( $\mu\text{g/mL}$ )	Volume EG Control Working Stock ( $\mu\text{L}$ )	Volume Makeup Methanol ( $\mu\text{L}$ )
1	120	200 (low)	60	30
2	1500	2000 (high)	75	15

Two levels of Positive Control Blood are analyzed in duplicate with each quantitative assay.

j. **Qualitative Analysis:**

For qualitative analysis, a negative control (f), and one Level 1 and one Level 2 Positive Control (i) will be analyzed along with unknown samples.

k. **System Suitability Check:**

An additional Level 1 control will be analyzed prior to unknown analysis to verify instrument performance.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure that may be used at the bench by the chemist performing the procedure.

- a. Label tubes for each sample, calibrator and control.
- b. Add 0.1 mL of blood to a snap-cap centrifuge tube. Case samples are prepared in duplicate for quantitative analysis. Additionally, prepare a negative control and calibrator samples as described in Section 6. Have a second analyst prepare positive control samples as described in Section 6.
- c. Add 90 µL of makeup methanol to all case samples and negative control(s).
- d. Add 25 µL of d<sub>4</sub>-EG Internal Standard Solution (400 µg/mL) to each case sample, calibrator and control.
- e. Add 400 µL of Phenylboronic Acid Derivatizing Reagent.
- f. Cap each tube and vortex-mix for 10 seconds.
- g. Centrifuge at 10,000 rpm for 3 minutes.
- h. Remove 20 µL of the acetone layer to a labeled 10 cc headspace vial and immediately cap.
- i. Analyze by HS-GC/MS(EI) after verifying instrument performance

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions that may be used at the bench by the chemist performing the procedure.

### 9.1 Headspace Sampler Parameters

incubation temperature	125°C	syringe temperature	145°C
incubation time	5 min	injection volume	0.1 mL
agitator speed	300 RPM	sample fill rate	0.050 mL/sec
agitation timing	10 sec on 1 sec off	sample fill strokes	3
use syringe dedicated to high temperatures		sample injection speed	0.25 mL/sec
		syringe flush time	300 sec

## 9.2 GC Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	50°C	inlet temperature	250°C	type	DB5MS
hold 1	0 min	injection mode	Split	length	30 m
ramp 1	20°C/min	split	20:1	internal diameter	0.25 mm
temperature 2	120°C	carrier gas	helium	film thickness	0.25 µm
ramp 2	60°C/min	carrier mode	constant flow		
temperature 3	280°C	carrier flow	1.2 mL/min		
hold 2	3.84 min				

## 9.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	280°C
scan range	35 - 200 m/z	quad temperature	150°C
		solvent delay	4.0 min

## 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this procedure. In general, compound identification will be based on comparison of the chromatography and mass spectrometry for the Calibrator Sample or Positive Control. In most cases, all of the below should be met in order to identify ethylene glycol within a biological specimen.

### 10.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak in a known sample analyzed on the same system in the same analytical runs. Additionally, the following two criteria should be met.

#### 10.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of an extracted Positive Control.

#### 10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10-fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank injected just prior to that sample.

## 10.2 Mass Spectrometry

The following ions are characteristic of the phenylboronic acid derivative of ethylene glycol: 148, 118, and 91. The mass spectrum of the derivatized ethylene glycol should match that of an extracted Positive Control or calibrator. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 11 Calculations

Ethylene glycol is quantitated by calculating the area of derivatized EG to the area of its internal standard (148:152) and plotting these ratios against concentration. Linear regression is used to find the best fit line through the data using 1/x weighting. For additional guidance in performing quantitations, see the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

Results in this method are calculated in the units  $\mu\text{g/mL}$ . Results can also be reported in  $\text{mg/dL}$ . In order to convert from  $\mu\text{g/mL}$  to  $\text{mg/dL}$ , the decimal place is moved once to the left. For example,  $500 \mu\text{g/mL} = 50.0 \text{ mg/dL}$ .

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

The measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- a. Accuracy: Range of -2.73% to +0.50% at three measured concentrations
- b. Calibration Range: 40 – 1800  $\mu\text{g/mL}$
- c. Limit of Detection: 40  $\mu\text{g/mL}$
- d. Precision: Range of 8.73% – 11.24% at three measured concentrations

- e. Processed Sample Stability: Not thoroughly evaluated; samples should be analyzed on the day of preparation.
- f. Interferences: None known. Laboratory experiments have demonstrated that diethylene glycol, triethylene glycol, propylene glycol, glycerol and 1,4-butanediol do not interfere with the method.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Quality Control for Toxicology Examinations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*FBI Laboratory Safety Manual*.

A. Ehlers, C. Morris and M.D. Krasowski. “A rapid analysis of plasma/serum ethylene and propylene glycol by headspace gas chromatography”. *SpringerPlus*, 2 (2013) 1-6.

T. Hlozek, M. Bursova and R. Cabala. “Fast determination of ethylene glycol, 1,2-propylene glycol and glycolic acid in blood serum and urine for emergency and clinical toxicology by GC-FID”. *Talanta*, 130 (2014) 470-414.

W.H. Porter and A. Auansakul. “Gas-chromatographic determination of ethylene glycol in serum”. *Clinical Chemistry*, 28 (1982) 75-78.

R.H. Williams, S.M. Shah, J.A. Maggiore and T.B. Erickson. “Simultaneous detection and quantitation of diethylene glycol, ethylene glycol, and the toxic alcohols in serum using capillary column gas chromatography”. *Journal of Analytical Toxicology*, 24 (2000) 621-626.



Rev. #	Issue Date	History
0	06/03/16	New document
1	11/15/16	Updated internal standard preparation in Section 6g. Subsequently updated step 8d and bench sheet. Updated syringe flush time in Table 9.1.
2	01/10/19	To account for qualitative analysis, the Title and Sections 1, 2, and 4 were updated. Updated Section 2 scope statement. Corrected typo at 5i (ug to mg). 6j was added. Added a system suitability check to 6k. Added 8g centrifuge step. Added clarification to 8i. Removed "subunit" from header, signature lines, and references.

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**Approval**

Chemistry Unit Chief:

Date: 01/09/2019

Tox Technical Leader:

Date: 01/09/2019

**QA Approval**

Quality Manager:

Date: 01/09/2019

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## Analysis of Plant Toxins

### 1 Introduction

Plants contain a variety of chemicals and compounds, many of which can be toxic. Examples include alkaloids such as gelsemine (*Gelsemium*) and glycosides such as digoxin/digitoxin (*Digitalis*), oleandrin (*Nerium*), and cerberin (*Cerbera*).

### 2 Scope

Analyses	<input checked="" type="checkbox"/> Screening <input checked="" type="checkbox"/> Confirmation <input type="checkbox"/> Quantitation
Matrices	Whole blood (0.2 mL per extraction).
Analytes	Digoxin, digitoxin, cerberin, oleandrin, gelsemine.
Personnel	This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

Specimens are diluted and adjusted to basic pH through a combination of aqueous buffers and organic solvent. The resulting solution is mixed and centrifuged. The supernatant is applied to a supported liquid extraction (SLE) column. Organic solvents are used to elute the analytes from the column. The eluent is concentrated, reconstituted and filtered. The prepared extract is analyzed by UPLC-HRMS/MS (ultra- performance liquid chromatography-high resolution tandem mass spectrometry). Three acquisition modes are utilized: full scan (FS; 35,000 resolution), selected ion monitoring (SIM; 35,000 resolution), and tandem mass spectrometry (MS<sup>2</sup>; 17,500 resolution).

#### 4 Procedure

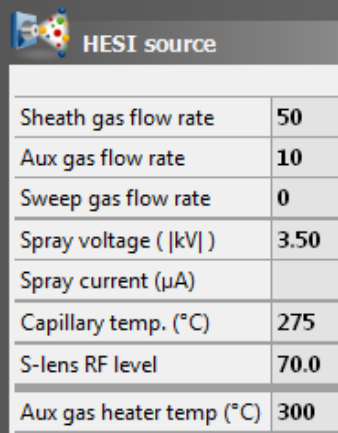
Step	Activity	Material	Reference/Lot
4.1	Materials required per sample: 2 mL Eppendorf tube (1), SLE+ 400 µL cartridge (1), 12 x 75 mm glass tube (1), 0.2 µm centrifugal filter (1), ALS vial (1)		
4.2	Thaw a control set (maintained at -20°C). (0, 1 and 10 ng/mL Controls, 200 µL each; System Suitability Sample (S <sup>3</sup> ), 10 ng/mL)	<a href="#">Control Lots, S<sup>3</sup></a>	[     ] <sub>4</sub>
4.3	Aliquot 200 µL of each case specimen into a 2 mL Eppendorf tube.		
4.4	Add 100 µL of Sample Buffer to each tube. (0.1 M sodium phosphate, pH 6.8)	<a href="#">Sample Buffer</a>	[     ]
4.5	Add 50 µL of Internal Standard Solution (ISS)	<a href="#">ISS</a>	[     ]
4.6	Add 50 µL of pH Modifier. Cap vial. (scan NH <sub>4</sub> OH)	<a href="#">pH Modifier</a>	[     ]
4.7	Vortex at 2000 rpm for 5 minutes at ambient temperature.		
4.8	Centrifuge at 10,000 rpm for 5 minutes at ambient temperature.		
4.9	Load Biotage SLE+ 400 µL cartridges onto positive pressure manifold. Place 12 x 75 mm tubes beneath.	<a href="#">Biotage SLE+ 400 µL</a>	[     ]
4.10	Apply 300 µL of supernatant to SLE+ cartridge		
4.11	Apply a short pulse of maximum nitrogen pressure to load sample onto cartridge. Wait 5 minutes.		
4.12	Apply 750 µL of Elution Solvent 1 to each cartridge (95:5 dichloromethane:isopropanol). Wait 5 minutes.	<a href="#">Elution Solvent 1</a>	[     ]
4.13	Apply 750 µL of Elution Solvent 1 to each cartridge. Wait 5 minutes. Apply low nitrogen flow for ~ 30 seconds to elute Elution Solvent 1.		
4.14	Apply 750 µL of Elution Solvent 2 to each cartridge (MTBE). Wait 5 minutes.	<a href="#">Elution Solvent 2</a>	[     ]
4.15	Apply 750 µL of Elution Solvent 2 to each cartridge. Wait 5 minutes. Apply low nitrogen flow for ~ 30 seconds to elute Elution Solvent 2.		
4.16	Evaporate eluent to dryness at 45°C. Let cool for 5 min.		
4.17	Reconstitute with 100 µL of Reconstitution Solvent to the bottom of the 12 x 75 mm tube. Vortex well.	<a href="#">Reconstitution Solvent</a>	[     ]
4.18	Transfer 100 µL extract to 0.2 µm centrifugal filter. Centrifuge at 10,000 rpm for 5 minutes.	<a href="#">Costar 0.2 µm filter</a>	[     ]
4.19	Transfer extract to Waters ALS vial with 250 µL insert. Cap with Waters pre-slit 12 x 32 mm vial cap.		
4.20	Analyze 20 µL of extract using the parameters in Section 5		

## 5 Instrument Parameters

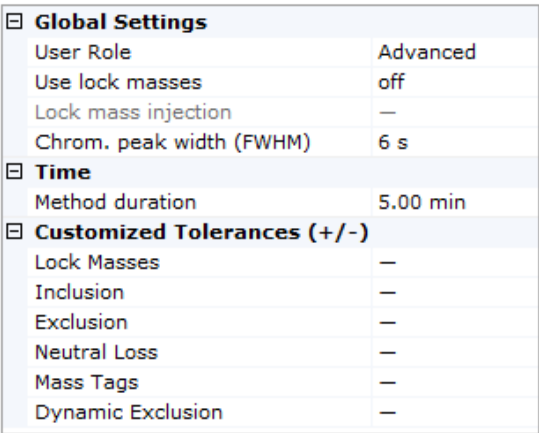
Thermo Fisher Q-Exactive with Waters Acquity I-Class UPLC System

### 5.1 Mass Spectrometry

#### 5.1.1 Heated Electrospray Ionization, Global Settings and Tune File

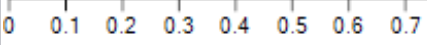


HESI source	
Sheath gas flow rate	50
Aux gas flow rate	10
Sweep gas flow rate	0
Spray voltage (  kV  )	3.50
Spray current (µA)	
Capillary temp. (°C)	275
S-lens RF level	70.0
Aux gas heater temp (°C)	300




<b>Global Settings</b>	
User Role	Advanced
Use lock masses	off
Lock mass injection	—
Chrom. peak width (FWHM)	6 s
<b>Time</b>	
Method duration	5.00 min
<b>Customized Tolerances (+/-)</b>	
Lock Masses	—
Inclusion	—
Exclusion	—
Neutral Loss	—
Mass Tags	—
Dynamic Exclusion	—

C:\Xcalibur\methods\TOX350.mstune



#### 5.1.2 Inclusion List

Method editor — Inclusion List										
File Edit Help									Done 	
	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	MSX ID	Comment
▶ 1	323.17540	C20H22N2O2	+ H	1	Positive	1.40	2.00	60		gelsemine
2	798.46343	C41H64O14	+ NH4	1	Positive	2.90	3.10	10		digoxin
3	577.33711	C32H48O9	+ H	1	Positive	3.32	3.62	10		cerberin and oleandrin
4	782.46852	C41H64O13	+ NH4	1	Positive	3.58	3.90	10		digitoxin
* 5										

The start/stop times listed are nominal. Due to normal column aging and variation in mobile phase preparation, small adjustments to the start and stop times may be required based upon the system suitability sample results.

### 5.1.3 Scan Events

#### Properties of Full MS — SIM

<b>General</b>	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
<b>Full MS — SIM</b>	
Microscans	1
Resolution	35,000
AGC target	5e5
Maximum IT	50 ms
Number of scan ranges	1
Scan range	300 to 840 m/z
Spectrum data type	Profile



#### Properties of Targeted-SIM

<b>General</b>	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Inclusion	on
<b>SIM</b>	
Microscans	1
Resolution	35,000
AGC target	2e5
Maximum IT	200 ms
MSX count	1
Isolation window	1.0 m/z
Isolation offset	0.0 m/z
Spectrum data type	Profile



#### Properties of PRM

<b>General</b>	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Default charge state	1
Inclusion	on
<b>MS<sup>2</sup></b>	
Microscans	1
Resolution	17,500
AGC target	2e5
Maximum IT	100 ms
Loop count	1
MSX count	1
MSX isochronous ITs	on
Isolation window	1.0 m/z
Isolation offset	0.0 m/z
Fixed first mass	—
(N)CE / stepped (N)CE	nce: 35
Spectrum data type	Profile



## 5.2 Liquid Chromatograph (LC) Parameters

### 5.2.1 LC Materials

Component	Description	Reference/Lot
<a href="#">Solvent A1</a>	5mM ammonium formate in water	
<a href="#">Solvent B1</a>	Methanol	
<a href="#">Solvent A2</a>	Methanol:Water 50:50	
<a href="#">Solvent B2</a>	Acetonitrile	
<a href="#">Weak Needle Wash (WNW)</a>	Methanol:Water 10:90	
<a href="#">Strong Needle Wash (SNW)</a>	Methanol:Acetonitrile:Water:Isopropanol 45:40:10:5	
<a href="#">Seal Wash (SW)</a>	Acetonitrile:Water 10:90	
UPLC Column	Waters Acquity UPLC HSS C18 1.8 $\mu$ m, 2.1 x 100 mm	

### 5.2.2 Solvent Manager

ACQ-SM ACQ-BSM

### Binary Solvent Manager

General | Analog Out | Events

**Solvents**

A1 5mM ammonium format

B1 Methanol

**Pressure Limits**

Low: 0 psi

High: 15000 psi

**Seal Wash:** ?

2.0 min

**Gradient**

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	Initial	0.200	50.0	50.0	Initial
2	0.50	0.200	50.0	50.0	6
3	2.50	0.200	5.0	95.0	6
4	3.00	0.200	5.0	95.0	6
5	3.05	0.200	50.0	50.0	6
6	5.00	0.200	50.0	50.0	6
7					

**Gradient Start:**

☒ At injection

☐ Before injection

☐ After injection

0 uL



### 5.2.3 Sample Manager

ACQ-SM | ACQ-BSM

## Sample Manager

General | Events

Wash Solvents

Weak Wash Name:  
10/90 methanol/water

Strong Wash Name:  
45/40/10/5 Strong Wash

Weak Wash Volume:  
1200  $\mu$ L

Strong Wash Volume:  
800  $\mu$ L

Max Sample Volume: 15.00  $\mu$ L

Comment:

Temperature Control

Column: 50.0  $^{\circ}$ C    Alarm Band: ☐  $\pm$  5.0  $^{\circ}$ C

Sample: 14.0  $^{\circ}$ C    ☐  $\pm$  5  $^{\circ}$ C

Loop Offline:  
Disable min

☐ Load Ahead

Active Preheater:  
Enabled

Advanced...

## 6 Equipment/Materials/Reagents

### 6.1 Chemicals and Consumables

Item	Supplier*	Description	Part Number*
Eppendorf Tubes	Eppendorf	Safe-Lock Tubes 2.0mL (polypropylene)	0030 120.094
SLE Cartridge	Biotage	Isolute SLE+, 400 $\mu$ L sample volume	820-0055-B-500
Glass Tube	Fisher	Disposable Culture Tube 12x75 mm	14-961-26
Centrifugal Filter	Corning	Costar Spin-X HPLC 0.2 $\mu$ m with nylon filter	8169
ALS Vials	Waters	Screw Top Vial, 12x32 mm, PTFE/Silicone pre-slit cap (with 250 $\mu$ L insert)	186000307C
Water	Fisher	Optima, LC-MS grade (mobile phase and Reconstitution Solvent)	W6-4

Water	In-house	18 mΩ, deionized	n/a
Methanol	Thermo Scientific	UPLC-MS grade (mobile phase preparation)	A458
Methanol	Fisher	Optima LC-MS grade (sample preparation and solvents)	A4 54-4
Acetonitrile	Fisher	Optima LC-MS grade	A955-5
Isopropanol	Fisher	Optima grade	A4 51
Ammonium formate	Fisher	Optima LC-MS grade	A115
Dichloromethane	Fisher	Optima grade	D151-1
MTBE (Elution Solvent 2)	Sigma-Aldrich	Chromasolv, 99.9%	202 57
Sodium phosphate, monobasic, monohydrate	Fisher	Certified ACS	S3 69
Sodium phosphate, dibasic, heptahydrate	Fisher	Certified ACS	S3 73
Ammonium hydroxide	Fisher	ACS Plus	A669S
Negative Control Matrix	Cliniqa	Blood	n/a
*use of an equivalent product is allowable			

## 6.2 Prepared Mixtures and Solvents

Depending upon the batch size, the absolute amounts may be adjusted so long as the ratios of components are maintained.

### 6.2.1 Sample Buffer (0.1 M sodium phosphate buffer, pH 6.8)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 50 mL
2	Add	40 mL	deionized water
3	Add	656 mg	sodium phosphate, dibasic, heptahydrate
4	Add	352 mg	sodium phosphate, monobasic, monohydrate
5	QS	50 mL	deionized water
6	Mix		
7	Transfer		amber glass
8	Store		refrigerated
	Stability		1 month
	Prepares	50 mL	(500 samples)

## 6.2.2 pH Modifier (2% ammonium hydroxide)

Step	Action	Amount	Component/Information
1	Acquire	1	eppendorf Tube, 2 mL polypropylene
2	Add	2.0 mL	deionized water
3	Add	41 µL	ammonium hydroxide
4	Mix		
5	Store		in tube
	Stability		1 day
	Prepares	2 mL	(40 samples)

## 6.2.3 Elution Solvent 1 (95:5 dichloromethane:isopropanol)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 100 mL
2	Add	57 mL	dichloromethane
3	Add	3 mL	isopropanol
4	Mix		
5	Transfer		amber glass
6	Store		ambient
	Stability		1 year
	Prepares	60 mL	(40 samples)

## 6.2.4 Reconstitution Solvent, Solvent A2 (50:50 methanol:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 25 mL
2	Add	12.5 mL	water (Optima LC-MS)
3	Add	12.5 mL	methanol (UPLC-MS grade)
4	Mix		
5	Transfer		glass
6	Store		ambient or refrigerated or frozen
	Stability		6 months
	Prepares	25 mL	(250 samples)

### 6.2.5 Solvent A1 (5mM ammonium formate in water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	250 mL	water (Optima LC-MS)
3	Add	79 mg	ammonium formate (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Store		ambient or refrigerated
	Stability		10 days
	Prepares	250 mL	

### 6.2.6 Weak Needle Wash (WNW) (10:90 methanol:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	225 mL	water (Optima LC-MS)
3	Add	25 mL	methanol (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Store		ambient
	Stability		3 months
	Prepares	250 mL	

### 6.2.7 Strong Needle Wash (SNW) (45:40:10:5 Methanol:Acetonitrile:Water:Isopropanol)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 500 mL
2	Add	225 mL	methanol (Optima LC-MS)
3	Add	200 mL	acetonitrile (Optima LC-MS)
4	Add	50 mL	water (Optima LC-MS)
5	Add	25 mL	isopropanol (Optima)
6	Mix		
7	Transfer		mobile phase bottle, glass
8	Store		ambient
	Stability		1 year
	Prepares	500 mL	

## 6.2.8 Seal Wash (SW) (10:90 acetonitrile:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	225 mL	water (Optima LC-MS)
3	Add	25 mL	acetonitrile (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Store		ambient
	Stability		3 months
	Prepares	250 mL	

## 7 Standards and Controls

### 7.1 Primary Standards

Analyte	Supplier*	Description	Part Number*
Cerberin	Santa Cruz Biotechnology	1 mg powder	SC-480467
Digoxin	Cerilliant	1.0 mg/mL in methanol	D-029
Digitoxin	Cerilliant	1.0 mg/mL in methanol	D-067
Oleandrin	PhytoLab	10 mg powder	89744
Gelsemine	PhytoLab	10 mg powder	80457
Digoxin-d3	Cayman Chemicals	1 mg powder	10010657
*Use of an equivalent product is allowable. Store at about -20°C. Stability determined by manufacturer			

### 7.2 Primary Standards in Methanol from Solid

For the standards in section 7.1 that are in solid form, perform a dilution to yield a 1.0 mg/mL solution in methanol. For example, remove 1.0 mg of the oleandrin primary standard and add 1.0 mL of methanol. Store at about -20°C in amber glass.

### 7.3 Intermediate Standards (10 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	50 µL	of each 1.0 mg/mL primary standard (excluding digoxin-d3)*
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		amber glass
7	Store		about -20°C
	Stability		2 years
*Make a separate Intermediate Standard containing digoxin-d3 only (internal standard)			

### 7.4 Working Standard (0.25 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	125 µL	of Intermediate Standard (Section 7.3)
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		amber glass
7	Store		about -20°C
	Stability		2 years

### 7.5 Controls (0, 1 and 10 ng/mL in matrix)

Prepare controls according to the table below. Mix each bulk control solution for 30 minutes prior to pipetting into Eppendorf centrifuge tubes (0.2 mL portions each). Store at about -20°C. Stable for two years.

Control Level	Working Standard (Section 7.4) µg/mL	Addition Volume µL	Matrix Volume mL	Concentration ng/mL
Negative	0.25	0	5	0
1 ng/mL	0.25	20	5	1
10 ng/mL	0.25	200	5	10

## 7.6 Internal Standard Solution (80 ng/mL in methanol)(ISS)

Aliquot 40 µL of the digoxin-d3 10 µg/mL solution (from Section 7.3) to a 5 mL glass volumetric flask. QS with methanol (Optima LC-MS). Store at about -20°C in amber glass. Stable for two years.

## 7.7 System Suitability Sample (S<sup>3</sup>)(10 ng/mL)

Prepare the S<sup>3</sup> portions according to the table below.

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	1.7 mL	methanol (Optima LC-MS)
3	Add	200 µL	of Working Standard (Section 7.4)
4	Add	625 µL	of ISS (Section 7.6)
5	QS	5 mL	water (Optima LC-MS)
6	Mix		
7	Transfer		eppendorf vials in 0.2 mL portions
8	Store		about -20°C along with controls
	Stability		2 years

## 8 Decision Criteria

In order for a chromatographic peak to be used for identification, the following criteria must be met:

Retention Time	Mass Accuracy	Signal To Noise	Preceding Negative Sample Response
± 5 % of concurrent standard or extracted control	± 5 mmu	≥ 3	≤ 10

### 8.1 Analyte Specific Decision Criteria

Analyte	Scan Mode	Retention Time†	Adduct / Fragment	m/z
Digoxin	SIM	2.99	M+NH <sub>4</sub>	798.463
	MS <sup>2</sup>	2.99	Fragment	651.373
			Fragment	97.065
			Fragment	391.247
	MS <sup>2</sup> spectra are concentration dependent. Refer to TOX104.			
		2.99	M+NH <sub>4</sub>	798.463



	Full Scan*		M+H	781.436
	<i>*The inclusion of full scan data is optional. Digoxin undergoes in-source fragmentation, as well as forms multiple adducts.</i>			
Digitoxin	SIM	3.65	M+NH <sub>4</sub>	782.469
	MS <sup>2</sup>	3.65	Fragment	635.380
			Fragment	97.065
			Fragment	375.253
	<i>MS<sup>2</sup> spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	3.65	M+NH <sub>4</sub>	782.469
	<i>*The inclusion of full scan data is optional. Digitoxin forms primarily the ammonium adduct.</i>			
Cerberin	SIM	3.53	M+H	577.337
	MS <sup>2</sup>	3.53	Fragment	203.091
			Fragment	171.065
	<i>MS<sup>2</sup> spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	3.53	M+H	577.337
			M+NH <sub>4</sub>	594.364
	<i>*The inclusion of full scan data is optional. Cerberin forms primarily the protonated adduct as well as an ammonium adduct at a lower abundance.</i>			
Oleandrin	SIM	3.41	M+H	577.337
	MS <sup>2</sup>	3.41	Fragment	373.237
			Fragment	433.258
			Fragment	113.060
	<i>MS<sup>2</sup> spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	3.41	M+H	577.337
		M+NH <sub>4</sub>	594.364	
	<i>*The inclusion of full scan data is optional. Oleandrin forms primarily the protonated adduct as well as an ammonium adduct at a lower abundance.</i>			
Gelsemine	SIM	1.63	M+H	323.175
	MS <sup>2</sup>	1.63	Fragment	70.065
			Fragment	236.106
			Fragment	195.067
	<i>MS<sup>2</sup> spectra are concentration dependent. Refer to TOX104.</i>			
	Full Scan*	1.63	M+H	577.337
	<i>*The inclusion of full scan data is optional. Gelsemine does not form additional adducts.</i>			
Digoxin-d3	Full Scan*	2.98	M+NH <sub>4</sub>	801.482

† The retention times listed are nominal. Due to normal column aging and variation in mobile phase preparation, small adjustments to the start and stop times may be required based upon the system suitability sample results.



## 8.2 Batch Acceptance

### 8.2.1 Control Criteria

Target analytes will not be detected in the Negative Control. The S<sup>3</sup>, 1 and 10 ng/mL Positive Control will have all target analytes identified. Either a positive control or an unextracted standard may be used for mass spec/ion ratios comparisons as needed. For an individual case, the target analytes required may vary.

### 8.2.2 Internal Standard

The internal standard will be recovered via full scan for each control and unknown sample.

### 8.2.3 Planned Action on QC Failure

Refer to TOX101 for potential responses to QC failure(s).

## 9 Limitations

### 9.1 Limit of Detection (LOD)

Analyte	Matrix	LOD (ng/mL)
Digoxin	Blood	0.5
Digitoxin	Blood	1
Cerberin	Blood	0.1
Oleandrin	Blood	0.1
Gelsemine	Blood	0.1

### 9.2 Interferences, Isomers, and Interpretation

No interferences identified. Cerberin and oleandrin are isotopomeric isomers. Baseline or near baseline resolution of these two analytes is required to differentiate on the basis of the protonated ion alone. However, the analytes do have different tandem mass spectra. While digoxin (and digitoxin, to a lesser extent) are available as highly purified preparations for medical use, other plant toxins are often present in unprocessed or less purified forms. Potential poisonings from these types of scenarios may generate multiple analytes and metabolites that may be similar in structure and mass spectra to validated analytes. A combination of full scan, SIM, and MS<sup>2</sup> analyses may be used to investigate potential additional analytes of interest.

## 10 Sampling

Not applicable.

## 11 Calculations

Not applicable

## 12 Measurement Uncertainty

Not applicable.

## 13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 14 References

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Rev. #	Issue Date	History
0	07/09/2019	New document

**Approval**

Redacted - Signatures on File

Acting Toxicology  
Technical Leader:

Date: 07/03/2019

Chemistry Unit Chief:

Date: 07/003/2019

**QA Approval**

Quality Manager:

Date: 07/03/2019

## **Solid Phase Extraction of Cocaine and Metabolites from Biological Specimens**

### **1 Introduction**

Cocaine is a naturally occurring stimulant that is found in the leaves of the *Erythroxylon coca* plant. The primary metabolites of cocaine in humans are benzoylecgonine and methylecgonine. Cocaethylene is another biotransformation product of cocaine that is produced when cocaine and ethanol are used together.

### **2 Scope**

This procedure is used to confirm and quantitate cocaine (COC), benzoylecgonine (BE) and cocaethylene (CE) in biological specimens; it is also used to identify methylecgonine (EME), but results are not reported quantitatively. It is derived from "Cocaine and Benzoylecgonine in Serum, Plasma, or Whole Blood" which is published in the Clean Screen Application Manual by Worldwide Monitoring. The published extraction procedure of biofluids is essentially followed intact, but analysis is accomplished by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI -MS/MS).

### **3 Principle**

Biological specimens are assayed for the presence of cocaine and metabolites. Specimens are mixed with an internal standard solution containing the deuterated analogs of the analytes of interest. The specimens are prepared for solid phase extraction (SPE) via centrifugation and/or dilution. Extractions are accomplished through the use of Clean Screen DAU SPE cartridges. Cocaine and metabolites are eluted from the SPE cartridge using a mixed solvent system of methylene chloride, isopropanol, and ammonium hydroxide. The eluent is taken to dryness, reconstituted in unbuffered LC mobile phase and analyzed directly by LC-ESI-MS.

### **4 Specimens**

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or a prepared tissue homogenate (1:1 in deionized water). Typically, 1 mL of specimen is used for a screen or for qualitative analysis. For quantitative analysis, one – 1.0 mL aliquot and one – 0.5 mL aliquot will be analyzed. In instances where sample volume is limited or there is reason to suspect a sample of being a strong positive, (due to case history or a saturated immunoassay response) a smaller volume of specimen, diluted to 1.0 mL with deionized water, may be used.

## 5 Equipment/Materials/Reagents

- a. 12 x 75 mm test tubes
- b. 16 x 100 mm test tubes
- c. Vortexer
- d. Centrifuge
- e. Clean Screen DAU SPE Cartridges (United Chemical Technologies, Bristol, PA)
- f. Solid phase extraction vacuum manifold or positive pressure manifold
- g. Liquid Chromatograph-Mass Spectrometer capable of data dependent tandem operation in the electrospray ionization mode
- h. Grace 5  $\mu$ m particle silica HPLC column, 2.1 x 150 mm (or equivalent)
- i. Miscellaneous routine laboratory glassware and supplies
- j. Deionized water
- k. Methanol (HPLC grade)
- l. Acetonitrile (HPLC grade)
- m. 0.1 M Sodium Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify  $5.8 < \text{pH} < 6.1$ . Store refrigerated in glass. Stable 2 months.
- n. SPE Elution Solvent (78:20:2 Methylene Chloride:Isopropanol:Ammonia):  
Combine 20 mL HPLC grade isopropanol with 2 mL concentrated ammonium hydroxide and mix well. Add 78 mL HPLC grade methylene chloride and mix well. Store in glass at room temperature. Prepare fresh.
- o. 0.1 M Hydrochloric Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.8 mL concentrated hydrochloric acid. Bring to 96 mL with deionized water and mix well. Store in glass at room temperature. Stable 6 months.
- p. 95:5 Methanol:Water:  
Combine 95 mL methanol (HPLC grade) and 5 mL deionized water in a graduated cylinder.

Mix well. Store in glass or plastic at room temperature. Stable 12 months.

- q. LC Mobile Phase (95:5:0.03 Methanol:Water:Ammonia):  
Combine 950 mL HPLC grade methanol and 50 mL deionized water. Mix well. Add 0.3 mL concentrated ammonium hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 2 weeks.
- r. pH paper
- s. Evaporator with nitrogen
- t. Rotator

## 6 Standards and Controls

- a. d<sub>3</sub>-Cocaine Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- b. d<sub>3</sub>-Methylecgonine Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- c. d<sub>3</sub>-Cocaethylene Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- d. d<sub>8</sub>-Benzoylecgonine Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- e. Internal Standard Working Mixture (d<sub>3</sub>-Cocaine, d<sub>3</sub>-Methylecgonine, d<sub>3</sub>-Cocaethylene, and d<sub>8</sub>-Benzoylecgonine - 10 µg/mL):  
To a 10-mL volumetric flask, add 5 mL of acetonitrile and 1 mL each of the d<sub>3</sub>-cocaine, d<sub>3</sub>-methylecgonine, d<sub>3</sub>-cocaethylene and d<sub>8</sub>-benzoylecgonine stock standards. Add 20 µL of 0.1 M HCl. Dilute to the mark with acetonitrile. Mix well. Store below 0°C. Stable for at least 2 years.
- f. Cocaine Stock Standard (1.0 mg/mL):  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- g. Methylecgonine Stock Standard (1.0 mg/mL):

Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.

- h. **Cocaethylene Stock Standard (1.0 mg/mL):**  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- i. **Benzoyllecgonine Stock Standard (1.0 mg/mL):**  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- j. **Intermediate Calibration Standard (Cocaine and Benzoyllecgonine - 20 µg/mL; Cocaethylene - 10 µg/mL):**  
To a 10-mL volumetric flask, add 200 µL each of the Cocaine and Benzoyllecgonine Stock Standards, and 100 µL of the Cocaethylene Stock Standard. Add 50 µL of 0.1 M HCl. Dilute to the mark with acetonitrile. Mix well. Store below 0°C. Stable for at least one year.
- k. **Working Calibration Standard (Cocaine and Benzoyllecgonine - 2 µg/mL; Cocaethylene - 1 µg/mL):**  
Add 1.0 mL of the Intermediate Calibration Standard to a 10-mL volumetric flask. Add 50 µL of 0.1 M HCl. Dilute to the mark with deionized water. Mix well. Prepare fresh.

Table 1 shows the concentrations and volumes used for preparation of typical calibrators.

Table 1: Typical Calibrator Preparation for Quantitations

Calibrator Level (ng/mL)	Volume of Matrix (mL)	Volume of Working Calibration Standard (mL)
0	1.0	0
50/25	1.0	0.025
100/50	1.0	0.050
400/200	1.0	0.200
700/350	1.0	0.350
1000/500	1.0	0.500

- l. **Intermediate Control Standard (Cocaine and Benzoyllecgonine - 80 µg/mL; Cocaethylene and Methylecgonine - 40 µg/mL):**  
To a 10-mL volumetric flask, add 800 µL each of the Cocaine and Benzoyllecgonine Stock Standards, and 400 µL each of the Cocaethylene and Methylecgonine Stock Standards. Add 50 µL of 0.1 M HCl. Dilute to the mark with acetonitrile. Mix well. Store below 0°C. Stable for at least one year.



- m. Working Control Standard (Cocaine and Benzoylecgonine – 8 µg/mL; Cocaethylene and Methylecgonine – 4 µg/mL):  
Add 1.0 mL of the Intermediate Calibration Standard to a 10-mL volumetric flask. Add 50 µL of 0.1 M HCl. Dilute to the mark with deionized water. Mix well. Prepare fresh.
- n. Positive Control:  
Prepared in-house on the day of extraction as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Typical control concentrations are 160 and 800 ng/mL for benzoylecgonine and cocaine and 80 and 400 ng/mL for cocaethylene and methylecgonine. A Positive Control is extracted and analyzed with every quantitative assay. The Positive Control will be matrix matched, when possible. (The deuterated analogs also serve as qualitative positive controls for each specimen.)
- Typical Positive Control Preparation:
- |              | µL Working Control Standard to add to 1 mL blood |
|--------------|--|
| Low Control  | 20   |
| High Control | 100  |
- o. Negative Control:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Clinical Controls International, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control is extracted and analyzed with every quantitative assay. The Negative Control will be matrix matched, when possible.
- p. LC/MS Performance Standard (1 µg/mL each of cocaine, benzoylecgonine, cocaethylene, and methylecgonine):  
To a 25 mL volumetric flask, add 25 µL each of the benzoylecgonine, cocaethylene, cocaine, and methylecgonine stock standards. Fill to the mark with acetonitrile. Store in glass below 0°C. Stable for at least one year. A 5 µL portion of this mixture is analyzed by LC/MS/MS under the instrumental conditions given in Section 10 of this procedure each day before the instrument is used for case samples.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To properly labeled 16 x 100 mm test tubes add 1 mL of control or biological fluid or 1 g of prepared tissue homogenate.
- b. Prepare calibration curves as shown in Table 1 above.
- c. Add 25  $\mu$ L of the Internal Standard Working Mixture. This will result in working concentrations of 250 ng/mL of each deuterated analog.
- d. Bring all samples to approximately 5 mL with deionized water and vortex.
- e. Whole blood and tissue homogenates (skip to step f. for urine, serum, or vitreous specimens) should stand for 5 minutes before centrifuging for 10 minutes. Discard any resulting pellet.
- f. Add 2 mL of 0.1 M phosphate buffer to each specimen. Vortex.
- g. Verify pH of each specimen is  $6.0 \pm 0.5$ .
- h. Prepare SPE cartridges.
- i. Pre-rinse SPE extraction cartridge by adding 3 mL of Elution Solvent followed by 3 mL of methanol.
- j. Condition column with 3 mL of deionized water followed by 1 mL of 0.1 M phosphate buffer.
- k. Load sample on SPE cartridge.
- l. Rinse column with 2 mL of deionized water, 2 mL of 0.1 M hydrochloric acid, and 3 mL of methanol.
- m. Dry column under full vacuum for 90 seconds.
- n. Apply 3 mL of Elution Solvent and collect eluent in 12 x 75 mm test tubes.
- o. Evaporate to dryness under nitrogen at 40°C.
- p. Reconstitute the residue in 100  $\mu$ L of 95:5 methanol:water.

- q. Prior to analysis of any case samples by LC/MS/MS, analyze 5 µL of the LC/MS Performance Standard using the instrumental parameters that follow in order to verify that the system is working properly. If the decision criteria in Section 11.1 of this procedure are met, proceed to step r. Otherwise, perform any appropriate instrument maintenance.
- r. Inject 5 µL of the extract into the LC/MS operated in positive ion electrospray ionization mode.

## 9 Instrumental Conditions

Following are the operating parameters for the instruments used in this procedure. Appendix 2 contains an abbreviated version of instrumental parameters used in this procedure that may be used by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Parameters		Column Parameters	
composition	95:5:0.03 methanol: water: ammonia	type	silica
isocratic flow	0.3 mL/min	length	150 mm
run time	15 min	internal diameter	2.1 mm
temperature	ambient	particle size	5 µm
		column temp	30°C

## 9.2 Mass Spectrometer Parameters

Segment #1 (0 - 5 min)			
Scan Event #1		Scan Event#2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	full scan; unit resolution	scan mode	product ion MS/MS; unit resolution
scan range	260 - 360 m/z	precursor ions	290, 304 and 318 m/z
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		collision energy	45% relative
		isolation width	1.5 AMU
		product scan range	software control
Segment #2 (5 – 15 min)			
Scan Event #1		Scan Event#2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	full scan; unit resolution	scan mode	product ion MS/MS; unit resolution
scan range	170 - 230 m/z	precursor ion	200 m/z
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		collision energy	40% relative
		isolation width	1.5 AMU
		product scan range	70 - 230 m/z

## 10 Decision Criteria

### 10.1 LC/MS Performance Standard Decision Criteria

#### 10.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each analyte in the performance standard should generate Gaussian shaped chromatographic peaks. The following molecular ions should be traced for each analyte: cocaine – 304, methylecgonine – 200, cocaethylene – 318, benzoylecgonine – 290.

The retention times of the 4 analytes should be within  $\pm 5\%$  of the previous run of the performance standard. Minor changes in mobile phase percentage may account for slight retention time shifts.

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

### **10.1.2 Mass Spectrometry**

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the four analytes in the performance standard should be present. The following molecular ions should be present as the base peak for each analyte: cocaine – 304, methylecgonine – 200, cocaethylene – 318, benzoylecgonine – 290.

### **10.2 Batch Acceptance Criteria**

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All analytes should be detected in the Positive Control. Each Quantitative Positive Control should quantitate within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for more information.

### **10.3 Analyte Decision Criteria**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, the criteria in sections 11.2.1 through 11.2.3 should be met in order to identify cocaine, benzoylecgonine, methylecgonine or cocaethylene within a biological specimen:

#### **10.3.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

##### **10.3.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

##### **10.3.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

### 10.3.2 Analyte Stability

In order to ensure that there has been no significant hydrolysis of cocaine during the sample preparation and analysis, ensure that any peak for d<sub>3</sub>-benzoylecgonine (trace m/z 293 in full scan) is less than 1% of the area for d<sub>8</sub>-benzoylecgonine (trace of m/z 298). If this criterion is not met, results may still be used for qualitative identification of the various analytes of interest, but no quantitative results from the assay should be reported.

Note: If the d<sub>8</sub>-benzoylecgonine has appreciable amounts of d<sub>3</sub>-benzoylecgonine in it (which happens in an occasional lot; the user will know this is the case if all samples in a batch fail the stability test), further steps may be necessary.

1. With the batch, analyze a portion of the 10 ppm Internal Standard working solution 10-fold diluted in 95/5 methanol water.
2. Measure the d<sub>3</sub>-benzoylecgonine to d<sub>8</sub>-benzoylecgonine ratio in this sample.
3. The d<sub>3</sub>-benzoylecgonine to d<sub>8</sub>-benzoylecgonine ratio in the extracted samples may not exceed this ratio by more than 0.01 (1% absolute).

### 10.3.3 Mass Spectrometry

The MS/MS fragmentation spectra should meet the following independent criteria for each compound identified.

- a. Cocaine: (fragments of m/z 304) The base peak should be m/z 182, with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 150.
- b. Cocaethylene: (fragments of m/z 318) The base peak should be m/z 196, with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for m/z 150.
- c. Benzoylecgonine: (fragments of m/z 290) The base peak should be m/z 168, with no other fragment more than 15% of the base peak intensity. Additionally, there should be chromatographically detectable traces for both m/z 150 and m/z 272.
- d. Methylecgonine: (fragments of m/z 200) The base peak should be m/z 182, with no other fragment more than 15% of the base peak intensity. Additionally, there should be chromatographically detectable traces for both m/z 82 and m/z 156.

### 10.4 Reporting Cocaine

To report cocaine qualitatively based upon this method, the area of the M+H peak for cocaine must be greater than or equal to 5% of the M+1 peak for benzoylecgonine. Quantitative results for cocaine that are 5% or less than the benzoylecgonine amount measured may be reported if the

results for cocaine are above 50 ng/mL.

## 11 Calculations

Linear regression analysis with 1/x weighting is performed for cocaine, benzoylecgonine and cocaethylene quantitation. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices for calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- a. Linearity: Cocaine: 50 - 1000 ng/mL  
 Benzoylecgonine: 50 - 1000 ng/mL  
 Cocaethylene: 25 - 500 ng/mL
- b. Limit of Detection: Cocaine: 10 ng/mL, or lower  
 Benzoylecgonine: 10 ng/mL, or lower  
 Methylecgonine: 5 ng/mL, or lower  
 Cocaethylene: 5 ng/mL, or lower

- c. Bias:

	%; at 75/50 ng/mL	%; at 500/250 ng/mL	%; at 800/400 ng/mL
COC	3.07	-0.32	-1.00
BE	-1.41	-2.52	-3.22
CE	15.61	7.95	5.11

d. Repeatability:

	%; at 75/50 ng/mL	%; at 500/250 ng/mL	%; at 800/400 ng/mL
COC	4.63	3.25	5.14
BE	2.50	3.23	2.35
CE	5.81	4.35	4.32

e. Intermediate Precision:

	%; at 75/50 ng/mL	%; at 500/250 ng/mL	%; at 800/400 ng/mL
COC	8.46	3.33	5.63
BE	4.31	4.11	2.40
CE	6.71	5.46	6.73

f. Interferences: None known. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Solid Phase Extraction Application Manual*; United Chemical Technologies. Lewistown, PA, 2004; pp. 29-31.

Mule, S. J.; Casella, G. A. *J. Anal. Toxicol.* 1988, 12, 153-155.

Jeanville, P.M.; Estape, E.S.; et al. *J. Am. Soc. Mass Spectrom.* 2000, 11, 257-263.

Jeanville, P.M.; Estape, E.S.; et al. *J. Anal. Toxicol.* 2001, 25, 69-75.

*FBI Laboratory Safety Manual*.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

FBI Laboratory Chemistry Unit - Instrument Operation and Support Subunit SOP Manual.



Rev. #	Issue Date	History
4	02/27/13	In 5h, updated column brand name. Added cocaine reporting criteria in 11.3.
5	10/01/14	Added instructions for preparation of reagents in Section 5. Added a second source for standards in Sections 6f, 6h and 6i. Updated calibration and control preparation instructions in Section 6j – 6n. Moved Table 1 to Section 6k, removed Calibration Section (7) and renumbered subsequent sections. In Section 8d, updated step involving addition of water to samples for clarity. Removed reference to high flow cartridges from 8h. Added Section 10.2 and renumbered subsequent sections. Added text to 10.3.2 to explain what to do if the d <sub>8</sub> -benzoylegonine standard is contaminated with d <sub>3</sub> -benzoylegonine. Removed reagent preparation instructions from Appendix 1. Reformatted Appendix 2 to include all pertinent instrumental parameters.
6	09/28/15	Removed references to Tox 103 (reagent SOP) in Sections 5 and 15. In Section 5.q, updated mobile phase expiration to 2 weeks. Removed methylecgonine from 6.j and 6.k since it is not quantitated. Updated worksheet in Appendix 1.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the SPE Cocaine Procedure for bench use.**

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**Appendix 2: Abbreviated instrumental parameters for the SPE Cocaine Procedure for bench use.**

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## **Benzodiazepines and Metabolites from Biological Fluids by LC/MS (ESI)**

### **1 Introduction**

Benzodiazepines are one of the most commonly prescribed classes of drugs in the United States. They are also frequently abused. Although immunoassays and other screening techniques may be very sensitive for some benzodiazepines, they may not provide enough cross reactivity for detecting the wide variety of benzodiazepines available today in this country, and abroad. See the *ELISA Screening* standard operating procedure (Tox 209) and the individual immunoassay kit inserts for limits of detection and cross-reactivity information.

This procedure qualitatively detects and confirms benzodiazepines and their metabolites in biological fluids. It is derived from "Flunitrazepam, 7-Aminoflunitrazepam & Desmethyflunitrazepam in Urine for GC/MS Confirmations Using 200 mg CLEAN SCREEN DAU Extraction Column" published by United Chemical Technologies, Inc. The published extraction procedure is essentially followed intact to prepare the crude extract. The extract is analyzed without derivatization by liquid chromatography/electrospray high resolution mass spectrometry LC/MS(ESI) in full scan, MS/MS, or data dependent scanning mode.

### **2 Scope**

This procedure allows for the screening and confirmation of benzodiazepines in biological fluids.

### **3 Principle**

Biological specimens are qualitatively assayed for benzodiazepines and their metabolites. Specimens are mixed with deuterated internal standards. Samples are extracted using CLEAN SCREEN<sup>®</sup> DAU solid phase extraction cartridges. Benzodiazepines and their metabolites are eluted using an elution solvent of ethyl acetate and ammonium hydroxide. The eluent is dried, reconstituted, and analyzed by LC/MS(ESI). MS analysis may be full scan, MS/MS, or by data dependent scanning.

### **4 Specimens**

This procedure uses a biological fluid such as: blood, serum, plasma, urine, vitreous humor, or tissue homogenate (1:1). When available, 2.5 mL of urine or 1 mL of blood are used.

## 5 Equipment/Materials/Reagents

- a. Screw-top test tubes (16 x 125 mm or 16 x 100 mm) with caps and teflon inserts
- b. Analytical balance
- c. Adjustable pipettes (5 - 50  $\mu$ L and 0.5 - 5 mL) with disposable tips
- d. Vortex Mixer
- e. Centrifuge
- f. Heating block
- g. CLEAN SCREEN DAU Solid Phase Extraction Cartridges (200 mg /10 mL)
- h. Vacuum Manifold or Positive Pressure Manifold
- i. 10 x 75 mm or 12 x 75mm test tubes
- j. Evaporator with nitrogen
- k. Deionized water (18 M $\Omega$ )
- l.  $\beta$ -Glucuronidase – 2M Units; from abalone (Campbell Science, Rockford, IL)
- m. 100 mM Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify 5.8<pH<6.1. Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months
- n. Sodium Acetate Buffer (0.1 M):  
To a 250-mL volumetric flask, add 200 mL deionized water, and 1.464 g sodium acetate trihydrate. Mix well to dissolve. Add 0.81 mL glacial acetic acid, and mix well. Verify 4.0<pH<5.0. Bring to the mark with deionized water. Store refrigerated in glass. Stable 3 months.
- o. Wash Solvent (20% Acetonitrile/0.1 M Phosphate Buffer):  
Combine 80 mL 0.1 M phosphate buffer (pH 6) with 20 mL acetonitrile and mix well. Store in glass at room temperature. Stable 2 months.
- p. Elution Solvent (Ethyl Acetate with 2% Ammonium Hydroxide):  
Combine 49 mL ethyl acetate with 1 mL concentrated ammonium hydroxide and mix well. Store in glass at room temperature. Prepare fresh.
- q. Methanol (HPLC grade)

- r. Hexane (Pesticide grade)
- s. 15-cm x 2.1-mm x 5- $\mu$ m Alltech Alltima C18 (or equivalent) LC analytical column
- t. HPLC-Kingdon trap Fourier transform MS instrument with at least 7500 mass axis resolution capable of data-dependent dynamic MSMS
- u. LC Mobile Phase – Benzodiazepines (60:40:0.03 v:v:v methanol:water:ammonia): Combine 300 mL HPLC grade methanol and 200 mL deionized water. Mix well. Add 0.15 mL concentrated ammonium hydroxide and mix gently. Verify pH>8. Store in glass at room temperature. Stable 2 weeks.
- v. Routine laboratory supplies, including disposable glass pipets, autosampler vials with caps, spatulas, graduated cylinders, test tube racks, pH paper etc.

## 6 Standards and Controls

- a. d<sub>5</sub>-Alprazolam Stock Standard (100  $\mu$ g/mL):  
Purchased from Cerilliant Corporation, or another approved vendor. Storage conditions and stability determined by manufacturer.
- b. d<sub>7</sub>-Flunitrazepam Stock Standard (100  $\mu$ g/mL):  
Purchased from Cerilliant Corporation, or another approved vendor. Storage conditions and stability determined by manufacturer.
- c. d<sub>7</sub>-7-Aminoflunitrazepam Stock Standard (100  $\mu$ g/mL):  
Purchased from Cerilliant Corporation, or another approved vendor. Storage conditions and stability determined by manufacturer.
- d. d<sub>5</sub>-Diazepam Stock Standard (100  $\mu$ g/mL):  
Purchased from Cerilliant Corporation, or another approved vendor. Storage conditions and stability determined by manufacturer.
- e. Benzodiazepine Internal Standard Working Solution (0.5  $\mu$ g/mL):  
To a 100-mL volumetric flask, add 0.5 mL each of the following Stock Standards: d<sub>5</sub>-Alprazolam d<sub>7</sub>-Flunitrazepam, d<sub>7</sub>-7-Aminoflunitrazepam and d<sub>5</sub>-Diazepam. Bring to volume with deionized water. Mix well. Store in glass or plastic. Stable for at least 2 years frozen, or at least 1 year refrigerated.
- f. d<sub>5</sub>-Oxazepam Glucuronide Stock Standard (100  $\mu$ g/mL):  
Purchased from Cerilliant Corporation, or another approved vendor. Storage conditions and stability determined by manufacturer.
- g. Hydrolysis Check Internal Standard (2  $\mu$ g/mL d<sub>5</sub>-Oxazepam equivalent):  
To a 10-mL volumetric flask, add 0.32 mL of the d<sub>5</sub>-Oxazepam Glucuronide Stock

Standard. Bring to volume with acetonitrile. Store at  $<0^{\circ}\text{C}$  in plastic or glass. Stable for at least 6 months.

- h. Benzodiazepine Multi-Component Mixture-8 Stock Standard (250 ug/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam): Purchased from Cerilliant Corporation, or another approved vendor. Storage conditions and stability determined by manufacturer.
- i. Benzodiazepine Multi-Component Mixture-8 Stock Standard Working Solution (5 ug/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam):  
To a 50 mL volumetric flask add 1.0 mL of Benzodiazepine Multi-component Mixture-8 Stock Stock Standard and dilute to the mark with acetonitrile. Store below  $0^{\circ}\text{C}$  in glass or plastic. Stable for at least 2 years.
- j. LC/MS Performance Standard (2.5 ug/mL of alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam):  
Combine equal volumes (i.e. 0.1 mL each) of the Benzodiazepine Multi-Component Mixture-8 Stock Standard Working Solution and deionized water. Prepare daily, as needed.
- k. Drug Standards:  
The following are purchased as 0.1 or 1.0 mg/mL solutions from Cerilliant Corporation, or another approved vendor. Storage conditions and stability are determined by the manufacturer:
  - $\alpha$ -hydroxyalprazolam (0.1 mg/mL)
  - 7-aminoclonazepam (0.1 mg/mL)
  - Midazolam (1 mg/mL)
  - Nordiazepam (1 mg/mL)
- l. Four Working Drug Standard Mix (5  $\mu\text{g/mL}$  each):  
The drug standards in 6k. above are diluted by adding 50  $\mu\text{L}$  of each 1 mg/mL standard and 500  $\mu\text{L}$  of each 0.1 mg/mL standard to a 10-mL volumetric flask and bringing to the mark with acetonitrile. Store below  $0^{\circ}\text{C}$  in glass or plastic. Stable for at least 2 years.
- m. Negative Control Urine:  
Prepared in-house or purchased from an appropriate vendor. Stable for 6 months when refrigerated. A Negative Control Urine sample will be extracted and analyzed with every urine assay.
- n. Positive Control Spike Mix (0.5  $\mu\text{g/mL}$  each alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam,  $\alpha$ -hydroxyalprazolam, 7-aminoclonazepam, midazolam and nordiazepam):  
Add 2.5 mL each of the Benzodiazepine Multi-Component Mixture-8 Stock Standard Working Solution (6.i above) and the Four Working Drug Standard Mix (6.l above) to a 25-mL volumetric flask and bring to the mark with acetonitrile. Store below  $0^{\circ}\text{C}$  in glass or plastic. Stable for at least 2 years.

- o. Positive Control Urine Specimen (25 ng/mL each alprazolam,  $\alpha$ -hydroxy-alprazolam, clonazepam, 7-aminoclonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam and temazepam):

Add 0.125 mL of the Positive Control Spike Mix to 2.5 mL of Negative Control Urine on the day of sample preparation.

A Positive Control Urine sample will be extracted and analyzed with every urine assay.

- p. Negative Control Blood:  
Purchased from Cliniqa or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

- q. Positive Control Blood (25 ng/mL each alprazolam,  $\alpha$ -hydroxy-alprazolam, clonazepam, 7-aminoclonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam and temazepam):

Add 0.050 mL of the Positive Control Spike Mix to 1.0 mL of Negative Control Blood on the day of sample preparation.

A Positive Control Blood sample will be extracted and analyzed with every blood assay.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 8.1 Sample Preparation for Blood Specimens

- a. Into properly-labeled test tubes (16 x 100 mm or 16 x 125 mm) add one (1) mL of control or questioned blood specimens.
- b. Add 40  $\mu$ L of the Benzodiazepine Internal Standard Working Solution to each specimen. This yields a final concentration of 20 ng/mL for each deuterated analog.
- c. Add 7 mL of 100 mM phosphate buffer. Vortex.
- d. Centrifuge at high speed for at least 15 minutes.
- e. Transfer supernatant liquid to a clean test tube, leaving any solids behind.



- f. Bring volume up to 9 mL with deionized water.
- g. Verify pH of specimen is in the range of  $6 \pm 0.5$ .

## 8.2 Sample Preparation for Urine Specimens

- a. Into properly-labeled test tubes (16 x 100 mm or 16 x 125 mm) add 2.5 mL of control or questioned urine specimens.
- b. Add 100  $\mu$ L of Benzodiazepine Internal Standard Working Solution to each specimen. This yields a final concentration of 20 ng/mL for each deuterated analog.
- c. Enzymatically hydrolyze the sample as follows.
  - 1. Add 25  $\mu$ L of the Hydrolysis Check Internal Standard to each sample and control to be hydrolyzed. This yields a 20 ng/mL concentration of d<sub>5</sub>-oxazepam.
  - 2. Adjust pH to  $4.5 \pm 0.5$  with 2 mL of sodium acetate buffer (0.1 M) coupled with the addition of 50  $\mu$ L of 6-glucuronidase from abalone.
  - 3. Vortex, cap, and incubate 30 minutes at 70°C.
  - 4. Cool to room temperature.
- d. Add 3 mL of 100 mM phosphate buffer to each tube. Vortex.
- e. Centrifuge at high speed for at least 10 minutes.
- f. Transfer supernatant liquid to a clean test tube, leaving any solids behind.
- g. Verify pH of specimen is in the range of  $6 \pm 0.5$ .

## 8.3 Solid Phase Extraction (applicable to blood and urine samples)

- a. Pre-rinse SPE extraction cartridge by adding 3 mL of methanol at 1-2 mL/minute.
- b. Condition column with 3 mL of deionized water by adding 2 mL of 100 mM phosphate buffer. Do not allow sorbent to dry.
- c. Load sample on SPE cartridge at approximately 1-2 mL/minute. Do not allow sorbent to dry.
- d. Wash column with 2 mL of deionized water and 2 mL of Wash Solvent.
- e. Dry column for 1 minute.

- f. Wash column with 2 mL of hexane.
- g. Dry column for 1 minute.
- h. Rinse column with 2 mL of deionized water.
- i. Dry column for 1 minute.
- j. Elute with 2.5 mL of Elution Solvent at approximately 0.5 mL/minute.
- k. Evaporate eluent at  $\leq 40^{\circ}\text{C}$  to dryness. Do not overdry.
- l. Reconstitute extracts with 50 - 100  $\mu\text{L}$  of mobile phase. Vortex.
- m. Analyze LC/MS Performance Standard by LC/MS(ESI) (10  $\mu\text{L}$ ), using the full scan instrumental conditions that follow. If the decision criteria in Section 11.1 of this procedure are met, proceed to step n. Otherwise, perform any appropriate instrument maintenance.
- n. Analyze 5-10  $\mu\text{L}$  of the extracts by LC/MS(ESI) using the instrumental conditions that follow. MS analysis can be full scan, MS/MS, or data dependent.

## 9 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

### 9.1 Liquid Chromatograph Parameters

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

Mobile Phase Parameters		Column Parameters	
composition	60:40:0.03 methanol: water: ammonia	type	Xterra C-18
isocratic flow	0.2 mL/min	length	15 cm
run time	35 min	internal diameter	2.1 mm
temperature	35°C	particle size	5 $\mu\text{m}$
Autosampler Parameters			
Injection Volume	5-10 $\mu\text{L}$	Temperature	15°C

## 9.2 Mass Spectral Parameters

Scan Event #1		Scan Events #2-5 (optional)	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
resolution	7500	resolution	unit mass
scan mode	full scan	scan mode	product ion MS/MS
scan range	200 - 400 m/z	precursor ion	1 <sup>st</sup> , 2 <sup>nd</sup> , 3 <sup>rd</sup> and 4 <sup>th</sup> most intense from list below
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		collision energy	40 (relative)
		isolation width	2 AMU
		product scan range	software control
		threshold	500 counts
		dynamic exclusion	disabled

Precursor Ion List (7500 mass resolution - centroid – $\pm 0.02$ m/z trigger window)
252.11, 266.13, 271.06, 271.10, 282.09, 284.12, 285.08, 286.07, 287.06, 289.05, 289.11, 295.07, 296.10, 300.08, 300.09, 301.07, 309.09, 314.09, 315.09, 316.01, 316.05, 321.02, 325.08, 325.11, 326.08, 330.09, 335.04, 342.08, 343.05, 343.08, 348.97, 353.07, 359.05, 388.16 m/z
Any subset of the above target precursor ions may be selected for confirmation of a given analyte or group of analytes, based on the information in Table 1.

## 10 Decision Criteria

### 10.1 LC/MS Performance Standard Decision Criteria

#### 10.1.1 Chromatography

In order for the LC to be considered in good operating condition, molecular ion traces for each benzodiazepine in the performance standard should generate Gaussian shaped chromatographic peaks. (The following molecular ions should be traced for each benzodiazepine: clonazepam – 316, nitrazepam – 282, flunitrazepam – 314, lorazepam – 321, oxazepam – 287, alprazolam – 309, temazepam – 301, and diazepam – 285.)

The retention times of the 8 analytes should be within  $\pm 5\%$  of the previous run of the performance standard. (Minor changes in mobile phase percentage may account for slight retention time shifts.)

The areas of each chromatographic molecular ion peak in the performance standard should be comparable (within 50% - 200%) to the previous run of the performance standard.

#### 10.1.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the eight analytes in the performance standard should be present. (The following molecular ions should be present as the base peak for each analyte: clonazepam – 316.05, nitrazepam –

282.09, flunitrazepam – 314.09, lorazepam – 321.02, oxazepam – 287.06, alprazolam – 309.09, temazepam – 301.07, and diazepam – 285.08.)

## **10.2 Unknown Sample Decision Criteria**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

### **10.2.1 Batch Acceptance**

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected in the LC/MS data. If a targeted run is being performed for a limited set of target drugs, only those need be detected in the Positive Control(s).

### **10.2.2 Unknown Sample Acceptance**

All internal standards should be detected in the sample.

### **10.2.3 Unknown Sample Compound Identification**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control.

#### **10.2.3.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

##### **10.2.3.1.1 Retention Time**

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

##### **10.2.3.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just

prior to the sample.

### 10.2.3.3 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted positive control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance. Table 1 lists benzodiazepines along with approximate retention time, base peak and MS/MS fragment peaks. The measured mass(es) of the analyte of interest should match those for a reference standard or an extracted positive control to within 0.005 m/z units.

Table 1: Benzodiazepines and Metabolites: LC/MS/MS Information

Drug/Metabolite	Retention Time	Base Peak (7500)	Primary Fragment Ions (Note #1)
<i>Internal Standards</i>			
<i>d7-7aminoflunitrazepam</i>	2.29	291.163	N/A
<i>d7-flunitrazepam</i>	3.97	321.137	N/A
<i>d5-alprazolam</i>	4.82	314.122	N/A
<i>d5-oxazepam</i>	4.82	292.090	N/A
<i>d5-diazepam</i>	7.85	290.110	N/A
7-aminoclonazepam	2.12	286.074	121, 222, 250
7-aminonitrazepam	2.15	252.113	121, 149, 224
7-aminoflunitrazepam	2.22	284.119	135, 163, 264
7-aminonimetazepam	2.37	266.129	135, 163, 238
3-hydroxyflunitrazepam	3.25	330.088	284, 312 (Note #2)
n-desmethyflunitrazepam	3.26	300.078	214, 254, 272
demoxepam	3.37	287.059	180, 258, 270
clonazepam	3.46	316.048	251, 270, 288
bromazepam	3.46	316.008	209, 261, 288
nitrazepam	3.69	282.087	176, 236, 254
alpha-hydroxytriazolam	3.81	359.046	176, 261, 331
n-desmethyloclobazam	3.86	287.059	245 (Note #3)
flunitrazepam	4.03	314.094	240, 268, 286
alpha-hydroxyalprazolam	4.21	325.085	227, 279, 297
estazolam	4.31	295.075	192, 260, 267
nimetazepam	4.33	296.103	222, 250, 268
clobazam	4.41	301.075	259 (Note #3)
triazolam	4.59	343.051	279, 308, 315
lorazepam	4.74	321.019	275, 303 (Note #2)
norchlordiazepoxide	4.82	286.074	241, 255, 269
alprazolam	4.91	309.090	241, 274, 281
oxazepam	4.91	287.058	241, 269 (Note #2)
desalkylflurazepam	5.28	289.054	140, 226, 261
etizolam	5.40	343.077	314, 307, 326
temazepam	5.57	301.074	255, 283 (Note #2)
alpha-hydroxymidazolam	5.61	342.080	203, 324 (Note #2)

Drug/Metabolite	Retention Time	Base Peak (7500)	Primary Fragment Ions (Note #1)
lormetazepam	5.73	335.035	289, 317 (Note #2)
chlordiazepoxide	6.28	300.090	241, 269, 283
phenazepam	6.53	348.972	184, 242, 321
nordiazepam	6.80	271.063	140, 208, 243
n-ethyloxazepam	7.10	315.090	242, 269, 297
diazepam	7.96	285.079	154, 222, 257
midazolam	7.78	326.085	244, 285, 291
halazepam	11.61	353.066	222, 290, 325
tetrazepam	14.30	289.111	225, 232, 261
flurazepam	15.38	388.159	315, 317 (Note #2)
prazepam	15.40	325.111	255, 271, 297
medazepam	24.03	271.100	180, 242, 254

Note #1 – These are the suggested ions for use in the evaluation of mass spectral ion ratios per the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104). Other diagnostic ions may be substituted.

Note #2 – These compounds each show only two significant fragment ions under the instrumental conditions used in this method. The absence of any additional fragment ions with intensity greater than 5% of the base peak may be treated as a third ion match for the purposes of SOP Tox 104.

Note #3 – These compounds each show only one significant fragment ion under the instrumental conditions used in this method. Data from other analytical methods should be used as the basis of primary identification for these analytes. Alternatively, as a minor deviation, collision energy could be optimized to produce a multi-ion fragment spectrum for one of these analytes.

## 11 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

- a. Limits of Detection:  
Approximately 1-2 ng/mL for blood and 1-5 ng/mL for urine when screened using Data Dependent Scan method. Detection limits can be decreased through targeted analysis. See Validation Binder for details of LODs for 10 analytes on different instruments.
- b. Interferences: None known. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.
- c. Cautionary Statement: Oxazepam is known to be unstable in methanol.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed.; Chemical Toxicology Institute, Foster City, California, 1995.

LeBeau, M.A.; Montgomery, M.A.; Wagner, J.R.; Miller, M.L. *J For Sci.* 2000, 45, 1133-1141.

Malik-Wolf, B., Vorce, S., Holler, J., Bosy, T., *J Anal Toxicol*, 2014, 38 (3), 171-176.

Flunitrazepam, 7-aminoflunitrazepam & desmethyflunitrazepam in urine for GC/MS confirmations using 200 mg CLEAN SCREEN<sup>®</sup> extraction column. *Clean Screen Application Manual*, Worldwide Monitoring.

*ELISA Screening* (Tox 209); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology

Subunit SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit –  
Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual.*



Rev. #	Issue Date	History
5	12/23/13	Updated internal standards in Section 6 and changed supplier in 6.o. Subsequently updated 9.1.b., 9.2.b. and 9.2.c.1 and Appendix 1. Replaced FT-tandem-MS analysis with multiple-event unit mass tandem MS analysis in section 10.2. Subsequently updated and revised Table 1. Retitled 11.2 and 11.2.3. Added 11.2.1 and 11.2.2. Revised Appendix 2.
6	11/20/14	In Section 5, removed reference to Reagent SOP and added instructions for preparation of reagents. Changed enzyme to abalone source in Section 5l. This caused updates to Section 8.2.c, bench sheet, and the addition of a new acetate buffer reagent in 5n and a new reference in Section 15. Updated expiration dates in 6e. Deleted Calibration Section and renumbered subsequent sections. In Section 8.2c, changed to make hydrolysis not optional, which caused removal of the footnote. Added autosampler parameters to 9.1. Removed comment about not performing ion ratios in 10.2.3.3.
7	06/08/15	In 5u, updated mobile phase expiration date to 2 weeks. In 6g, updated storage conditions. Updated positive control preparation in Sections 6k-l, 6n-o, 6q, and Appendix 1. Added etizolam to Section 9.2, Table 1, and Appendix 2.

**Approval**

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**Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use  
(Pg 1 of 2).**

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**Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use  
(Pg 2 of 2).**

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**Appendix 2: Abbreviated version of Benzodiazepine Instrumental Conditions for bench use.**

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## **GHB and GBL from Biological Fluids by Headspace GC/MS(EI)**

### **1 Introduction**

Gamma-hydroxybutyrate (GHB) and its precursors, gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD), have become popular recreational substances in the United States. This is primarily due to their sedative and euphoric properties, but they are also abused as steroid alternatives by some bodybuilders. Furthermore, these chemicals have been implicated in a number of cases of drug-facilitated sexual assault.

Both GBL and 1,4-BD are rapidly metabolized to GHB *in vivo*, thus the pharmacological effects of both mimic those of GHB. The conversion is so rapid that in most cases it is not necessary to analyze for GBL and 1,4-BD. However, in overdose cases a GHB finding may be explored for the possibility of GBL or 1,4-BD involvement. This procedure allows for the analysis of a specimen for GHB and GBL.

There are a number of analytical complications surrounding GHB. One such complication is its existence as an endogenous product in humans. In postmortem specimens, the GHB levels in blood may be elevated to such a degree that apparent endogenous levels of GHB may overlap those reported to be lethal. An added complication is the pH-driven interconversion between GHB and GBL. Thus, caution should be employed in the interpretation of GHB findings.

### **2 Scope**

This procedure allows for the screening and confirmation of GHB in urine specimens and aqueous samples. When appropriate controls are simultaneously analyzed, it can be used to screen blood samples for elevated levels of GHB. It also provides a method for the differentiation of GHB from GBL.

### **3 Principle**

For the initial screen, d<sub>6</sub>-GHB is added to the specimen as the internal standard. Since 10 µg/mL is the administrative cutoff for urine specimens, a 10 µg/mL GHB Positive Control is also extracted. The samples are treated with concentrated sulfuric acid and heat (for conversion of GHB to GBL). Methylene chloride is used to extract the GBL from the biological matrix. The organic extracts are concentrated and transferred to headspace autosampler vials. The vials are heated and the headspace is analyzed by gas chromatography/mass spectrometry (GC/MS). This method measures the total amount of GHB and GBL in a specimen. For urine specimens, if the ratio of the GHB peak area/internal standard peak area in the questioned sample approaches the ratio of the GHB peak area/internal standard peak area in the 10 µg/mL Positive Control, the sample is

presumed positive. For aqueous specimens, any amount of GHB detected is considered positive, although care should be given to the interpretation of the findings. If GBL is suspected in a sample, it may be analyzed without acid hydrolysis and with  $\alpha$ -methylene GBL as the internal standard. This procedure may be repeated to confirm positive samples, and serves as a framework for quantitative analysis with proper validation.

#### 4 Specimens

This procedure can be performed on a biological fluid such as blood, serum, plasma, urine, or vitreous humor. 2.0 mL of specimen are used in order to perform the screen and the confirmation.

#### 5 Equipment/Materials/Reagents

- a. Gas Chromatograph / Mass Spectrometer operating in electron impact (EI) mode equipped with a headspace autosampler and a 30 m x 0.25 mm x 1.4  $\mu$ m film DB-624 (or equivalent) column
- b. Centrifuge
- c. Vortex mixer
- d. Heating block
- e. Evaporator with nitrogen
- f. Balance
- g. Adjustable pipetters (0.01 - 1 mL) with appropriate tips
- h. 16 x 100 mm screw-top test tubes with caps
- i. 10 mL conical-bottom screw-top centrifuge tubes with caps
- j. 10 mL and 100 mL volumetric flasks
- k. Routine laboratory supplies, including disposable glass pipets, autosampler vials with caps, spatulas, graduated cylinders, test tube racks, etc.
- l. Concentrated Sulfuric Acid (Reagent Grade)
- m. Methylene Chloride (HPLC Grade)

- n. Methanol (HPLC Grade)
- o. Deionized water
- p. Rotater
- q. 20-mL headspace vials with magnetic caps

## 6 Standards and Controls

- a. GHB Na Stock Standard (1.0 mg/mL):  
Purchased from Cerilliant Corporation or another approved supplier as the sodium salt. Storage and stability determined by manufacturer.
- b. GHB Na Working Standard (0.1 mg/ml):  
Prepare by adding 1.0 mL of the GHB Na Stock Standard to a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated in glass. Stable for at least one year.
- c. GBL Stock Standard (1.0 mg/mL):  
Purchased from Cerilliant Corporation or another approved supplier. Storage and stability determined by manufacturer.
- d. d<sub>6</sub>-GHB Na Internal Standard (d<sub>6</sub>-Gammahydroxybutyrate Sodium Salt; 100 µg/mL OR 1 mg/mL):  
Purchased from Cerilliant Corporation or another approved supplier. Storage and stability determined by manufacturer.
- e. α Methylene-GBL:  
Purchased from Sigma or another approved supplier. Storage and stability determined by manufacturer.
- f. α Methylene-GBL Internal Standard (0.1 mg/mL):  
To a 100-mL volumetric flask, add 10.0 mg of Alpha Methylene- Gammabutyrolactone. Bring volume to the mark with methanol. Store refrigerated in glass or plastic. Stable for at least 1 year.
- g. Negative Control:  
Since GHB is endogenous, the most appropriate Negative Control is a deionized water sample or synthetic urine (Surine, obtained from Dyna-Tek, Inc., Lenexa, KS). Stability and storage of Surine are determined by the manufacturer.

However, a true matrix matched Negative Control (urine or blood) is also analyzed.

Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or prepared in-house from an appropriate blank specimen. Blood and urine will be stored refrigerated, frozen or obtained fresh. Stability determined by manufacturer.

A Negative Control will be analyzed with every assay.

- h. Positive GHB Controls (5 and 10 µg/mL):  
For urine screens, Positive Urine Controls at 5 and 10 µg/mL are analyzed. These are prepared fresh by adding 60 and 120 µL of the GHB Na Working Standard to 1 mL aliquots of Surine.

At least one Positive GHB Control will be analyzed with every GHB assay.

- i. Positive GBL Control (10 µg/mL):  
Prepared fresh by adding 10 µL of the GBL Stock Standard (1.0 mg/mL) to 1 mL of Surine.

A Positive Control (GBL) will be analyzed with every GBL assay.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the Examiner or Chemist performing the procedure.

- a. Add 1 mL specimen or control to a properly labeled 16 x 100 mm screw-top test tube.
- b. Add 10 µg d<sub>6</sub>-GHB Na (10 µL of the 1.0 mg/mL d<sub>6</sub>-GHB Na Internal Standard or 100 µL of the 100 µg/mL d<sub>6</sub>-GHB Na Internal Standard) to each tube<sup>1</sup>.
- c. Add 150 µL of concentrated sulfuric acid to all tubes, cap, vortex, and place them in a heating block at approximately 70°C for 5 minutes. Cool to room temperature.
- d. Add 4 mL of methylene chloride to each tube. Extract by rotation for 5 minutes; centrifuge for 5 minutes.
- e. Remove aqueous (top) layer to waste. Transfer organic (bottom) layer to an appropriately labeled conical bottom tube.

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<sup>1</sup> To screen for GBL, add 20 µL of the 0.1 mg/mL α-methylene-GBL Internal Standard instead of d<sub>6</sub>-GHB, and skip to step d.



- f. Concentrate organic layer to approximately 75  $\mu$ L in the evaporator under nitrogen at approximately 35°C. Do not let the organic layer go to dryness.
- g. Transfer concentrated organic to a 20-mL headspace vial and cap.
- h. Analyze specimens by headspace GC/MS with the instrumental parameters listed later in this procedure.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Headspace Sampler Parameters

incubation temperature	100°C	syringe temperature	110°C
incubation time	15 min	sample fill volume	1.0 mL
agitator speed	300 RPM	sample fill rate	0.5 mL/sec
agitation timing	10 sec on 1 sec off	sample fill strokes	4
cycle time	20 min	sample injection speed	1.0 mL/sec
		syringe flush time	4.0 min

### 9.2 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature 1	50°C	type	DB-624	inlet temp.	150°C
hold 1	3 min	length	30 m	injection mode	split
ramp 1	20°C/min	internal diameter	0.25 mm	carrier gas	helium
temperature 2	150°C	film thickness	1.4 $\mu$ m	carrier mode	constant flow
hold 2	7 min			flow	0.87 mL/min
total run time	15 min			split ratio	10:1

### 9.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	260°C
scan range	35 - 200 AMU	quadrupole temperature	150°C
multiplier offset	+200 V	solvent delay	5 min

## **10 Decision Criteria**

### **10.1 GHB Screen: Determining if Further Analysis is Required**

When screening a urine sample to determine if a toxicologically significant amount of GHB is present, calculate the ratio of the GHB peak area/internal standard peak area in all samples. (This may be done automatically using the software on the instrument, or manually.) If the area ratio in the questioned urine sample approaches the area ratio in the 10 µg/mL Positive Control, the sample is presumed positive. Blood samples are not routinely analyzed by this procedure, but a level estimated to be above 2 µg/mL should be investigated further.

### **10.2 Decision Criteria for Analytical Data**

The following criteria are used as guides in determining the acceptability of the data produced in this assay. In general, compound identification should be based on comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control. In most cases, all of the below should be met in order to identify GHB or GBL within a biological specimen.

#### **10.2.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

##### **10.2.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from the injection of a reference standard, calibrator, or extracted Positive Control of GHB or GBL.

##### **10.2.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank injected just prior to that sample.

##### **10.2.2 Mass Spectrometry**

The mass spectrum of the analyte of interest should match that of the appropriate reference

standard, calibrator, or extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 11 Calculations

To calculate the ratio of the GHB peak area to the internal standard peak area, reconstructed ion chromatograms are traced for m/z 86 (GHB) and m/z 92 (d<sub>6</sub>-GHB). The traces are integrated, resulting in an area for each peak. The area of the 86 peak divided by the area of the 92 peak is the peak area ratio used in Section 11.1 of the Decision Criteria Section of this procedure.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

- a. Limit of Detection: Although levels of GHB less than 5 µg/mL can be analytically identified in urine samples, the limit of detection is administratively set at 5 µg/mL in urine samples.
- b. Interferences: None known. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.

## 14 Precautionary Statement

Care should be taken in interpretation of GHB levels. GHB is a naturally occurring product in the body. Further, studies have shown that GHB is elevated in blood collection tubes containing citrate. Exercise care in reporting and interpreting low values of GHB.

When analyzing specimens from living persons, in most cases, amounts of GHB in blood below 2 µg/mL, and/or amounts of GHB in urine below 10 µg/mL should not be reported as positive.

GHB levels may be elevated in postmortem blood samples and/or unpreserved blood samples. Therefore, a positive GHB finding in a postmortem blood sample should always be confirmed in a second specimen such as urine or vitreous humor.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed.; Chemical Toxicology Institute, Foster City, California, 1995.

LeBeau, M.A.; Christenson, R.H.; Levine, B.; Darwin, W.D.; Huestis, M.A. *J. Anal. Tox.* 2002, 26, 340-346.

LeBeau, M.A.; Montgomery, M.A.; Jufer, R.A.; Miller, M.L. *J. Anal. Tox.* 2000, 24, 383-384.

LeBeau, M.A.; Miller, M.L.; Levine, B. *For. Sci. Int.* 2000, 119, 161-167.

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Pan, Y.M., et al. *Proceedings of the American Academy of Forensic Sciences Annual Meeting*, Orlando, Florida, February 15-20, 1999. p 271.

Stevens. *J For Sci.* 1999; 44(1), 231-2.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Division Safety Manual.*

Rev. #	Issue Date	History
3	09/19/12	Removed reference to 1,4-BD SOP in Section 1 (SOP archived). Removed all references to quantitative analysis of GHB and GBL in Sections 2, 3, 9 (9.1 header designating screen and entire 9.2 removed), 12, 13, 14 and 17. Updated GHB and GBL stock standards in Sections 6 a, b and c to Cerilliant. Updated Control preparation in Sections 6 g and h to include two levels of GHB control and not to require volumetric flask since assay qualitative only now. In Section 9 b, added footnote for GBL screening. Updated decision criteria for the screen in 11.1. Updated chromatography decision criteria in Section 11.2.1. Added note about possibility of elevated GHB levels in unpreserved blood in Section 15.
4	07/09/14	In Section 6.f, added sources for Negative Control blood. Fixed typo in Section 10.2 (flow rate). On Appendix 1, added option for GBL analysis and a spot to record dry down temperature. Reformatted Appendix 2 to include all pertinent instrumental parameters.
5	04/20/15	Changed GBL internal standard to $\alpha$ -methylene-GBL since d <sub>6</sub> -GBL is no longer commercially available. This caused updates to the following Sections: 3, 6.e., 6.f., 8.b.(footnote), and Appendix 1. In Section 5, removed reference to Tox 103 (reagent SOP). Removed Calibration Section. Renamed Measurement Uncertainty Section.

**Approval**

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**Appendix 1: Abbreviated version of the GHB Procedure for bench use.**

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**Appendix 2: Abbreviated version of Instrumental Parameters for bench use.**

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## **Quaternary Amine Analysis by LC/MS/MS**

### **1 Introduction**

Mivacurium is a short-acting, non-depolarizing neuromuscular blocking agent of the quaternary amine class. It is sold as mivacurium chloride under the trade name Mivacron<sup>®</sup> by GlaxoSmithKline. Mivacurium exists as three stereoisomers. Plasma cholinesterases rapidly hydrolyze mivacurium to inactive alcohol and ester metabolites. Formation of these products also occurs in solution via hydrolysis, but this is minimized in an acidic environment.

Vecuronium and rocuronium are intermediate-acting, non-depolarizing neuromuscular blocking agents of the quaternary amine class. Vecuronium bromide (Norcuron<sup>®</sup>) is known to metabolize in vivo and also to convert in vitro to desalkyl metabolites. Rocuronium also forms a desalkyl metabolite, but this has not yet been analyzed in the FBI Laboratory. Rocuronium bromide is sold as Zemuron<sup>®</sup>.

Doxacurium is long-acting, non-depolarizing neuromuscular blocking agent of the quaternary amine class. Doxacurium chloride is sold as Nuromax<sup>®</sup>. Doxacurium is also unstable in solution, and converts to ester and alcohol breakdown products.

### **2 Scope**

This protocol allows for the identification of mivacurium and metabolites, vecuronium and its primary breakdown product, doxacurium and its breakdown products, and rocuronium in biological samples (i.e., blood, urine and tissue homogenates), liquids, and syringe residues.

### **3 Principle**

Samples are screened and confirmed for quaternary amines and their breakdown products by liquid chromatography tandem mass spectrometry (LC/MS/MS).

### **4 Specimens**

This procedure may be used to analyze pharmaceutical formulations, liquids, syringe residues, and biological fluids, such as blood, urine, and tissue homogenates. When available, 1 mL of a



biological fluid is used for the screen, and a separate 1 mL aliquot is used for the confirmation.

## 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Liquid Chromatograph/Mass Spectrometer (triple quadrupole or ion trap) (LC/MS/MS) equipped with a LiChrospher 60 RP-Select B (15 cm x 2.1 mm x 5  $\mu$ m particle) (obtained from Alltech Associates, Inc.) column
- b. Centrifuge
- c. Vortex mixer
- d. Vacuum solid phase extraction (SPE) manifold
- e. Vacuum solvent filtration system with 0.45  $\mu$ m filters
- f. Tissue homogenizer
- g. Evaporator with nitrogen
- h. Balance accurate to at least  $\pm 0.2$  mg
- i. Volumetric flasks (10 mL and 1 L)
- j. Adjustable pipetters (0.01 - 5 mL) with appropriate tips
- k. Maxi-Clean IC-RP SPE Cartridges (0.05 mL bed volume) - obtained from Alltech Associates, Inc.
- l. 16 x 150 mm screw-top test tubes, with caps
- m. 12 x 75 mm culture tubes, with caps
- n. Routine laboratory supplies, including disposable glass pipets, spatulas, test tube racks, graduated cylinders, etc.

- o. pH paper (acidic range)
- p. Acetonitrile (HPLC grade)
- q. Methanol (HPLC grade)
- r. Deionized water (DI H<sub>2</sub>O)
- s. Dilute Hydrochloric Acid (HCl) (2 mM)
- t. Acetonitrile:Deionized Water (1:1)
- u. LC Mobile Phase - Mivacurium MSMS (40:60 v:v acetonitrile:0.025% methanesulfonic acid)
- v. Methanesulfonic acid (MSA)
- w. 0.015% Methanesulfonic Acid:  
Combine 0.15 mL MSA and deionized water in a graduated cylinder. Bring to the 1-L mark with deionized water. Mix well.

## 6 Standards and Controls

- a. Mivacurium (Miv) Chloride:  
Obtained from GlaxoSmithKline, Inc., or another approved supplier. Storage and stability determined by manufacturer.
- b. Mivacurium Alcohol Compound (MIV-OH):  
Obtained from GlaxoSmithKline, Inc., or another approved supplier. Storage and stability determined by manufacturer.
- c. Mivacurium Ester Compound (MIV-Ester):  
Obtained from GlaxoSmithKline, Inc., or another approved supplier. Storage and stability determined by manufacturer.
- d. d-Tubocurarine Chloride:  
Purchased from Sigma-Aldrich Chemical Company or another approved supplier. Storage and stability determined by manufacturer.

- e. Vecuronium (Vec) Bromide:  
Purchased from United States Pharmacopeia, or another approved supplier. Storage and stability determined by manufacturer.
- f. 3-desalkylvecuronium (Vec-OH or ORG 7268):  
Obtained from N.V. Organon, a part of Schering Plough Corporation, The Netherlands, or another approved supplier. Storage and stability determined by manufacturer.
- g. Rocuronium (Roc) Bromide:  
Purchased from Sigma-Aldrich Chemical Company or another approved supplier. Storage and stability determined by manufacturer.
- h. Doxacurium (Dox) Chloride:  
Obtained from Glaxo Wellcome, or another approved supplier. Storage and stability determined by manufacturer.
- i. Mivacurium Standard Stock Solution (1.0 mg/mL):  
Weigh 10.7 mg mivacurium chloride into a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass or plastic. Stable at least one month.
- j. Mivacurium Ester Metabolite Standard Stock Solution (1.0 mg/mL):  
Weigh 10.0 mg mivacurium ester compound into a 10-mL volumetric flask. Bring to the mark with acetonitrile. Store refrigerated in glass or plastic. Stable at least one month.
- k. Mivacurium Alcohol Metabolite Stock Standard Solution (1.0 mg/mL):  
Weigh 10.0 mg mivacurium alcohol compound into a 10-mL volumetric flask. Bring to the mark with acetonitrile. Store refrigerated in glass or plastic. Stable at least one month.
- l. Mivacurium and Metabolites Working Standard Solution (10 µg/mL mivacurium, mivacurium ester, and mivacurium alcohol):  
Combine 100 µL Mivacurium Stock Standard Solution, 100 µL Mivacurium Ester Stock Standard Solution, and 100 µL Mivacurium Alcohol Stock Standard Solution in a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- m. Mivacurium and Metabolites Dilute Working Solution (1.0 µg/mL mivacurium, mivacurium ester, and mivacurium alcohol):  
Combine 10 µL Mivacurium Stock Standard Solution, 10 µL Mivacurium Ester Stock Standard Solution, and 10 µL Mivacurium Alcohol Stock Standard Solution in a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at

least one month.

- n. d-Tubocurarine Chloride Stock Solution (1.0 mg/mL):  
Weigh 10.0 mg d-Tubocurarine Chloride into a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated in glass. Stable at least 1 month.
- o. d-Tubocurarine Internal Standard Solution (1.0 µg/mL):  
Add 10 µL d-Tubocurarine Chloride Stock Solution to a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated in glass. Stable at least one month.
- p. Vecuronium Stock Solution (1.0 mg/mL):  
Weigh 11.4 mg vecuronium bromide into a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- q. Vecuronium Working Solution (1.0 µg/mL):  
Add 10 µL Vecuronium Stock Solution to a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- r. 3-desalkylvecuronium Stock Solution (1.0 mg/mL):  
Weigh 10.0 mg 3-desalkylvecuronium into a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- s. 3-desalkylvecuronium Working Solution (1.0 µg/mL):  
Add 10 µL 3-desalkylvecuronium Stock Solution to a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- r. Rocuronium Stock Solution (1.0 mg/mL):  
Weigh 11.5 mg rocuronium bromide into a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- s. Rocuronium Working Solution (1.0 µg/mL):  
Add 10 µL Rocuronium Stock Solution to a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- t. Doxacurium Stock Solution<sup>1</sup> (1.0 mg/mL):  
Weigh 10.6 mg doxacurium chloride into a 10-mL volumetric flask. Bring to the mark

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<sup>1</sup> A reference standard of the doxacurium breakdown product is not commercially available, so the doxacurium solution may be allowed to sit at room temperature for a period of time to allow for the hydrolysis of doxacurium to its main breakdown products.

with dilute HCl or deionized water. Store refrigerated in glass. Stable at least one month.

- u. Doxacurium Working Solution (1.0 µg/mL):  
Add 10 µL Doxacurium Stock Solution to a 10-mL volumetric flask. Bring to the mark with dilute HCl. Store refrigerated in glass. Stable at least one month.
- v. Negative Control:  
Prepared from blank urine (obtained in house) or negative whole blood (obtained from Clinia or another approved supplier). Dilute HCl is an appropriate Negative Control when analyzing liquids and syringes. A Negative Control will be analyzed with every assay. The Negative Control will be matrix matched when possible.
- w. Positive Control (100 ng/mL):  
Prepared in-house by adding 100 µL of the appropriate 1.0 µg/mL Working Standard(s) to 1 mL of an appropriate matrix. Prepare fresh weekly. A Positive Control will be analyzed with every assay. When possible, the Positive Control will be matrix matched.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Pharmaceutical Formulations, Liquids, and Syringe Residues

- a. Dilute liquid samples with an appropriate amount of dilute HCl<sup>2</sup>. Rinse syringes with

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<sup>2</sup> The degree of dilution should be such to reach a working concentration that fits within the validated concentrations defined later in this procedure. It should be recognized that numerous dilutions may be required to reach such a concentration.

minimal dilute HCl.

- b. Pipette 1 mL of the sample and 100  $\mu$ L of the d-Tubocurarine Chloride Internal Standard Solution into an autosampler vial. Prepare Positive and Negative Controls in a similar fashion.
- c. Analyze samples by LC/MS/MS.

## 9.2 Biological Specimens

- a. Add 1 mL of each sample or control to a clean 16 x 150 mm test tube. For tissue samples, prepare a 1:1 homogenate in deionized water, and analyze 1 g of the homogenate.
- b. Add 100  $\mu$ L of the d-Tubocurarine Chloride Internal Standard Solution and vortex thoroughly<sup>3</sup>.
- c. Add 2 mL acetonitrile dropwise, while vortexing.
- d. Vortex for at least 1 minute.
- e. Centrifuge samples, and transfer the supernatant to a clean 12 x 75 mm culture tube<sup>4</sup>.
- f. Prepare solid phase extraction cartridges by conditioning with 2.5 mL methanol followed by 2.5 mL acetonitrile:deionized water (1:1).
- g. Pour supernatant over column reservoir, and collect eluent no faster than 1 mL/minute.
- h. Apply full vacuum after sample has eluted to collect the last few drops.
- i. Dry eluent under nitrogen at approximately 60°C.
- j. Reconstitute samples in 100  $\mu$ L LC Mobile Phase - Mivacurium MSMS.
- k. Analyze 5  $\mu$ L of each extract by LC/MS/MS.

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<sup>3</sup> If tubocurarine is expected in a sample, a different quaternary amine should be chosen as the internal standard, following appropriate validation for tubocurarine.

<sup>4</sup> When screening samples for rocuronium, proceed to step i., and skip the solid phase extraction step.

## 10 Instrumental Conditions

### 10.1 Liquid Chromatograph Parameters for Mivacurium, Doxacurium and Vecuronium

Mobile Phase Parameters		Column Parameters	
composition	40:60 acetonitrile: 0.025% MSA <sup>5</sup>	type	Licrospher 60 RP-Select B
isocratic flow	0.3 mL/min	length	15 cm
run time	11 min	internal diameter	2.1 mm
temperature	ambient	particle size	5 µm

### 10.2 Liquid Chromatograph Parameters for Rocuronium

Mobile Phase Parameters			Column Parameters	
Time (min)	% Acetonitrile	% MSA (0.015%)	type	Licrospher 60 RP-Select B
0	10	90	length	15 cm
1	10	90	internal diameter	2.1 mm
10	75	25	particle size	5 µm
15	75	25		
16	10	90		
21	10	90		

### 10.3 Mass Spectrometer Parameters for Mivacurium

Segment #1 (0 – 3.2 min)			
Scan Event #1		Scan Event #2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	product ion MS/MS	scan mode	product ion MS/MS
precursor ion	609 AMU	precursor ion	446 AMU
collision energy	31% relative	collision energy	32% relative
product scan range	180 - 700 AMU	product scan range	120 – 470 AMU

<sup>5</sup>To improve chromatography for vecuronium and 3-desalkylvecuronium, 17% of 0.015% MSA solution may be mixed with the Mivacurium Mobile Phase by the LC pump. Additionally, the run time may be reduced to 10 minutes.

Segment #2 (3.2 – 6 min)		Segment #3 (6 – 11 min)	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	product ion MS/MS	scan mode	product ion MS/MS
precursor ion	600 AMU	precursor ion	514 AMU
collision energy	36% relative	collision energy	36% relative
product scan range	165 - 610 AMU	product scan range	140 - 800 AMU
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.			

#### 10.4 Mass Spectrometer Parameters for Doxacurium and its Breakdown Products

Scan Event #1		Scan Event #2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	product ion MS/MS	scan mode	product ion MS/MS
precursor ion	609 AMU	precursor ion	517 AMU
collision energy	31% relative	collision energy	30%
product scan range	180 - 700 AMU	product scan range	140 – 1250 AMU
Scan Event #3		Scan Event #4	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	product ion MS/MS	scan mode	product ion MS/MS
precursor ion	576 AMU	precursor ion	476 AMU
collision energy	29%	collision energy	29%
product scan range	155 – 600 AMU	product scan range	130 – 600 AMU
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.			

#### 10.5 Mass Spectrometer Parameters for Vecuronium and 3-desacetylvecuronium

Scan Event #1		Scan Event #2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	full scan MS	scan mode	product ion MS/MS
scan range	400 – 700 AMU	precursor ion	557 AMU
		collision energy	25%
		product scan range	150 – 600 AMU



Scan Event #3		Scan Event #4	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	product ion MS/MS	scan mode	product ion MS/MS
precursor ion	515 AMU	precursor ion	609 AMU
collision energy	25%	collision energy	25%
product scan range	140 – 530 AMU	product scan range	165 – 630 AMU
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.			

## 10.6 Mass Spectrometer Parameters for Rocuronium

Scan Event #1		Scan Event #2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	full scan MS	scan mode	product ion MS/MS
scan range	400 – 700 AMU	precursor ion	609 AMU
		collision energy	31% relative
		product scan range	180 - 700 AMU
Scan Event #3			
ionization mode	electrospray (+)		
scan mode	product ion MS/MS		
precursor ion	529 AMU		
collision energy	25%		
product scan range	145 – 530 AMU		
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.			

## 11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control. In most cases, all of the below should be met in order to identify a quaternary amine and/or its breakdown products:

## 11.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### 11.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute) obtained from injection of a reference standard, calibrator, or extracted Positive Control of mivacurium or one of its metabolites.

### 11.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. For LC/MS/MS, this determination may be made from the reconstructed ion chromatograph for the product ion of greatest abundance. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank injected just prior to that sample.

## 11.2 Mass Spectrometry

The product mass spectrum of the analyte of interest should match that of an appropriate reference standard or extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 12 Calculations

Not applicable.

## 13 Uncertainty of Measurement

Not applicable.

## 14 Limitations

- a. Limits of Detection: 5 ng/mL for MIV-OH in blood  
25 ng/mL for MIV-Ester and Miv in blood  
10 ng/mL for Vec in blood  
50 ng/mL for Vec in liver  
1 ng/mL for Vec-OH in blood  
20 ng/g for Vec-OH in liver  
1 ng/mL for Roc in blood  
10 ng/mL for Dox in blood
- b. Specificity: No commonly encountered drugs or components of biological matrices have been found to interfere with this procedure.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

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*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
0	06/21/06	New document that replaces a previous document also titled " <i>Mivacurium and Metabolites Analysis by LC/UV/FLU and LC/MS/MS</i> ".
1	01/11/10	Removed LC/UV/FLU screen, and all references to quantitative analysis. Updated entire SOP to add doxacurium, doxacurium breakdown products, vecuronium, vecuronium breakdown product, and rocuronium. Reduced amount of internal standard in steps 9.1b and 9.2b.
2	01/03/13	Updated Chromatography Decision Criteria in 11.1.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Quaternary Amine Procedure for bench use.**

Redacted - Form on File

## **Solid Phase Extraction of Opioids from Biologicals with Analysis by LC-Tandem MS (High Resolution)**

### **1 Introduction**

Opioids are a class of substances that include natural, semi-synthetic and synthetic alkaloidal agents derived from opium or substances which have morphine-like activity. Naturally occurring opioids such as morphine and codeine are typically referred to as opiates. Heroin (diacetylmorphine) is a semi-synthetic opioid that is synthesized by the acetylation of morphine (MOR). In humans, heroin is rapidly metabolized to 6-monoacetylmorphine (6-MAM) and morphine. Morphine is further metabolized to N-desmethyilmorphine (NorM). Codeine (COD) is the 3-methyl ether derivative of morphine, and is metabolized to morphine and N-desmethylocodeine (NorC). Among the more common synthetic opioids are oxycodone (OXYC; Oxycontin), hydrocodone (HC; Vicodin), hydromorphone (HM; Dilaudid), and dihydrocodeine (DHC; Drocode). Hydrocodone is biotransformed to hydromorphone, while oxycodone is metabolized to oxymorphone (OXYM) and N-desmethyloxycodone (NorOxyc), and dihydrocodeine is converted to dihydromorphone (DHM). The conversion of opioids to glucuronide conjugates is a common metabolic transformation. Conjugated opioids are difficult to extract and chromatograph in a single fraction with unconjugated opioids. Therefore, analysis of the total concentration of an opioid that is present in conjugated and unconjugated form requires a hydrolysis step to cleave the conjugates.

### **2 Scope**

This procedure allows for the screening and confirmation of morphine, codeine, hydromorphone, hydrocodone, oxymorphone, oxycodone, 6-acetylmorphine, normorphine, norcodeine, noroxycodone, dihydromorphone, and dihydrocodeine in biological specimens. It also provides a method of quantitative analysis for the first seven of these compounds. This document applies to Chemistry Unit caseworking personnel who perform toxicology analyses.

### **3 Principle**

Biological specimens are qualitatively screened and/or quantitated for opioids by this method. Since most opioids are biotransformed to form a glucuronide conjugate during metabolism, these conjugates need to be hydrolyzed to obtain "total" opioid concentrations. The hydrolysis occurs by cleaving the drug-conjugate with the enzyme  $\beta$ -glucuronidase. Analysis without hydrolysis yields "free" opioid concentrations. Analysis with hydrolysis yields "total" opioid concentrations. Specimens are mixed with internal standards, adjusted to a slightly acidic pH, and extracted using mixed mode hydrophobic/cation exchange solid phase extraction cartridges. Target drugs are eluted using a mixed solvent system of methylene chloride, isopropanol, and ammonium

hydroxide. The eluent is taken to dryness and reconstituted prior to analysis by Liquid chromatography-tandem mass spectrometry (LC-Tandem MS) High Resolution.

#### **4 Specimens**

This procedure can be used for assaying biological specimens such as blood, serum, plasma, urine, bile, gastric contents, vitreous humor, or a previously prepared tissue homogenate. When available, 1 mL of biofluid or 2 g of a prepared tissue homogenate (1:1) is used in the assay. Blood, bile, gastric content, and tissue homogenate samples are centrifuged prior to analysis. Urine, vitreous humor, plasma, or serum specimens can be directly extracted. Total opiate analysis requires that specimens such as blood, urine, and bile be enzymatically hydrolyzed prior to analysis. In instances where specimen volume is altered (e.g., to improve sensitivity or account for limited specimen volume), appropriate modifications may be made to this procedure.

#### **5 Equipment/Materials/Reagents**

- a. Binary (or higher) liquid chromatograph coupled to an electrospray ion trap mass spectrometer capable of at least 15000 resolution (for example, Orbitrap)
- b. Xterra Phenyl LC column: 150 x 2.1 mm. 5  $\mu$ m d<sub>p</sub>, with 2  $\mu$ m titanium prefilter
- c. Test tubes (16 x 125 mm screw-top, 16 x 100 mm and 12 x 75 mm culture, or comparable)
- d. Centrifuge
- e. Heating block
- f. Vortex mixer
- g. Solid phase extraction manifold (vacuum or positive pressure)
- h. CLEAN SCREEN DAU solid phase extraction (SPE) cartridges (200 mg x 10 mL)
- i. Evaporator with nitrogen
- j. Homogenizer (for tissue or similar specimens)
- k.  $\beta$ -Glucuronidase (Type H-2 from Helix Pomatia; 100,000+ units/mL)
- l. Sodium acetate trihydrate (reagent grade)



- m. Hydrochloric acid, concentrated (12 M) (ACS grade)
- n. 1 N Hydrochloric Acid: To a 100-mL graduated cylinder, add 80 mL deionized water. Add 8 mL concentrated hydrochloric acid and mix well. Bring to 96 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- o. Sodium acetate buffer (1.1 M): To a 100-mL volumetric flask, add 14.95 g sodium acetate trihydrate, 60 mL deionized water, and 2.2 mL glacial acetic acid. Mix well to dissolve, and bring to volume with deionized water. Verify  $5 < \text{pH} < 6$ . Store refrigerated in glass. Stable 2 months.
- p. Water (Optima grade and deionized)
- q. 0.1 M, pH 6 Phosphate buffer: To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify  $5.8 < \text{pH} < 6.1$ . Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.
- r. 1:1 Methanol:Water: Combine 50 mL methanol with 50 mL water (both Optima grade) and mix well. Store in glass at room temperature. Stable 12 months.
- s. Methanol (HPLC and Optima grades)
- t. Acetic acid, glacial (17 M) (ACS grade)
- u. 0.1 M Acetic acid: To a 100-mL graduated cylinder, add 80 mL deionized water and 0.5 mL glacial acetic acid. Mix well and bring to 85 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- v. Ammonium formate
- w. Acetonitrile (Optima grade)
- x. 0.5  $\mu\text{m}$  PTFE membrane filter
- y. Methylene chloride (HPLC grade)
- z. Isopropanol (HPLC grade)
- aa. Ammonium hydroxide (concentrated, reagent grade)
- bb. Formic Acid (reagent grade)

- cc. SPE elution solvent (78:20:2 methylene chloride:isopropanol:ammonia): Combine 20 mL HPLC grade isopropanol with 2 mL concentrated ammonium hydroxide and mix well. Add 78 mL HPLC grade methylene chloride and mix well. Store in glass at room temperature. To be prepared fresh.
- dd. Reconstitution solvent (5:95 methanol:water): Combine 5 mL water with 95 mL methanol (both Optima grade) and mix well. Store in glass at room temperature. Stable for 6 months.
- ee. LC mobile phase 1 (95:5:0.05 10 mM ammonium formate : acetonitrile : formic acid): Dissolve 630 mg of ammonium formate in 1 L of Optima grade water. Remove 50 mL of this solution, save for LC Mobile Phase #2, and add 50 mL of acetonitrile. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Add 500 µL formic acid and mix well. Store in glass at room temperature. Stable for 1 months.
- ff. LC mobile phase 2 (5:95:0.05 10 mM ammonium formate : acetonitrile : formic acid): Add 25 mL of the aqueous formate solution from the preparation of LC mobile phase #1 to 475 mL of acetonitrile. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Add 250 µL formic acid and mix well. Store in glass at room temperature. Stable for 1 months.
- gg. Common laboratory supplies such as volumetric flasks, autosampler vials, pipette tips, etc.

## 6 Standards and Controls<sup>1</sup>

- a. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant or another approved supplier. Stability and storage conditions are determined by the manufacturer.

d <sub>3</sub> -Morphine	d <sub>6</sub> -Oxycodone
d <sub>6</sub> -Codeine	d <sub>3</sub> -Hydromorphone
d <sub>3</sub> -Oxymorphone	d <sub>3</sub> -Hydrocodone
d <sub>3</sub> -6-MAM	

- b. Internal Standards Working Solution (4 µg/mL or 1 µg/mL, depending on analyte): Mix 1 mL each of the d<sub>3</sub>-Morphine and the d<sub>6</sub>-Codeine Stock Solutions with 250 µL each of the d<sub>3</sub>-Hydromorphone, d<sub>3</sub>-Hydrocodone, d<sub>3</sub>-Oxymorphone, and d<sub>6</sub>-Oxycodone Stock Solutions. Dilute with 1:1 methanol:water to a final volume of 25 mL. Store at <0°C in glass. Stable for at least 1 year.

<sup>1</sup> Working solutions may be made at different volumes by scaling components if necessary.

- c. d<sub>3</sub>-6-MAM Working Solution (2 µg/mL):  
Dilute 500 µL of the d<sub>3</sub>-6-MAM stock solution to 25 mL in acetonitrile. Store in glass at <0°C. Stable for 6 months.
- d. Standard Stock Solutions (1 mg/mL) may be purchased for Cerilliant (typically used for calibrators) and from Lipomed (typically used for controls) or another approved supplier. Stability and storage conditions are determined by the manufacturer.

Morphine	Oxymorphone	Norcodeine
Codeine	Oxycodone	Noroxycodone
Hydromorphone	6-MAM	Dihydromorphone
Hydrocodone	Normorphine	Dihydrocodeine
Morphine-3-β-glucuronide or Morphine-6-β-glucuronide (0.1 mg/mL)		

- e. Column Performance Evaluation Mix (0.5 µg/mL each component)  
Mix 50 µL each of the morphine, hydromorphone, oxycodone, dihydrocodeine, and norcodeine stock standards. Dilute to 100 mL with Reconstitution solvent (5:95 methanol:water) and mix well. Store refrigerated in glass. Stable for at least one year. A 5 µL portion of this solution is analyzed before each day's samples, in order to confirm acceptable instrument performance.
- f. Control Working Solution #1 (3.5 or 0.7 µg/mL, depending on component):  
Mix 175 µL each of the Morphine and Codeine Stock Solutions with 35 µL each of the Hydromorphone, Hydrocodone, Oxymorphone, and Oxycodone Stock Solutions. Dilute with 1:1 methanol:water to a final volume of 50 mL. Store in glass at <0°C. Stable for at least 1 year.
- g. Control Working Solution #2 (1 µg/mL):  
Dilute 50 µL of the 6-MAM Stock Solution with acetonitrile to a final volume of 50 mL. Store in glass at <0°C. Stable for 6 months.
- h. Control Working Solution #3 (1 µg/mL each component):  
Dilute 50 µL each of the Normorphine, Norcodeine, Noroxycodone, Dihydromorphone, and Dihydrocodeine Stock Solutions with 1:1 methanol:water to a final volume of 50 mL. Store in glass at <0°C. Stable for at least 1 year.
- i. Control Working Solution #4 (2.5 µg/mL):  
Dilute 250 µL of the Morphine-β-glucuronide Stock Solution with 1:1 methanol:water to a final volume of 10 mL. Store in glass at <0°C. Stable for 6 months.
- j. Calibration Working Solution #1 (20 or 4 µg/mL, depending on component):  
Mix 1.0 mL each of the Morphine and Codeine Stock Solutions with 200 µL each of the Hydromorphone, Hydrocodone, Oxymorphone, and Oxycodone Stock Solutions and

dilute with 1:1 methanol:water to a final volume of 50 mL. Store in glass at <0°C. Stable for at least 1 year.

- k. Calibration Working Solution #2 (1 or 0.2 µg/mL, depending on component):  
Dilute 2.5 mL of the Calibration Working Solution #1 to 50 mL with 1:1 methanol:water. Store in glass at <0°C. Stable for at least 1 year.
- l. Calibration Working Solution #3 (2.5 µg/mL):  
Dilute 125 µL of the 6-MAM Stock Solution with acetonitrile to a final volume of 50 mL. Store in glass at <0°C. Stable for 6 months.
- m. Calibration Working Solution #4 (0.5 µg/mL):  
Dilute 10 mL of the Calibration Working Solution #3 with acetonitrile to a final volume of 50 mL. Store in glass at <0°C. Stable for 6 months.

Table 1: Blood Calibrator Preparation

Volume of Matrix (mL)	Volume of Cal Solution #1 (µL)	Volume of Cal Solution #2 (µL)	Volume of Cal Solution #3 (µL)*	Volume of Cal Solution #4 (µL)*
Level 1 – 25 ng/mL morphine and codeine, 5 ng/mL for all others				
0.95	0	25**	0	10
Level 2 – 50 ng/mL morphine and codeine, 10 ng/mL for all others				
0.95	0	50	0	20
Level 3 – 100 ng/mL morphine and codeine, 20 ng/mL for all others				
0.85	0	100	0	40
Level 4 – 300 ng/mL morphine and codeine, 40 ng/mL 6-MAM, 60 ng/mL for all others				
0.95	15	0	16	0
Level 5 – 500 ng/mL morphine and codeine, 60 ng/mL 6-MAM, 100 ng/mL for all others				
0.95	25	0	24	0
Level 6 – 700 ng/mL morphine and codeine, 80 ng/mL 6-MAM, 140 ng/mL for all others				
0.95	35	0	32	0
Level 7 – 1000 ng/mL morphine and codeine, 100 ng/mL 6-MAM, 200 ng/mL for all others				
0.90	50	0	40	0

\* - Calibration solutions #3 and #4 should not be added to samples that will be subjected to hydrolysis.

\*\* - This calibrator will be outside the linear range for hydromorphone.

- n. Negative Control: Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay. When possible, the negative control will be matrix matched.

When samples are analyzed in a batch using hydrolysis, a Negative Control will be hydrolyzed, extracted and analyzed.

- o. **Positive Control:** These are normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as circumstances dictate. Storage and stability determined by manufacturer. Normally prepared by adding the amounts of Control Working Solution to 1 mL matrix as directed in Table 1 below. Quantitative controls are typically prepared in duplicate. When possible, the Positive Control will be matrix matched. Additionally, deuterated analog internal standards serve as a qualitative positive control for each individual specimen.

Table 2: Opiate Control Preparation

Volume of Control Solution #1 (µL)	Volume of Control Solution #2 (µL)*	Volume of Control Solution #3 (µL)*	Volume of Control Solution #4 (µL)
Qualitative Blood or Urine Control (245 ng/mL morphine and codeine, 49 ng/mL for all other target analytes)			
70	49	49	0
Low Quantitative Blood Control (70 ng/mL morphine and codeine, 15 ng/mL for 6-MAM, and 14 ng/mL all other quantitated analytes)			
20	15	0	0
High Quantitative Blood Control (770 ng/mL morphine and codeine, 80 ng/mL 6-MAM, 154 ng/mL for all other quantitated analytes)			
220	80	0	0
Hydrolysis Control (250 ng/mL morphine-glucuronide 154 ng/mL morphine)**			
0	0	0	100

\* - Control solution #2 should not be added to samples that will be subjected to hydrolysis.

\*\* - The Hydrolysis Control is analyzed whenever hydrolysis is performed on case specimens to ensure that the enzyme is working properly.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Measure out 1 mL of bio fluid or 2 g of a 1:1 tissue homogenate into a labeled 16 x 125

mm screw-top test tube. For quantitation, case samples and Positive Controls are typically analyzed in duplicate. When performing hydrolysis on case samples, a set of hydrolyzed Negative and Positive Controls will be analyzed. (Ensure that no 6-MAM is added to Positive Controls when performing hydrolysis.)

- b. Add 50  $\mu$ L of the Internal Standards Working Solution to the specimen and vortex.<sup>2</sup>
- c. For "total" opiate assays: Enzymatically hydrolyze the sample by adjusting the pH to approximately 5.2 with 1 mL of 1.1 M sodium acetate buffer coupled with the addition of 30  $\mu$ L of  $\beta$ -glucuronidase. Vortex. Incubate overnight at approximately 37°C.
- d. For "free" opiate assays: Add 25  $\mu$ L of the d<sub>3</sub>-6-MAM Working Solution and 1 mL of deionized water and vortex.
- e. Add 4 mL of 0.1 M phosphate buffer and vortex. Verify that the pH is between 5.5 and 6.5.
- f. For blood and tissue specimens: Centrifuge at high speed for 15 minutes. Transfer supernatant to a clean 16 x 100 mm culture tube, leaving solid material behind.
- g. Pre-rinse SPE extraction cartridge by adding 3 mL of methanol (HPLC grade) at 1 mL/minute.
- h. Condition cartridge with 3 mL of deionized water followed by 1 mL of 0.1 M phosphate buffer at 1 mL/minute. Do not allow sorbent to dry.
- i. Load sample on SPE cartridge at 1-2 mL/minute. Do not allow sorbent to dry.
- j. Wash cartridge with 3 mL of deionized water, 1 mL of 0.1 M acetic acid, and 3 mL of methanol (Optima grade) (each at 1-2 mL/minute).
- k. Dry cartridge under full vacuum for 3 minutes.
- l. Apply 3 mL of SPE Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm culture tubes.
- m. Evaporate to dryness under nitrogen at 40EC.
- n. Reconstitute the dry residue in 100  $\mu$ L of reconstitution solvent (5:95 methanol:water) and analyze 5  $\mu$ L portions by LC-electrospray-tandem MS with the conditions given in

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<sup>2</sup>Other internal standards may be substituted at relevant concentrations if deemed appropriate.

section 10. Be sure to analyze an injection of a solvent blank under the column wash conditions specified in Section 10.3 of this procedure at least every 15 analytical injections.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
2: 5:95:0.05 10 mM formate : acetonitrile : formic acid	total flow	0.25 mL/min		type	Phenyl (Xterra)
	time (min)	%2	%1	length	15 cm
1: 95:5:0.05 10 mM formate : acetonitrile : formic acid	0	0	100	internal diameter	2.1 mm
	2	0	100	particle size	5 µm
	6	20	80	temperature	30°C
	10	20	80		
	11	60	40		
	16	60	40		
	17	0	100		
	25	0	100		
	total time		25 min		

## 9.2 Mass Spectrometer Parameters

Source Parameters		
Mode: Electrospray	Spray Voltage: +5 kV	Capillary Temperature: 225°C
Sheath Gas: 25 (arb units)	Aux Gas: 12 (arb units)	Sweep Gas: 0 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Segment #1 (0-2 minutes) (1 scan event)		
Event #1	full scan m/z 200 – 370, 7500 resolution (minimum)	
Segment #2 (2-6.5 minutes) (3 scan events)		
Event #1	full scan m/z 200 - 370, 7500 resolution (minimum)	
Event #2	MS/MS data dependant scan (unit resolution)	collision energy: 30% (rel)
	precursor: most intense of m/z 272.13, 284.13, 286.14, 288.16	
	isolation width: 2.0 AMU	scan range: software control
Event #3	MS <sup>3</sup> product scan (unit resolution)	collision energy: see below
	precursor: m/z 302.2 (CE = 30%) > m/z 284.2 (CE = 30%)	
	isolation width: 2.0 AMU	scan range: m/z 75-320
Segment #3 (6.5-15 minutes) (4 scan events)		
Event #1	full scan m/z 200 - 370, 7500 resolution (minimum)	
Event #2	MS/MS data dependant scan (unit resolution)	collision energy: 25% (rel)
	precursor: most intense of m/z 284.13, 286.14, 298.14, 300.16, 302.18, 328.15	
	isolation width: 2.0 AMU	scan range: software control
Event #3	MS <sup>3</sup> product scan (unit resolution)	collision energy: see below
	precursor: m/z 316.2 (CE = 25%) > m/z 298.2 (CE = 35%)	
	isolation width: 2.0 AMU	scan range: m/z 80-330
Event #4	MS <sup>3</sup> product scan (unit resolution)	collision energy: see below
	precursor: m/z 302.2 (CE = 30%) > m/z 284.2 (CE = 30%)	
	isolation width: 2.0 AMU	scan range: m/z 75-320
Segment #4 (15-25 minutes) (1 scan event)		
Event #1	full scan m/z 200 – 370, 7500 resolution (minimum)	



**9.3 Column Washing** – At least once every 15 injections, the column will be washed under the following conditions to keep the analytical column in good working order.

Mobile Phase Compositions	Flow Parameters			Column Parameters	
A: 5:95:0.05 10 mM formate : acetonitrile : formic acid	total flow	0.25 mL/min		type	Phenyl (Xterra)
	time (min)	%A	%B	length	15 cm
B: 95:5:0.05 10 mM formate : acetonitrile : formic acid	0	0	100	internal diameter	2.1 mm
	1	0	100	particle size	5 µm
Mass Spectrometer	4	90	10	temperature	30°C
As above, but only one segment with one scan event throughout the analysis: full scan from m/z 200 to m/z 370.	14	90	10		
	17	0	100		
	25	0	100		
	total time		25 min		

## 10 Decision Criteria

### 10.1 Batch Acceptance Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All intended analytes should be present in the Positive Control. Each Quantitative Positive Control shall quantitate within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for more information.

### 10.2 Sample Acceptance Criteria

#### 10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

##### 10.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute,

as appropriate) obtained from injection of a reference standard, calibrator, or Positive Control.

Table 3: Retention Time Data

Compound Name	RRT (to d <sub>3</sub> -morphine)
d <sub>3</sub> -Morphine	RT ~ 4 min
Morphine	1.0
Codeine	2.0
6-MA M	2.1
Oxycodone	2.1
Oxymorphone	1.1
Hydrocodone	2.2
Hydromorphone	1.4
Normorphine	0.8
Norcodeine	2.0
Noroxycodone	2.1
Dihydromorphone	0.9
Dihydrocodeine	2.0

Note: Norhydrocodone (M+H 286.144) elutes with a RRT of 2.3.

#### 10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

#### 10.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance. Mass spectral fragments of commonly encountered opioids are listed in Table 4. Under the listing of preferred tandem MS product ions, the normal base peak is listed in bold text. Other significant ions may be substituted for preferred ions if uncorrectable interference exists for that ion.

Table 4: Mass Spectrometry Data:

Compound Name	Quantitation Ion(s) from Full Scan MS	Precursor Ion for MS <sup>2</sup> or Precursor Chain for MS <sup>3</sup>	Preferred Tandem MS Product Ions
Morphine	286.144	286.14	183, <b>201</b> , 211
d <sub>3</sub> -Morphine	289.163	NA	NA
Codeine	300.159	300.16	<b>215</b> , 225, 282
d <sub>6</sub> -Codeine	306.197	NA	NA
6-MA M	328.154	328.15	193, <b>211</b> , 268
d <sub>3</sub> -6-MA M	331.173	NA	NA
Oxycodone	316.154, 298.144*	316.2 > 298.2*	187, 241, <b>256</b>
d <sub>6</sub> -Oxycodone	322.192, 304.151*	NA	NA
Oxymorphone	302.139	302.2 > 284.2	199, 227, <b>242</b>
d <sub>3</sub> -Oxymorphone	305.158	NA	NA
Hydrocodone	300.159	300.16	<b>199</b> , 241, 257
d <sub>3</sub> -Hydrocodone	303.178	NA	NA
Hydromorphone	286.144	286.14	<b>185</b> , 227, 243
d <sub>3</sub> -Hydromorphone	289.163	NA	NA
Normorphine	NA	272.13	201, 229, <b>254</b>
Norcodeine	NA	286.14	215, 225, <b>268</b>
Noroxycodone	NA	302.2 > 284.2*	187, <b>229</b> **
Dihydromorphone	NA	288.16	<b>187</b> , 213, 231
Dihydrocodeine	NA	302.18	<b>201</b> , 227, <b>245</b> ***

\* Oxycodone and noroxycodone both show large (M-18) fragments in their full scan mass spectra, with significant variation in the ion ratio dependant upon concentration. The instrument method is set to acquire MS/MS spectra of these fragments in addition to the MS<sup>3</sup> spectra of the pseudomolecular ion in case the pseudomolecular precursor is too weak to provide good spectral fidelity. The MS/MS and MS<sup>3</sup> spectra are qualitatively similar, but show different ion ratios.

\*\* Noroxycodone normally yields only two fragment ions of reasonable intensity in MS<sup>3</sup> analysis. A criterion of no other ions present at >15% of the base peak may be used as additional criteria for the presence of this compound.

\*\*\* Either m/z 201 or m/z 245 may be the base peak for MS/MS of dihydrocodeine, depending upon the specific sample.

## 11 Calculations

Linear regression analysis with equal or 1/x weighting is performed for all analytes except codeine using a  $\pm 20$  mmu extracted ion mass window in the full scan high resolution data. For codeine, calibration is performed using the quadratic log-log fit. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- precision of the pipette used to deliver the sample
- precision of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

a. Method Performance Parameters:

LOD Limit of Detection; LLOQ Lower Limit of Quantitation

Compound	LOD in Blood (ng/mL)	LOD in Urine (ng/mL)	LLOQ (ng/mL)	Linear Range (ng/mL)	Accuracy (% bias)	Precision (% intermed)
Morphine	10	25	25	25-1000	-0.8	3.5 to 8.6
Codeine	5	10	25	25-1000	+16.5	4.5 to 10.6
Hydromorphone	5	10	10	10-200	-2.8	5.0 to 9.0
Hydrocodone	2	5	5	5-200	+1.6	4.8 to 7.2
Oxymorphone	2	5	5	5-200	-2.0	9.7 to 12.7
Oxycodone	1	2	5	5-200	-1.1	4.9 to 10.2
6-MAM	2	10	5	5-100	-13.5	4.3 to 6.8
Normorphine	5	10	<i>Not evaluated.</i>			
Norcodeine	10	10				
Noroxycodone	5	10				
Dihydromorphone	10	10				
Dihydrocodeine	5	5				

b. Interferences: Grossly decomposed or putrefied samples may affect both detection and quantitation limits. Very high levels of codeine (>1 µg/mL) may interfere with accurate quantitation of oxycodone, and very high levels of naltrexone may interfere with accurate quantitation of oxycodone. In none of these cases will qualitative identification be compromised. High levels of naloxone may interfere with detection and quantitation of hydromorphone, but would not yield false positive results. A compound that is present in many blank blood samples has shown to interfere with

the quantitation of oxycodone and oxymorphone at unit mass resolution, but this compound can be resolved using high resolution.

- c. Other Considerations: The enzymatic hydrolysis procedure will convert a large fraction of any 6-MAM in a sample to free morphine. Appropriate care should be taken in interpreting total morphine concentration in any sample for which 6-MAM was detected in the free opioid analysis.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Moffat, A.C., *Isolation and Identification of Drugs*, 2nd ed., Pharmaceutical Press: London, 1986.

Edinboro, L. E., Backer, R. C., Poklis, A., “Direct Analysis of Opiates in Urine by Liquid Chromatography-Tandem Mass Spectrometry”, *Journal of Analytical Toxicology*, v. 29, pp. 704-710, 2005.

Al-Asmari, A. I., Anderson, R. A., “Method for Quantification of Opioids and Their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry”, *Journal of Analytical Toxicology*, v. 31, pp. 394-408, 2007.

*FBI Laboratory Safety Manual*.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

*Instrument Support SOP Manual*; FBI Laboratory Chemistry Unit.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

Rev. #	Issue Date	History
4	10/01/14	In sections 5 and 15, removed references to Tox 103. In 5.b, updated precolumn to prefilter. In Section 5, included recipes for reagents. In Section 5 (ee and ff) and Section 9.1, changed mobile phase designators from letters to numbers. In Section 6, combined standards into tables and renumbered rest of Section. Updated Positive Control scheme in Section 6 to cover far ends of the calibration curve and to include a Hydrolysis Control. Removed Calibration Section (Section 7) and renumbered subsequent sections. Moved calibrator preparation instructions to Section 6 (Table 1) and renames old Table 1 as Table 2. In 8.a and bench sheet, specified duplicate analysis for quantitation. In Section 11, added option for 1/x weighting. Reformatted Appendix 2 to include all pertinent instrumental parameters.
5	09/11/19	Updated Scope language. In section 5 (ee and ff) changed stability to 1 month. Added footnote to allow for preparation of different volumes of working solutions in Section 6. In 6.n., clarified when a Negative Control must be analyzed hydrolyzed. Updated qualitative control preparation instructions in Table 2. In Section 8 and bench sheet, clarified hydrolysis control analysis requirement. Updated codeine linearity calculations in Section 11. Removed references to "Subunit".

### **Approval**

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Acting Toxicology  
 Technical Leader:

Date: 09/09/2019

Chemistry Unit Chief:

Date: 09/09/2019

### **QA Approval**

Quality Manager:

Date: 09/09/2019

**Appendix 1: Abbreviated version of the Solid Phase Extraction of Opioids  
from Biologicals with Analysis by LC-Tandem MS for bench use.**

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**Appendix 2: Abbreviated version of the Opioids LC-Tandem MS Instrumental  
Parameters for bench use.**

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**Appendix 2: Abbreviated version of the Opioids LC-Tandem MS Instrumental  
Parameters for bench use. (continued)**

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## **Chloral Hydrate Metabolites by Headspace GC/MS(EI)**

### **1 Introduction**

Chloral hydrate, an older hypnotic agent, rapidly metabolizes in the body to trichloroethanol (TCE) which is excreted unchanged and as a glucuronide conjugate. TCE is further metabolized to trichloroacetic acid (TCA), which is also excreted in the urine. TCA has a very long elimination half life, and can be detected in the urine days after chloral hydrate consumption. Chloral hydrate is infrequently prescribed, but may be used in a drug-facilitated assault.

### **2 Scope**

This procedure allows for the screening and confirmation of TCE and TCA in urine.

### **3 Principle**

Urine is mixed in a headspace vial with sulfuric acid and dimethyl sulfate. The dimethyl sulfate is used to form the methyl ester derivative of TCA. The sulfuric acid cleaves any glucuronide conjugated TCE and aids in the derivatization of TCA. Vials are heated at 60°C for three hours, and the headspace is analyzed by GC/MS(EI) simultaneously in SIM and Scan modes.

### **4 Specimens**

This procedure uses 1 mL of urine.

### **5 Equipment/Materials/Reagents**

- a. Hewlett Packard Gas Chromatograph/Mass Spectrometer equipped with a headspace autosampler and a 30 m x 0.25 mm x 1.4 µm DB-624 column, or equivalent
- b. 20-mL disposable headspace vials, magnetic caps, and crimper
- c. Pipetters and disposable tips
- d. Sulfuric Acid (Reagent grade, or better)
- e. Dimethyl Sulfate (99.8% pure)
- f. Disposable syringes with hypodermic needles

## 6 Standards and Controls

- a. 2,2,2-Trichloroethanol (99+%)
- b. Trichloroacetic acid (Sigma Ultra, 99.0%)
- c. TCE Working Standard Solution (1.0 mg/mL):  
Add 65  $\mu$ L trichloroethanol to about 90 mL deionized water in a 100-mL volumetric flask. Dilute to the mark with deionized water and mix thoroughly. Store refrigerated in a tightly sealed glass or plastic container. Stable for 2 months.
- d. TCA Working Standard Solution (1.0 mg/mL):  
Add 10 mg trichloroacetic acid to a 10-mL volumetric flask. Dilute to the mark with deionized water and mix thoroughly. Store refrigerated in a tightly sealed glass or plastic container. Stable for 2 months.
- e. Negative Control Urine:  
Obtain in house. Store refrigerated in plastic. Stable for at least one year. A Negative Control Urine sample will be analyzed with every assay.
- f. Positive Control Urine:  
To 1 mL aliquots of urine, add 20  $\mu$ L of the TCE and TCA Working Standard Solutions to prepare a 20 PPM Positive Control Sample, or 100  $\mu$ L of each Working Standard Solution to prepare a 100 PPM Positive Control Sample. At least one Positive Control Urine sample will be analyzed with every assay.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Into properly labeled 20-mL headspace vials add 1 mL of urine. Positive controls may be prepared directly in the headspace vial.
- b. Immediately cap.
- c. Add 0.5 mL concentrated sulfuric acid through the cap using a disposable syringe with a hypodermic needle. Add 0.1 mL dimethyl sulfate through the cap using a disposable syringe with a hypodermic needle.
- d. Analyze specimens by headspace GC/MS(EI) after confirming that the instrument is calibrated and in proper working condition.

## 10 Instrumental Conditions

### 10.1 Headspace Sampler Parameters

incubation temperature	60°C	syringe temperature	90°C
incubation time	180 min	sample fill volume	2.5 mL
agitator speed	300 RPM	sample fill rate	1.0 mL/sec
agitation timing	10 sec on 1 sec off	sample fill strokes	5
injection volume	1.0 mL	sample injection speed	1.0 mL/sec
		syringe flush time	240 sec

## 10.2 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature 1	50°C	type	DB-624	inlet temp.	150°C
hold 1	3 min	length	30 m	injection mode	split
ramp 1	10°C/min	internal diameter	0.25 mm	carrier gas	ultrapure helium
temperature 2	250°C	film thickness	1.4 µm	carrier mode	constant pressure
hold 2	15 min			pressure	6.54 psi
total run time	38 min			split ratio	10:1

## 10.3 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	Scan/SIM	transfer line temperature	260°C
scan range	29 - 200 AMU	quadrupole temperature	150°C
multiplier offset	+106 V	solvent delay	4 min
SIM group 1 (4-12 min)	59, 82, 117, 119	dwel times	20
SIM group 2 (12-38 min)	31, 49, 77, 113		

## 11 Decision Criteria

The criteria in sections 11.1 through 11.2 are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or positive controls.

### 11.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### 11.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, or an extracted positive control. The relative retention times of the components should agree with those listed in the enclosed table within  $\pm 2\%$ . If not, the shift in relative retention times should be noted and appropriate corrections made when analyzing the data generated from case specimens.

Expected Retention Times for TCA-methyl ester and TCE

Analyte	Expected RT (min)
TCA-methyl ester	11.843
TCE	12.239

### 11.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

### 11.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard, calibrator, or Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* (Tox 104) standard operating procedure for further guidance.

### 12 Calculations

Not applicable.

### 13 Uncertainty of Measurement

Not applicable.

## 14 Limitations

a. Limits of Detection:

1 PPM for TCE  
0.5 PPM for TCA

b. Interferences:  
None known.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

Breimer, D.D., Ketelaars, H.C.J. and Van Rossum, J.M. "Gas Chromatographic Determination of Chloral Hydrate, Trichloroethanol and Trichloroacetic Acid in Blood and Urine Employing Headspace Analysis", *J Chrom*, 1974, 88, 55-63.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
0	04/17/09	New document.
1	08/23/12	Updated chromatography decision criteria in Section 11.1. Added expected retention times to Section 11.1.1.

**Approval**

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**Appendix 1: Abbreviated version of the Chloral Hydrate Metabolite Procedure for bench use.**

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## **Direct Solvent Extraction of Sympathomimetic Amines from Biological Samples**

### **1 Introduction**

Sympathomimetic amines (SMAs) are generally a class of synthetic phenethylamine-derived drugs often generically referred to as “amphetamines”. Almost all of these compounds show some degree of stimulant effects, but a wide variety of additional structure-dependent pharmacological effects can be seen in various compounds. These include pure stimulants (amphetamine and methamphetamine), decongestants (phenylpropanolamine and pseudoephedrine), anorexics (phentermine and fenfluramine), and hallucinogens (mescaline, one of the few relevant naturally occurring SMAs). Over the last few decades there has been particular interest in and concern over the widespread illicit use of various “designer” SMAs with combined stimulant and hallucinogenic properties. The “type specimen” of this class is 3,4-methylenedioxy-methamphetamine (MDMA or “ecstasy”), which was originally developed for possible use as an adjunct drug in psychotherapy, but now is one of the most widely used illicit drugs in teenage and young adult populations. Chemists in clandestine drug laboratories have developed a wide array of related compounds, including thioalkyl- and halogen-containing analogues, in attempts to stay ahead of drug scheduling regulations. In approximately 2010, several new designer amphetamines including methylone, mephedrone, and 3,4-methylenedioxypyrovalerone began appearing on the U.S. abused drug scene as “bath salts”.

### **2 Scope**

This procedure allows for screening and confirmation of a wide range of SMAs, and is currently validated for quantitation of amphetamine, methamphetamine, ephedrine / pseudoephedrine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), methylone, mephedrone and 3,4-methylenedioxypyrovalerone (MDPV). With appropriate validation, it may be used for quantitation of other detected SMAs.

### **3 Principle**

Biological specimens are qualitatively assayed and/or quantitated for SMAs. Specimens are mixed with an internal standard (normally a mixture of six deuterated SMAs), adjusted to a basic pH, and extracted with hexane. (When quantitating bath salt compounds, a mixture of deuterated bath salt compounds is used as the internal standard mixture.) The hexane is removed, acidified to prevent evaporation of volatile SMAs, and taken to dryness. The resulting residue is reconstituted in 10/90 methanol/water and analyzed by LC-ESI-MS with data dependant MS<sup>2</sup> and MS<sup>3</sup>. MS<sup>3</sup> detection is

included because some SMAs yield uninformative MS<sup>2</sup> spectra with limited information content. The extraction procedure is derived from work by Sadeghipour and Veuthey. The chromatographic and mass spectral procedures and parameters were developed in-house.

#### 4 Specimens

This procedure uses a biological sample such as: blood, serum, plasma, urine, gastric contents, vitreous humor, or a prepared tissue homogenate. When available, 0.5 mL of biological fluid or 1.0 g of tissue homogenate (1:1) is used in the assay. In instances where specimen volume is altered (e.g. to improve sensitivity or account for limited specimen volume), appropriate modifications to this procedure may be made.

#### 5 Equipment/Materials/Reagents

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. 16x100 mm screw-top tubes with Teflon-lined caps
- b. 12x75 mm culture tubes with polypropylene snap-tops
- c. Acetonitrile (Optima grade or better)
- d. Formic Acid (Puriss grade or better)
- e. Hexane (UV grade or better)
- f. Hydrochloric acid (ACS grade or better)
- g. Methanol (Optima grade or better)
- h. Sodium hydroxide (ACS grade or better)
- i. Water (Deionized and Optima or better grade)
- j. 4% Sodium hydroxide  
Dissolve 2 g sodium hydroxide in 50 mL deionized water. Store in plastic at room temperature. Stable for at least 6 months.

- k. Methanol:Hydrochloric Acid (4:1 v:v)  
Mix 20 mL methanol with 5 mL hydrochloric acid. Store in glass at room temperature.  
Stable for at least 1 month.
- l. Methanol:Water (10:90 v:v)  
Mix 5 mL methanol with 45 mL water (both Optima grade or better). Store in glass at room temperature. Stable for at least 1 year.
- m. 0.1% Formic acid in acetonitrile  
Vacuum filter 500 mL acetonitrile through a 5 µm PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.
- n. 0.1% Formic acid in water  
Vacuum filter 500 mL water (Optima grade or better) through a 5 µm PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.
- o. Vortex mixer, Rotator and Centrifuge
- p. Evaporator with nitrogen
- q. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
- r. Liquid Chromatograph-Ion Trap Mass Spectrometer
- s. HPLC Column (Xterra C18, 2.1 x 150 mm, 5 µm dp, with a 2.1 x 7.5 mm guard column; or equivalent)

## 6 Standards and Controls

- a. Internal Standard Stock Solutions (0.1 mg/mL) may be purchased from Cerilliant or other approved supplier. Stability and storage conditions are determined by the manufacturer.

d <sub>5</sub> -Amphetamine	d <sub>5</sub> -MDEA
d <sub>5</sub> -Methamphetamine	d <sub>3</sub> -Mephedrone
d <sub>5</sub> -MDA	d <sub>3</sub> -Methylone
d <sub>5</sub> -MDMA	d <sub>8</sub> -MDPV

- b. Internal Standard Working Solution (2 µg/mL each of common components):  
Combine 0.5 mL each of the d<sub>3</sub>-ephedrine, d<sub>5</sub>-amphetamine, d<sub>5</sub>-methamphetamine, d<sub>5</sub>-MDA, d<sub>5</sub>-MDMA, and d<sub>5</sub>-MDEA stock solutions in a 25 mL volumetric flask. Add 2

mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 2 years.

- c. Bath Salts Internal Standard Working Solution (2 µg/mL each of d<sub>3</sub>-mephedrone, d<sub>3</sub>-methyldone, and d<sub>8</sub>-MDPV):  
Combine 0.5 mL each of the d<sub>3</sub>-mephedrone, d<sub>3</sub>-methyldone, and d<sub>8</sub>-MDPV stock solutions in a 25 mL volumetric flask. Add 2 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 2 years.

- d. Standard Stock Solutions (1 mg/mL) may be purchased from Cerilliant (typically used for calibrators) and from Lipomed (typically used for controls) or another approved supplier. Stability and storage conditions are determined by the manufacturer.

Ephedrine	MDMA
MBDB (N-methylbenzodioxazolybutanamine, N-methyl-1-3,4-methylenedioxy-phenyl)-2-butanamine )	MDEA
Amphetamine	Mephedrone
Methamphetamine	Methyldone
MDA	MDPV

- e. Amine Mixture-6 (250 µg/mL each component):  
A methanol solution containing amphetamine, methamphetamine, phentermine, MDA, MDMA, and MDEA purchased from Cerilliant or another approved vendor. Stability and storage conditions are determined by the manufacturer.
- f. Column Performance Evaluation Mix (1 µg/mL each component):  
Combine 25 µL each of the MBDB and ephedrine stock solutions with 100 µL of the Amine Mixture-6 in a 25 mL volumetric flask. Add 2.4 mL methanol and bring to the mark with water (both Optima grade or better). Stable for at least 2 years. A 10 µL portion of this solution is analyzed before each day's samples, in order to confirm acceptable instrument performance.
- g. Control Working Solution (1 µg/mL each component):  
Mix 50 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 9.9 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 1 year. Note: Once verified, this solution can be parsed out into small containers for freezing.
- h. Bath Salts Control Working Solution (1 µg/mL each component):  
Mix 50 µL each of the mephedrone, methyldone, and MDPV stock solutions in a 50 mL volumetric flask. Add 9.9 mL methanol and bring to the mark with water (both Optima

grade or better). Store in glass at  $<0^{\circ}\text{C}$ . Stable for at least 1 year. Note: Once verified, this solution can be parsed out into small containers for freezing.

- i. Calibration Working Solution #1 ( $5\text{ }\mu\text{g/mL}$  each component):  
Mix  $250\text{ }\mu\text{L}$  each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a  $50\text{ mL}$  volumetric flask. Add  $8.5\text{ mL}$  methanol and bring to the mark with water (both Optima grade or better). Store in glass at  $<0^{\circ}\text{C}$ . Stable for at least 1 year. Note: Once verified, this solution can be parsed out into small containers for freezing.
- j. Calibration Working Solution #2 ( $0.5\text{ }\mu\text{g/mL}$  each component):  
Mix  $25\text{ }\mu\text{L}$  each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a  $50\text{ mL}$  volumetric flask. Add  $9.9\text{ mL}$  methanol and bring to the mark with water (both Optima grade or better). Store in glass at  $<0^{\circ}\text{C}$ . Stable for at least 1 year. Note: Once verified, this solution can be parsed out into small containers for freezing.
- k. Bath Salts Calibration Working Solution #3 ( $5\text{ }\mu\text{g/mL}$  each component):  
Mix  $250\text{ }\mu\text{L}$  each of the mephedrone, methylone and MDPV stock solutions in a  $50\text{ mL}$  volumetric flask. Add  $9.25\text{ mL}$  methanol and bring to the mark with water (both Optima grade or better). Store in glass at  $<0^{\circ}\text{C}$ . Stable for at least 1 year. Note: Once verified, this solution can be parsed out into small containers for freezing.
- l. Bath Salts Calibration Working Solution #4 ( $0.5\text{ }\mu\text{g/mL}$  each component):  
Mix  $25\text{ }\mu\text{L}$  each of the mephedrone, methylone and MDPV stock solutions in a  $50\text{ mL}$  volumetric flask. Add  $9.9\text{ mL}$  methanol and bring to the mark with water (both Optima grade or better). Store in glass at  $<0^{\circ}\text{C}$ . Stable for at least 1 year. Note: Once verified, this solution can be parsed out into small containers for freezing.
- m. Negative Control Blood and/or Urine:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or prepared in-house from an appropriate blank specimen. Blood and urine will be stored refrigerated, frozen or obtained fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay. When possible, the negative control will be matrix matched.
- n. Quantitative Positive Control Blood:  
This is normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as needed. Storage and stability determined by manufacturer. Normally prepared at concentrations of  $60$  and  $400\text{ ng/mL}$  by adding  $30$  and  $200\text{ }\mu\text{L}$  of the Control Working Solution to  $0.5\text{ mL}$  samples of Negative Control Blood on the day of extraction. Other levels and matrices may be used as circumstances dictate.

- o. **Bath Salts Quantitative Control Blood:**  
This is normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as needed. Storage and stability determined by manufacturer. Normally prepared at concentrations of 60 and 400 ng/mL by adding 30 and 200 µL of the Bath Salts Control Working Solution to 0.5 mL samples of Negative Control Blood on the day of extraction. Other levels and matrices may be used as circumstances dictate.
- p. **Qualitative Positive Control Blood or Urine:**  
This is normally prepared in-house, but may be purchased from an appropriate vendor as needed. Storage and stability determined by manufacturer. Normally prepared at a concentration of 200 ng/mL by spiking a 0.5 mL portion of negative control matrix with 100 µL of the Control Working Solution and the Bath Salts Control Working Solution. Other levels may be used as circumstances dictate. Additionally, deuterated analog internal standards serve as a qualitative positive control for each individual specimen.

## 7 Calibration

This procedure may be used quantitatively via construction of a multi-point calibration curve with equal weighting for the analyte(s) of interest following the *Guideline for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows typical concentrations and volumes for blood calibrators.

Table 1: Typical Blood Calibrator Preparation

Cal Level (ng/mL)	Blood Volume (mL)	Calibrator Working Solution #1 Volume (µL)	Calibrator Working Solution #2 Volume (µL)
25	0.5	0	25
50	0.45	0	50
75	0.45	0	75
100	0.4	0	100
250	0.5	25	0
500	0.45	50	0
750	0.45	75	0

Table 2: Bath Salts Blood Calibrator Preparation

Cal Level (ng/mL)	Blood Volume (mL)	Bath Salts Calibrator Working Solution #3 Volume (µL)	Bath Salts Calibrator Working Solution #4 Volume (µL)
25	0.5	0	25
50	0.45	0	50
75	0.45	0	75
100	0.4	0	100
250	0.5	25	0
500	0.45	50	0
750	0.45	75	0

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- To a properly labeled 16x100 mm screw-top tube add 0.5 mL of biological fluid or 1 g of a prepared tissue homogenate (1:1 in water). Also prepare Negative and Positive Controls as described in Section 6. For quantitation, prepare calibrators as described in Section 7, and prepare case samples in duplicate.
- Add 50 µL of the Internal Standards Working Solution, resulting in a concentration of 200 ng/mL for each internal standard. For bath salts quantitations, add 50 µL of the Bath Salts Internal Standard Working Solution.
- Add 0.2 mL of 4% sodium hydroxide to each sample and vortex briefly.
- Add 2 mL of hexane to each tube and extract for 20 minutes on a rotator. Centrifuge 10 minutes at a minimum of 3000 rpm. Use a wooden stick to break up any emulsions that develop, and recentrifuge if necessary.
- Transfer organic (top) layer to a 12x75 mm culture tube.
- Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.



- g. Evaporate to dryness under a gentle stream of nitrogen at approximately 40°C.
- h. Reconstitute the dried residue in 0.1 mL of 10:90 methanol:water.
- i. Analyze by 10 µL by LC-MS-ESI with data dependent tandem MS (DDS) with the conditions given below (Sections 10.1 and 10.2).

## 10 Instrumental Conditions

Appendix 2 contains a checklist of method parameters that should be used to verify proper instrumental conditions prior to analysis of case samples.

### 10.1 Liquid Chromatograph Parameters (Shimadzu Prominence, or equivalent)

Mobile Phase Compositions	Flow Parameters			Column Parameters	
1: 0.1% formic acid in acetonitrile	total flow	0.3 mL/min		type	C18
	time (min)	%1	%2	length	150 mm
2: 0.1% formic acid in water	0	7.5	92.5	internal diameter	2.1 mm
	5	7.5	92.5	particle size	5 μm
	20	60	40	temperature	40°C
	23	60	40	guard length	7.5 mm
	28	7.5	92.5	guard ID	2.1 mm
	32	7.5	92.5		
	total time		32 min		

## 10.2 Mass Spectrometer Parameters with DDS (Thermo / Finnigan LTQ, or equivalent)

Source Parameters			
Mode: Electrospray		Spray Voltage: +5 kV	Capillary Temperature: 250°C
Sheath Gas: 25 (arb units)		Aux Gas: 10 (arb units)	Sweep Gas: 0 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.			
1 Segment with 3 Scan Events			
Event #1	full scan m/z 125-350		
Event #2*	MS/MS data dependent scan		collision energy: 70 (rel)
	precursor: most intense ion from event #1, excluding m/z 141, 155, 169, 181, 185, 199, 211, 213 and 284, threshold = 1000 counts		
	isolation width: 2.0 AMU		scan range: software control
Event #3*	MS <sup>3</sup> data dependent scan		collision energy: 70 (rel)
	precursor: most intense neutral loss of 17, 18, 31, or 45 observed in event #2, threshold = 1000 counts		
	isolation width: 2.0 AMU		scan range: software control
Dynamic Exclusion Enabled for Data Dependent Scanning			
repeat count	10	repeat duration	30 seconds
exclusion list size	25	exclusion duration	30 seconds
expiration count	5	expiration threshold	s/n<5
exclusion width	-1 to +2 amu		

\*These events can also be limited to one mass or several masses for targeted analysis.

## 11 Decision Criteria

### 11.1 Batch Acceptance Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All intended analytes should be present in the Positive Control. Each Quantitative Positive Control shall quantitate within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for more information.

## **11.2 Sample Acceptance Criteria**

### **11.2.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **11.2.2 Retention Time**

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, calibrator, or Positive Control.

#### **11.2.3 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

#### **11.2.2 Mass Spectrometry (for Data Dependent Scanning Analysis)**

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for general criteria. Mass spectral fragments of all SMAs tested in validation and found to extract via this procedure are listed in Table 3. In most circumstances the MS<sup>2</sup> and MS<sup>3</sup> (when present) spectra in an unknown sample should have all the same significant ions as the spectra of the known analyte in a contemporaneously analyzed standard, control, or calibrator, and should not have any significant ions not present in the known spectrum. Additionally, for any compound in Table 3 with two primary ions listed for a given spectral level, the intensity ratio for those ions should meet the requirements given in the Tox 104 standard operating procedure.

Table 3: Mass Spectrometry Data for Sympathomimetic Amines (precursor ions in bold type when multiple ions listed)

Compound Name	Precursor from Full Scan MS	Primary MS <sup>2</sup> Product Ion(s)	Primary MS <sup>3</sup> Product Ions(s)
Amphetamine	136	119	91
Cathinone	150	132	117
Methamphetamine	150	119	91
Phentermine	150	133	91
Phenylpropanol amine	152	134	117
Ethylamphetamine	164	119	91
Methcathinone	164	146	131
(pseudo)Ephedrine	166	148	133, 117
PMA	166	149	121
Benzylpiperazine	177	91, 85	not triggered
Propylamphetamine	178	119, 91	not triggered
Mephedrone	178	160	not triggered
MDA	180	163	135, 133
PMMA	180	149	121
2C-H	182	165	150
Dimethoxyphenethylamine	182	165	150
4-MTA	182	165	137, 117
BDB	194	177	147, 133
MDMA	194	163	135, 133
Dimethoxy-amphetamine	196	179	151
Chlorophenyl piperazine	197, 199	154	not triggered
Methylone	208	190, 160	not triggered
MBDB	208	177	135
MDDMA	208	163	135, 133
MDEA	208	163	135, 133
DOM	210	193	178, 156
Mescaline	212	195	180
DOET	224	207	192, 179
Trimethoxy-amphetamine	226	209	194, 181
Trifluoromethyl	231	188	not triggered

Compound Name	Precursor from Full Scan MS	Primary MS <sup>2</sup> Product Ion(s)	Primary MS <sup>3</sup> Product Ions(s)
phenylpiperazine			
Fenfluramine	232	187, 159	159
Methylphenidate	234	84	not triggered
2-CT-2	242	225	210, 164
2-CT-4	256	239	197
2-CT-7	256	239	224, 197, 164
2C-B	260, 262	243	228, 164
DOB	274, 276	257	229, 178
MDPV	276	205, 175, 126	not triggered
2C-I	308	291	276, 164

## 12 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 13 Uncertainty of Measurement

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette or balance used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 14 Limitations

- Method Performance Parameters (Blood and Urine):  
 LOD = Limit of Detection; LLOQ = Lower Limit of Quantitation

Compound	LOD in Blood (ng/mL)	LOD in Urine (ng/mL)	LLOQ (ng/mL)	Linear Range (ng/mL)	Accuracy (average % bias)	Precision (average % intermediate)
Amphetamine	5	5	10*	10-750	+5.7	4.8
Methamphetamine	5	5	25	25-750	+0.6	4.2
(pseudo)Ephedrine	10	10	10*	10-750	+1.9	3.5
MDA	5	5	25	25-750	+5.1	4.5
MDMA	5	5	25	25-750	+3.9	3.9
MDEA	5	5	25	25-750	-0.7	5.2
Methylone	25	10	25	25-750	-10.9%	3.7
Mephedrone	25	10	25	25-750	-11.5%	3.8
MDPV	25	2	25	25-750	-3.4%	2.0

\*Although amphetamine and pseudo(ephedrine) have been validated to an LOQ of 10 ng/mL, for routine analysis, curves will be analyzed down to 25 ng/mL. Therefore, any results below the curve will be reported as less than 25 ng/mL (less than the lowest calibrator.)

b. **Interferences:** Grossly decomposed or putrefied samples may affect both detection and quantitation limits. High levels of PMMA may interfere with accurate quantitation of MDA, and high levels of BDB may interfere with accurate quantitation of MDMA. The following compound pairs will be difficult or impossible to differentiate by this procedure: ephedrine and pseudoephedrine; 4-chlorophenylpiperazine and 3-chlorophenylpiperazine.

c. **Other Considerations:** At concentrations below approximately 25 ng/mL, some analytes may show a strong signal in full MS extracted ion chromatograms, but show no tandem MS signal due to the interaction of data dependent scan conditions and dynamic exclusion parameters. If there is good reason to suspect that this has happened, the questioned sample should be reinjected with scan event #2 changed to target only the ion(s) of interest and dynamic exclusion disabled. This procedure is not able to distinguish different optical isomers of SMAs, and cannot distinguish between the diastereomeric compounds ephedrine and pseudoephedrine. The following phenethylamine-group compounds were tested and found to not be extractable via this procedure: HMA (hydroxymethoxyamphetamine), HHMA (hydroxymethamphetamine), HMMA (hydroxymethoxymethamphetamine), and salbutamol.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

Sadeghipour, F. and Veuthey, J., *Journal of Chromatography A*, v. 787 (1997), pp. 137-143

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual*.



Rev. #	Issue Date	History
2	03/08/12	Removed hair to its own SOP (Tox 428) and updated all affected sections. Added methylone, mephedrone and MDPV (i.e., bath salts) as validated analytes and updated all affected sections. Updated LC column brand in 5s. Allowed for frozen storage of blood and urine in 6dd. Changed Control Working Solution concentration from 10 to 1 µg/mL in 6 x and updated Quantitative Positive Control Levels in 6ee from 200 ng/mL to 60 and 400 ng/mL. Updated Chromatography Decision Criteria in 11.1.
3	07/09/14	In Section 6, combined stock solutions into tables, included Lipomed as a source for controls, and included option to freeze prepared calibrators and controls in small vials after verification. In Section 7, specified equal weighting for the calibration curve. In Section 9.a, specified duplicate analyses for quantitation. In 9.d, added recentrifuge step. In Section 9.i, specified injection volume. In Section 10.2, included option of targeted analysis. Added Section 11.1 and renumbered subsequent sections. In 14.a, explained that in routine analysis, calibration for amphetamine and (pseudo)ephedrine will be calibrated down to 25 ng/mL, despite the validated LOQ of 10 ng/mL. Added calibrator and control preparation to Appendix 1 and removed reagent instructions. Reformatted Appendix 2 to include all pertinent instrumental parameters.

**Approval**

Redacted - Signatures on File



**Appendix 1: Abbreviated version of the SMA procedure for bench use.**

Redacted - Form on File

**Appendix 2: Instrumentation parameters checklist for the SMA procedure.**

Redacted - Form on File

## **Synthetic Cannabinoid Metabolite Analysis by LC/MS**

### **1 Introduction**

Synthetic cannabinoids began appearing widely in the United States in 2010. This group of compounds has been termed synthetic cannabinoids because they bind to CB<sub>1</sub> and CB<sub>2</sub> receptors in the brain. The synthetic cannabinoids are typically sprayed onto herbs which are sold as incense. The incense is then smoked. Common street names for these compounds include Spice and K2. Effects of the synthetic cannabinoids mimic those of marijuana, and include euphoria, incoordination and hallucinations. In November 2010, the DEA expressed intent to control 5 synthetic cannabinoids as schedule I controlled substances. These compounds are: CP 47, 497; CP 47, 497-C8; JWH-018; JWH-073 and JWH-200.

### **2 Scope**

This procedure allows for the identification of possible metabolites of JWH-018 and JWH-073. Since the metabolism of these compounds has not been extensively studied, some of the metabolites in the assay may not appear in human urine samples following use of the synthetic cannabinoids.

### **3 Principle**

Urine samples are subjected to enzymatic hydrolysis and extracted via liquid/liquid extraction. Extracts are taken to dryness, reconstituted and analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) using high resolution and data dependent scanning.

### **4 Specimens**

This procedure uses 1 mL of urine for a screen, and a second mL of urine for a second, confirmatory analysis.

### **5 Equipment/Materials/Reagents**

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Volumetric glassware
- b. Adjustable Pipettes with disposable tips

- c. Centrifuge
- d. Vortex Mixer
- e. Rotating Mixer
- f. Evaporator with nitrogen
- g. Disposable glassware including culture tubes, screw-cap test tubes and autosampler vials
- h. Liquid chromatograph/mass spectrometer (capable of high resolution MS and data dependent MS/MS)
- i. Waters Xterra C18 MS LC Column: 100 x 3.0 mm, 3.5  $\mu$ m d<sub>p</sub> (or equivalent)
- j. Sodium Acetate Buffer (1.1 M)
- k.  $\beta$ -Glucuronidase - Type H-2 from *Helix pomatia*
- l. Acetonitrile (Optima Grade)
- m. Water (Optima Grade)
- n. Formic Acid (Optima Grade)
- o. Diethyl Ether (High purity grade)
- p. 200 Proof Ethanol sparged with nitrogen for at least 15 minutes
- q. Sodium Carbonate (98%, or better)
- r. Sodium Bicarbonate (98%, or better)
- s. Sodium Sulfate (98%, or better)
- t. 3.0 M Sodium Carbonate Buffer, pH 10:  
Combine 23.8 g sodium carbonate and 18.9 g sodium bicarbonate in a graduated cylinder and bring to the 150-mL mark with deionized water. Mix well. Store in glass or plastic at room temperature. Stable for at least two months.
- u. LC Mobile Phase 1 (0.1% Formic Acid in Water):  
Vacuum filter 500 mL water (Optima grade or better) through a 5  $\mu$ m PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

- v. LC Mobile Phase 2 (0.1% Formic Acid in Acetonitrile):  
Vacuum filter 500 mL acetonitrile through a 5 µm PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

## 6 Standards and Controls

- a. d<sub>3</sub>-THC-OH Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant or another approved vendor. Storage conditions and stability determined by manufacturer.
- b. Internal Standard Solution (2.0 µg/mL d<sub>3</sub>-THC-OH):  
To a 10-mL volumetric flask, add 0.2 mL of d<sub>3</sub>-THC-OH Stock Standard. Bring to volume with methanol. Mix well. Store in glass at less than 0°C. Stable for at least one year.
- c. JWH-018 2OH (1.0 mg/mL):  
JWH-018 2OH (JWH-018 2-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- d. JWH-018 4OH (1.0 mg/mL):  
JWH-018 4OH (JWH-018 4-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- e. JWH-018 5OH (1.0 mg/mL):  
JWH-018 5OH (JWH-018 5-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- f. JWH-018 6OH (1.0 mg/mL):  
JWH-018 6OH (JWH-018 6-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- g. JWH-018 7OH (1.0 mg/mL):  
JWH-018 7OH (JWH-018 7-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- h. JWH-018 Hydroxypentyl Metabolite (1.0 mg/mL):  
JWH-018 Hydroxypentyl (N-(5-Hydroxypentyl) Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol.

Store in glass at less than 0°C. Stable for at least one year.

- i. JWH-018 COOH Metabolite (1.0 mg/mL):  
JWH-018 COOH (N-Pentanoic Acid) Metabolite (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- j. JWH-073 4OH (1.0 mg/mL):  
JWH-073 4OH (JWH-073 4-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- k. JWH-073 5OH (1.0 mg/mL):  
JWH-073 5OH (JWH-073 5-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- l. JWH-073 6OH (1.0 mg/mL):  
JWH-073 6OH (JWH-073 6-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- m. JWH-073 7OH (1.0 mg/mL):  
JWH-073 7OH (JWH-073 7-Hydroxyindole Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- n. JWH-073 N-(5-Hydroxybutyl) Metabolite (1.0 mg/mL):  
JWH-073 N-(5-Hydroxybutyl) Metabolite (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- o. JWH-073 COOH (1.0 mg/mL):  
JWH-073 COOH (JWH-073 N-Butanoic Acid Metabolite) (1 mg) is purchased from Cayman Chemical or another approved vendor, then diluted with 1 mL sparged ethanol. Store in glass at less than 0°C. Stable for at least one year.
- p. JWH-018 Metabolites Intermediate Solution (10 µg/mL):  
To a 10-mL volumetric flask, add 0.10 mL each of the JWH-018 metabolites stock solutions. Bring to volume with sparged ethanol. Mix well. Store in glass at less than 0°C. Stable for at least 1 year.

- q. JWH-073 Metabolites Intermediate Solution (10 µg/mL):  
To a 10-mL volumetric flask, add 0.10 mL each of the JWH-073 metabolites stock solutions. Bring to volume with sparged ethanol. Mix well. Store in glass at less than 0°C. Stable for at least 1 year.
- r. JWH-018 and JWH-073 Metabolites Working Solution (1.0 µg/mL):  
To a 10-mL volumetric flask, add 1.0 mL of the JWH-018 and JWH-073 Metabolite Intermediate Solutions. Bring to volume with sparged ethanol. Mix well. Store in glass at less than 0°C. Stable for at least 1 year.
- s. Negative Control Urine:  
Prepared in-house or purchased from an appropriate vendor. Stable for 6 months when refrigerated. A Negative Control Urine sample will be extracted and analyzed with every urine assay.
- t. Positive Control Urine (50 ng/mL):  
Add 50 µL of the JWH-018 and JWH-073 Metabolites Working Solution to 0.95 mL of negative urine. Prepare fresh. A Positive Control Urine sample will be extracted and analyzed with every urine assay.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Add 1 mL of control or questioned urine specimen to properly labeled screw cap test tubes.
- b. Add 50 µL of the Internal Standard Solution (2.0 µg/mL d<sub>3</sub>-THC-OH).
- c. Adjust pH to 5.2 ± 0.5 with 1 mL of Sodium Acetate Buffer (1.1 M) coupled with the addition of 30 µL of β-glucuronidase.
- d. Vortex, cap, and incubate 4 to 6 hours at 37°C.

- e. Cool to room temperature.
- f. Add 1 mL of 3.0 M Carbonate Buffer to each tube. Vortex.
- g. Add 5 mL of diethyl ether to each tube.
- h. Add a spatula tip of sodium sulfate to each tube. Vortex.
- i. Extract via rotation for 20 minutes.
- j. Centrifuge at ~3000 rpm for at least 10 minutes.
- k. Transfer diethyl ether layer to a clean test tube.
- l. Evaporate to dryness at  $\leq 40^{\circ}\text{C}$
- m. Reconstitute with 100  $\mu\text{L}$  of sparged ethanol. Vortex.
- n. Analyze LC/MS Performance Standard (JWH-018 and JWH-073 Metabolites Working Solution (1.0  $\mu\text{g}/\text{mL}$ )) by LC/MS(ESI) (5  $\mu\text{L}$ ). If the decision criteria in Section 11.1 of this procedure are met, proceed to next step. Otherwise, perform any appropriate instrument maintenance.
- o. Analyze 10  $\mu\text{L}$  of the extracts by LC/MS(ESI) using the instrumental conditions that follow. MS analysis can be full scan, MS/MS, and/or data dependent.

## 10 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

Appendix 1 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.



## 10.1 Liquid Chromatograph Parameters

Mobile Phase Parameters			
initial composition	70% LC mobile phase #1		
	30% LC mobile phase #2		
initial hold	3 minutes		
composition at 15 minutes		90% LC mobile phase #2	
hold	15 minutes		
final composition at 30 minutes		30% LC mobile phase #2	
final hold	6 minutes	flowrate	0.3 mL/min
column temperature	30° C	runtime	37 minutes

Column Parameters	
type	Waters Xterra C18 MS, or equivalent
length	10 cm
internal diameter	3.0 mm
particle size	3.5 µm

## 10.2 Mass Spectral Parameters – LC/MS/MS

Scan Event #1		Scan Event #2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	FTMS res=7500 centroid	scan mode	FTMS res=7500 centroid MS/MS DDS
scan range	250 – 500 m/z	precursor (metabolites)	most intense of m/z 344.1655, 358.1449, 372.1604
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		isolation width	2.0
		collision energy	35%
		scan range	software control

## 11 Decision Criteria

### 11.1 LC/MS Performance Standard Decision Criteria

Peaks for each of the 13 analytes should be detectable. The 5 and 6 OH metabolites will not be baseline separated, but the peak that represents these two compounds should at least be split.

## 11.2 Analyte Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control.

### 11.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 11.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or an extracted Positive Control.

#### 11.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

### 11.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted positive control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 12 Calculations

Not applicable.

## 13 Uncertainty of Measurement

Not applicable.

## 14 Limitations

- a. Limits of Detection: 5 ng/mL for JWH-2OH; 1 ng/mL for all other analytes.
- b. Interferences: The 5 and 6 OH metabolites elute very closely, so their identification should be made cautiously. If the data does not clearly indicate which isomer is present, 5/6 OH may be reported if the inability to differentiate between the two isomers is explained in the report.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

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Rev. #	Issue Date	History
0	02/18/11	New document.
1	08/23/12	Updated chromatography decision criteria in Section 11.2.1. Updated retention time requirements in Section 11.2.1.1.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Synthetic Cannabinoid Procedure for bench use.**

Redacted - Form on File

## Quantitation of Acidic Drugs

### 1 Introduction

This procedure is used to quantitate and confirm seven common acidic/neutral drugs in biological fluids.

### 2 Scope

This procedure allows for quantitation and confirmation of butalbital, carbamazepine, carisoprodol, meprobamate, phenobarbital, phenytoin and secobarbital in biological fluids. It can also be used for quantitative analysis of additional acidic/neutral drugs, as per instructions in the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) and *Chemistry Unit Validation of Analytical Procedures* standard operating procedure (CUQA 11). Although this procedure is written in order to quantitate all seven drugs at once, standards and controls may be made up for a smaller subset of drugs if case needs dictate. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

Specimens are mixed with internal standards, adjusted to a slightly acidic pH, and extracted using Clean Screen DAU solid phase extraction cartridges. Acid/neutral drugs are eluted using a mixed solvent system of hexane and ethyl acetate. The eluent is taken to dryness and reconstituted prior to analysis by gas chromatography/mass spectrometry (GC/MS).

### 4 Specimens

This procedure is validated for whole blood. Typically, 2 x 0.5 mL samples are analyzed, but samples suspected to be above the procedure's linear range may be diluted before extraction if required.

### 5 Equipment/Materials/Reagents

- a. Gas Chromatograph/Mass Spectrometer equipped with a 30 m x 0.25 mm x 0.25  $\mu$ m Rtx-5MS (or equivalent) column
- b. Centrifuge
- c. Evaporator w/ Nitrogen

- d. Vortex mixer
- e. SPE Vacuum or Positive Pressure Manifold
- f. Desiccator
- g. Hexane (UV grade)
- h. Ethyl Acetate (HPLC grade)
- i. 16 x 100 mm culture tubes with polypropylene snap-tops
- j. 12 x 75 mm culture tubes with polypropylene snap-tops
- k. Clean Screen DAU<sup>®</sup> SPE cartridges (regular flow) - 200 milligrams
- l. Deionized water
- m. Methanol (HPLC grade)
- n. 0.1 M Sodium Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify  $5.8 < \text{pH} < 6.1$ , adjusting pH by addition of 0.1 M dibasic sodium phosphate (increases pH) or 0.1 M monobasic sodium phosphate (decreases pH) as necessary. Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.
- o. Elution Solvent (Hexane/Ethyl Acetate (50/50)):  
Combine 100 mL hexane and 100 mL ethyl acetate in a graduated cylinder. Mix to combine. Store in glass at room temperature. Stable for at least 3 months.
- p. 0.1 M Hydrochloric Acid:  
To a 100-mL graduated cylinder, add 80 mL deionized water and 0.8 mL concentrated hydrochloric acid. Bring to 96 mL with deionized water and mix well. Store in glass at room temperature. Stable 6 months.
- q. Mid-range pH paper
- r. 4:1 Chloroform:Methanol:  
Combine 40 mL GC<sup>2</sup> grade chloroform with 10 mL GC<sup>2</sup> methanol. Mix well. Store in brown glass at room temperature. Stable 1 month.

## 6 Standards and Controls

- a. d<sub>5</sub>-Butalbital Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules. Storage and stability determined by manufacturer.
- b. d<sub>10</sub>-Carbamazepine Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules. Storage and stability determined by manufacturer.
- c. d<sub>7</sub>-Carisoprodol Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules. Storage and stability determined by manufacturer.
- d. d<sub>7</sub>-Meprobamate Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules. Storage and stability determined by manufacturer.
- e. d<sub>5</sub>-Phenobarbital Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules; ring d<sub>5</sub>-Phenobarbital is purchased, not side chain d<sub>5</sub>-Phenobarbital. Storage and stability determined by manufacturer.
- f. d<sub>10</sub>-Phenytoin Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules. Storage and stability determined by manufacturer.
- g. d<sub>5</sub>-Secobarbital Stock Standard (100 µg/mL):  
Purchased from Cerilliant in 1 mL ampules. Storage and stability determined by manufacturer.
- h. Internal Standard Solution (4 µg/mL; 2 µg/mL d<sub>10</sub>-Phenytoin):  
Combine 1 mL d<sub>10</sub>-Phenytoin Stock Standard and 2 mLs each d<sub>5</sub>-Butalbital, d<sub>10</sub>-Carbamazepine, d<sub>7</sub>-Carisoprodol, d<sub>7</sub>-Meprobamate, d<sub>5</sub>-Phenobarbital and d<sub>5</sub>-Secobarbital Stock Standards in a 50-mL volumetric flask. Bring to the mark with deionized water and mix well. Store refrigerated in glass. Stable for at least one year.
- i. Butalbital Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Lipomed in 1 mL ampules. Storage and stability determined by manufacturer.
- j. Carbamazepine Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Grace in 1 mL ampules. Storage and stability determined by manufacturer.



- k. Carisoprodol Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Lipomed in 1 mL ampules. Storage and stability determined by manufacturer.
- l. Meprobamate Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Lipomed in 1 mL ampules. Storage and stability determined by manufacturer.
- m. Phenobarbital Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Lipomed in 1 mL ampules. Storage and stability determined by manufacturer.
- n. Phenytoin Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Lipomed in 1 mL ampules. Storage and stability determined by manufacturer.
- o. Secobarbital Stock Standard (1 mg/mL):  
Purchased from Cerilliant and Lipomed in 1 mL ampules. Storage and stability determined by manufacturer.
- p. Calibration Working Standard (WS) for Carisoprodol and Phenobarbital (10 µg/mL):  
Combine 100 µL of the Carisoprodol and Phenobarbital Stock Standards from Cerilliant in a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated or frozen in glass or plastic. Once solution is verified, it should be parsed out into small amounts to be defrosted with each use. Stable for at least one year.
- q. Calibration WS for Butalbital, Carbamazepine, Meprobamate, Phenytoin and Secobarbital (10 µg/mL):  
Combine 100 µL of the Butalbital, Carbamazepine, Meprobamate, Phenytoin and Secobarbital Stock Standards from Cerilliant in a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated or frozen in glass or plastic. Once solution is verified, it should be parsed out into small amounts to be defrosted with each use. Stable for at least one year.
- r. Control WS Solution (10 µg/mL):  
Combine 100 µL of the Butalbital, Carisoprodol, Meprobamate, Phenobarbital, Phenytoin and Secobarbital Stock Standards from Lipomed and 100 µL of the Carbamazepine Stock Standard from Grace in a 10-mL volumetric flask. Bring to the mark with deionized water. Store refrigerated or frozen in glass or plastic. Once solution is verified, it should be parsed out into small amounts to be defrosted with each use. Stable for at least one year.
- s. Negative Control Blood:

Purchased from Cliniq or an equivalent approved supplier, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay.

- t. Positive Control Blood (1.2 and 3.5 µg/mL):  
Prepared by spiking Negative Control Blood with the Control Working Standard Solution as directed in Table 2 below. Prepared the day of extraction.

This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows the amount of the Calibration Working Standard Solution to add to 0.5 mL of Negative Control Blood for calibrator preparation. Table 2 shows the amount of Control Working Standard Solution to add to 0.5 mL of Negative Control Blood for Positive Control Blood preparation. The "0" control level is the Negative Control Blood sample.

Table 1: Calibrator Preparation

Calibrator Level (µg/mL)	Calibrator WS Solution Volume (µL)
0.5/0.7*	25/35
1.0	50
2.0	100
3.0	150
5.0	250

Table 2: Control Preparation

Control Level (µg/mL)*	Control WS Solution Volume (µL)
0	0
1.2	60
4.0	175

\*Note: The lowest calibrator for carisoprodol and phenobarbital is 0.7 µg/mL, but it is 0.5 µg/mL for all other drugs. Therefore, 35 µL of the Carisoprodol and Phenobarbital WS Solution will be added to the low calibrator, while 25 µL of the other WS Solution will be added. Volumes for the other calibrators do not vary.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To properly labeled 16 x 100 mm culture tubes add 0.5 mL of sample. Prepare samples in duplicate, if specimen size allows. Prepare calibrators and controls as directed in Section 6 above. Samples may have to be diluted before analysis if the drug screen or the case history indicate a concentration above the procedure's linear range.

- b. Add 200  $\mu$ L of the Internal Standard Solution.
- c. Add 3 mL of 100 mM phosphate buffer. Bring all samples to approximately 5 mL volume total with deionized water. Vortex.
- d. Centrifuge at approximately 3000 rpm for at least 5 minutes. Transfer supernatant to a clean culture tube, leaving solid cellular material behind.
- e. Pre-rinse SPE extraction cartridges by adding 3 mL of methanol at 1 mL/minute.
- f. Condition cartridges with 3 mL of deionized water followed by 1 mL of 100 mM phosphate buffer at 1 mL/minute. Do not allow sorbent to dry.
- g. Load sample onto cartridge at 1-2 mL/minute. Do not allow sorbent to dry.
- h. Wash cartridge with 3 mL of deionized water followed by 1 mL of 0.1 M hydrochloric acid (each at 1-2 mL/minute).
- i. Dry cartridge under full vacuum for 1 minute.
- j. Wash cartridge with 1 mL of hexane (1-2 mL/minute).
- k. Apply 3 mL of Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm test tubes.
- l. Evaporate to dryness under nitrogen at 50°C.
- m. Reconstitute extracts in 50  $\mu$ L chloroform:methanol (4:1) and analyze 1  $\mu$ L by GC/MS(EI) following the instrumental parameters given below after confirming that the instrument is in proper working condition. Analyze negative control blood samples immediately prior to each case sample or case sample pair to account for carryover after concentrated samples.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of instrumental parameters used in this procedure. This checklist may be used by the examiner or chemist performing the procedure.

## 9.1 Gas Chromatograph Parameters

Oven Parameters		Inlet and Carrier Parameters		Column Parameters	
temperature 1	45°C	inlet temperature	220°C	type	DB-5MS
hold 1	1 min	injection mode	split	length	30 m
ramp 1	25°C/min	carrier gas	ultrapure helium	internal diameter	0.25 mm
temperature 2	150°C	carrier mode	constant flow	film thickness	0.25 µm
hold 2	2 min	carrier flow	1.2 mL/min		
ramp 2	15°C/min	split flow	12 mL/min		
temperature 3	280°C	split ratio	10:1		
hold 3	7 min	Injection volume	1 µL		
total run time	22.87 min				

## 9.2 Mass Spectrometer Parameters

ionization mode	electron impact (+)	source temperature	230°C
scan mode	full scan	transfer line temperature	280°C
scan range	35 – 400 AMU	solvent delay	3 min

## 10 Decision Criteria

In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control.

### 10.1 Unknown Sample Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

#### 10.1.1 Batch Acceptance

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the seven analytes in the Positive Control should be detected in the GC/MS data. High and low positive controls should fall within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for further guidance.

### **10.1.2 Unknown Sample Acceptance**

The Internal Standards (d<sub>10</sub>-Phenytoin, d<sub>5</sub>-Butalbital, d<sub>10</sub>-Carbamazepine, d<sub>7</sub>-Carisoprodol, d<sub>7</sub>-Meprobamate, d<sub>5</sub>-Phenobarbital and d<sub>5</sub>-Secobarbital) should be detectable in the GC/MS data.

#### **10.1.2.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **10.1.2.2 GC Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of an extracted Positive Control or extracted calibrator.

#### **10.1.2.3 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10-fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

#### **10.1.2.4 Mass Spectrometry**

The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

## **11 Calculations**

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

Table 3 below lists quantitation ion(s) for each analyte in this procedure.

Table 3

Analyte	Quant Ion(s)	IS Quant Ion(s)
Butalbital	167 + 168	173 + 186
Carbamazepine	193 + 236	203 + 246
Carisoprodol	158 + 245	165
Meprobamate	83 + 114 + 144	89 + 121
Phenobarbital	117 + 204 + 232	209
Phenytoin	180 + 209	189 + 219
Secobarbital	124 + 168	173 + 200

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- Linear range using equal weighting and a linear calibration model:  
 0.7 – 5 µg/mL for carisoprodol and phenobarbital  
 0.5 – 5 µg/mL for other analytes
- Limit of Detection and Lower Limit of Quantitation:  
 0.7 µg/mL for carisoprodol and phenobarbital  
 0.5 µg/mL for other analytes

- Bias (n=15):

Analyte	Low (1.2 µg/mL)	Mid (2.0 µg/mL)	High (3.5 µg/mL)
Butalbital	+5.68%	+5.87%	+7.28%
Carbamazepine	-0.72%	-0.13%	+1.47%
Carisoprodol	+0.11%	+0.09%	+4.01%



Meprobamate	+0.77%	-3.53%	-0.20%
Phenobarbital	+3.67%	+3.53%	-0.20%
Phenytoin	+1.99%	+0.04%	+0.70%
Secobarbital	-0.33%	-0.52%	+0.71%

d. Repeatability (n=15):

Analyte	Low (1.2 µg/mL)	Mid (2.0 µg/mL)	High (3.5 µg/mL)
Butalbital	2.55%	1.32%	6.32%
Carbamazepine	2.73%	3.51%	5.21%
Carisoprodol	2.95%	2.39%	3.06%
Meprobamate	3.36%	2.28%	4.97%
Phenobarbital	2.07%	3.18%	3.13%
Phenytoin	2.17%	3.46%	5.58%
Secobarbital	1.47%	2.55%	4.02%

e. Intermediate Precision (n 15):

Analyte	Low (1.2 µg/mL)	Mid (2.0 µg/mL)	High (3.5 µg/mL)
Butalbital	2.55%	3.15%	6.32%
Carbamazepine	4.75%	4.25%	5.21%
Carisoprodol	3.17%	4.15%	3.70%
Meprobamate	7.78%	3.94%	6.48%
Phenobarbital	3.63%	3.29%	3.87%
Phenytoin	2.54%	3.89%	6.33%
Secobarbital	1.63%	2.93%	4.07%

f. Interferences: None known. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.

g. Carryover: Minor carryover was observed for phenytoin and phenobarbital.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual.*



Rev. #	Issue Date	History
0	07/01/11	New document.
1	08/23/12	Updated chromatography decision criteria in Section 11.1.
2	01/27/14	In Section 6e, clarified type of ds-Phenobarbital to purchase. In Section 6i-o, added additional purchasing vendor. In Section 6p-r, updated vendor selection for calibrator and control solutions and parsing out of stock solutions. In Section 9l, specified to evaporate at 50°C. In Section 9m, specified injection volumes and to check instrument in proper working order. In Section 10.1, specified 1 µL injections. In Section 11, added Batch Acceptance Criteria (11.1.1), added Unknown Sample Acceptance Criteria (11.1.2), and renumbered remaining subsections (11.1.2.1 thru 11.1.2.4). In Appendix 1, edited calibrator table list from 4.0 to 3.0, and added injection volume. Added more detail to Appendix 2.
3	02/09/18	Updated Scope language. Updated scan range in Section 10.2. Removed “reasonable degree of scientific certainty” language from Section 11.1.2.4. Updated approval lines. Removed footer. Removed Calibration section (formerly 7), and renumbered. Fixed “Measurement Uncertainty.” Updated Section 6, Table 2 highest control to be 4 mcg/mL (formerly 3.5). In Section 5 and 15, deleted reference to TOX103, and updated items in Section 5: n, p. and r. Updated Appendices 1 and 2.

### **Approval**

Toxicology  
 Technical Lead:

Date: 02/08/2018

Chemistry Unit Chief:

Date: 02/08/2018

### **QA Approval**

Quality Manager:

Date: 02/08/2018

**Appendix 1: Abbreviated version of the Quantitation of Acidic Drugs Procedure  
for bench use.**

Redacted - Form on File

**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.**

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Redacted - Form on File

## Quantitation and Confirmation of Alkaline Drugs

### 1 Introduction

This procedure is used to quantitate common alkaline drugs in blood. It is also used to confirm common alkaline drugs in both blood and urine.

### 2 Scope

This procedure allows for quantitation and confirmation of amitriptyline, chlorpheniramine, chlorpromazine, citalopram, clomipramine, cyclobenzaprine, desipramine, dextromethorphan, doxylamine, diphenhydramine, doxepin, duloxetine, EDDP (methadone metabolite; 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), fentanyl, fluoxetine, imipramine, ketamine, meperidine, methadone, mirtazapine, nortoxepin, norfentanyl, norfluoxetine, normeperidine, norpropoxyphene, nortriptyline, paroxetine, PCP (phencyclidine), pheniramine, propoxyphene, propranolol, sertraline, tramadol, trazodone, trimipramine, venlafaxine, verapamil and zolpidem in blood. This procedure also allows for qualitative confirmation of brompheniramine, bupropion, clozapine, metoprolol, nortriptyline, quetiapine and thioridazine in blood, as well as confirmation of all analytes in urine. It can also be used for quantitative analysis of additional alkaline drugs, as per instructions in the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) and *FBI Laboratory Practices for Validating Chemical Procedures*.

### 3 Principle

Specimens are mixed with internal standard(s), adjusted to alkaline pH, and extracted into hexane. The hexane is taken to dryness and reconstituted prior to analysis by liquid chromatography/Fourier transform mass spectrometry (LC/FTMS).

### 4 Specimens

This procedure is validated for whole blood and urine. Typically, 2 x 0.5 mL samples are analyzed, but samples suspected to be above the procedure's linear range may be diluted before extraction.

### 5 Equipment/Materials/Reagents

- a. 16 x 100 mm screw-top tubes with Teflon-lined caps
- b. 12 x 75 mm culture tubes with polypropylene snap-tops

- c. Acetonitrile (Optima grade or better)
- d. Formic Acid (Puriss grade or better)
- e. Hexane (UV grade or better)
- f. Sodium hydroxide (ACS grade or better)
- g. Water (Deionized, 18 MΩ)
- h. Water (Optima or better grade)
- i. 4% Sodium hydroxide: Dissolve 2 g sodium hydroxide in 50 mL deionized water. Store in plastic at room temperature. Stable for at least 6 months.
- j. Centrifuge
- k. Evaporator w/ Nitrogen
- l. Vortex mixer
- m. Methanol:Water (10:90 v:v): Mix 5 mL methanol with 45 mL water (both Optima grade). Store in glass at room temperature. Stable 12 months.
- n. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
- o. Liquid Chromatograph - Mass Spectrometer capable of 15,000 resolution
- p. HPLC Column (Xterra C-18 MS, 3.0 x 150 mm, 3.5 μm dp; or equivalent)
- q. Mobile Phase A (Acetonitrile with 0.1% Formic Acid ): Combine 500 mL Optima grade water and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for 2 months.
- r. Mobile Phase B (Water with 0.1% Formic Acid ): Combine 500 mL Optima grade acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable 2 months.

## 6 Standards and Controls

- a. Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed, or an equivalent supplier. Typically, calibration material is purchased from Cerilliant and control material is purchased from Lipomed. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

Citalopram	EDDP
Normeperidine	Norsertaline

- b. Standard Stock Solutions (1.0 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed, or an equivalent supplier. Typically, calibration material is purchased from Cerilliant and control material is purchased from Lipomed. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

Amitriptyline	Fentanyl	PCP
Brompheniramine	Fluoxetine	Pheniramine
Bupropion	Imipramine	Propoxyphene
Chlorpheniramine	Ketamine	Propranolol
Chlorpromazine	Meperidine	Quetiapine
Clomipramine	Methadone	Sertraline
Clozapine	Metoprolol	Thioridazine
Cyclobenzaprine	Mirtazapine	Tramadol
Desipramine	Nordoxepin	Trazodone
Dextromethorphan	Norfentanyl	Trimipramine
Diphenhydramine	Norfluoxetine	Venlafaxine
Doxepin	Norpropoxyphene	Verapamil
Doxylamine	Nortriptyline	Zolpidem
Duloxetine	Paroxetine	

- c. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX) or an equivalent supplier. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

Amitriptyline-d <sub>3</sub>	Duloxetine-d <sub>3</sub>	Nortriptyline-d <sub>3</sub>
Chlorpheniramine-d <sub>6</sub>	EDDP-d <sub>3</sub>	Paroxetine-d <sub>6</sub>
Chlorpromazine-d <sub>3</sub>	Fentanyl-d <sub>5</sub>	PCP-d <sub>5</sub>
Citalopram-d <sub>6</sub>	Fluoxetine-d <sub>6</sub>	Pheniramine-d <sub>6</sub>
Clomipramine-d <sub>3</sub>	Imipramine-d <sub>3</sub>	Propoxyphene-d <sub>5</sub>
Cyclobenzaprine-d <sub>3</sub>	Ketamine-d <sub>4</sub>	Sertraline-d <sub>3</sub>
Desipramine-d <sub>3</sub>	Meperidine-d <sub>4</sub>	Tramadol-13C-d <sub>3</sub>
Dextromethorphan-d <sub>3</sub>	Methadone-d <sub>3</sub>	Trazodone-d <sub>6</sub>
Diphenhydramine-d <sub>3</sub>	Norfentanyl-d <sub>5</sub>	Trimipramine-d <sub>3</sub>

Doxepin-d <sub>3</sub>	Norfluoxetine-d <sub>6</sub>	Venlafaxine-d <sub>6</sub>
Doxylamine-d <sub>5</sub>	Normeperidine-d <sub>4</sub>	Zolpidem-d <sub>6</sub>
	Norpropoxyphene-d <sub>5</sub>	

- d. High Calibration Working Solution (5.0 µg/mL)<sup>1</sup>:  
Combine 0.5 mL of each 0.1 mg/mL Standard Stock Solution and 0.05 mL of each 1.0 mg/mL Standard Stock Solution in a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at least one year.
- e. Low Calibration Working Solution (WS) (0.5 µg/mL)<sup>1</sup>:  
Add 1.0 mL of the High Calibration Working Solution to a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at least one year.
- f. Low Dose Drugs Intermediate Calibration Solution (10 µg/mL)<sup>1,2</sup>:  
Combine 0.1 mL of each 1.0 mg/mL Standard Stock Solution in a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at least one year.
- g. Low Dose Drugs High Calibration Working Solution (1.0 µg/mL)<sup>1,2</sup>:  
Add 1.0 mL of the Low Dose Drugs Intermediate Calibration Solution to a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at least one year.
- h. Low Dose Drugs Low Calibration Working Solution (0.1 µg/mL)<sup>1,2</sup>:  
Add 1.0 mL of the Low Dose Drugs High Calibration Working Solution to a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at least one year.

<sup>1</sup> Working Solutions and Internal Standard Solutions may be made in groupings or individually, depending on case needs.

<sup>2</sup> The following drugs are validated at a lower concentration range than most of the drugs in the procedure, and therefore use Stock Solutions and Internal Standard Solutions at lower concentrations: cyclobenzaprine, fentanyl, norfentanyl, paroxetine, PCP and zolpidem. Brompheniramine is also in this group, but is validated for qualitative analysis only.

Tables 1-2 show the amount of the Calibration Working Solutions to add to 0.5 mL of Negative Control Blood for calibrator preparation.

Table 1: Calibrator Preparation for amitriptyline, chlorpheniramine, chlorpromazine, citalopram, clomipramine, desipramine, dextromethorphan, diphenhydramine, doxepin, duloxetine, EDDP, fluoxetine, imipramine, ketamine, meperidine, methadone, mirtazapine, nordoxepin, norfluoxetine, normeperidine, norpropoxyphene, nortriptyline, pheniramine, propoxyphene, propranolol, sertraline, tramadol, trimipramine, venlafaxine and verapamil

Calibrator Level (ng/mL)	High Cal WS (5 µg/mL) Volume (µL)	Low Cal WS (0.5 µg/mL) Volume (µL)
50*	-	50
100	-	100
250	25	-
500	50	-
750	75	-
1000**	100	-

\*The 50 ng/mL calibrator will not be analyzed for mirtazapine and propranolol, as it is out of the linear range for these analytes.

\*\*The 1000 ng/mL calibrator will not be analyzed for doxylamine and verapamil as it is out of the linear range for these analytes.

Table 2: Calibrator Preparation for cyclobenzaprine, fentanyl, norfentanyl, paroxetine, PCP and zolpidem

Calibrator Level (ng/mL)	Low Dose Drugs High Cal WS (1 µg/mL) Volume (µL)	Low Dose Drugs Low Cal WS (0.1 µg/mL) Volume (µL)
10	-	50
20	-	100
50	25	-
100	50	-
150	75	-
200	100	-

- i. Internal Standard Working Solution (3 µg/mL)<sup>1</sup>:  
Combine 0.3 mL of each Internal Standard Stock Solution in a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at least two years.
- j. Low Dose Drugs Internal Standard Working Solution (1 µg/mL)<sup>1,2</sup>:  
Combine 0.1 mL of each Internal Standard Stock Solution in a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated or frozen in glass. Stable for at



least two years.

- k. High Control Working Solution ( $4 \mu\text{g/mL}$ )<sup>1</sup>:  
Combine 0.4 mL of each 0.1 mg/mL Standard Stock Solution and 0.04 mL of each 1.0 mg/mL Standard Stock Solution in a 10-mL volumetric flask and bring to the mark with deionized water. Store refrigerated in glass. Stable for at least one year.
- l. Low Control Working Solution ( $0.4 \mu\text{g/mL}$ )<sup>1</sup>:  
Add 0.5 mL of the High Control Working Solution to a 5-mL volumetric flask and bring to the mark with deionized water. Store refrigerated in glass. Stable for at least one year.
- m. Low Dose Drugs High Control Working Solution ( $0.8 \mu\text{g/mL}$ )<sup>1,2</sup>:  
Add 1.0 mL of the High Control Working Solution to a 5-mL volumetric flask and bring to the mark with deionized water. Store refrigerated in glass. Stable for at least one year.
- n. Low Dose Drugs Low Control Working Solution ( $0.2 \mu\text{g/mL}$ )<sup>1,2</sup>:  
Add 0.25 mL of the High Control Working Solution to a 5-mL volumetric flask and bring to the mark with deionized water. Store refrigerated in glass. Stable for at least one year.
- o. Negative Control Blood:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or obtained in-house from a drug-free donor. Store refrigerated or frozen. Stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.
- p. Negative Control Urine:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or obtained in-house from a drug-free donor. Store refrigerated or frozen. Stability determined by manufacturer. A Negative Control Urine sample will be extracted and analyzed with every urine assay.
- q. Quantitative Positive Control Blood:  
This is normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as needed. A Quantitative Positive Control Blood sample will be extracted in duplicate with every quantitative assay. The Quantitative Positive Control Blood may be prepared for one or more analytes, depending on which analytes are being quantitated in a given analytical run. Quantitative Positive Controls will typically be prepared fresh from Control Working Solutions as described below:
  - 1. Low Control ( $24 \text{ ng/mL}$ ) for cyclobenzaprine, fentanyl, norfentanyl, paroxetine, PCP and zolpidem: Add 0.06 mL of the Low Dose Drugs Low Control Working Solution to 0.5 mL of Negative Control Blood.

2. Low Control (72 ng/mL) for amitriptyline, chlorpheniramine, chlorpromazine, citalopram, clomipramine, desipramine, dextromethorphan, diphenhydramine, doxepin, duloxetine, EDDP, fluoxetine, imipramine, ketamine, meperidine, methadone, nordoxepin, norfluoxetine, normeperidine, norpropoxyphene, nortriptyline, pheniramine, propoxyphene, sertraline, tramadol, trimipramine, venlafaxine and verapamil: Add 0.09 mL of the Low Control Working Solution to 0.5 mL of Negative Control Blood.
  3. Low Control (320 ng/mL) for mirtazapine and propranolol: Add 0.04 mL of the High Control Working Solution to 0.5 mL of Negative Control Blood.
  4. High Control (144 ng/mL) for cyclobenzaprine, fentanyl, norfentanyl, paroxetine, PCP and zolpidem: Add 0.09 mL of the Low Dose Drugs High Control Working Solution to 0.5 mL of Negative Control Blood.
  5. High Control (680 ng/mL) for amitriptyline, chlorpheniramine, chlorpromazine, citalopram, clomipramine, desipramine, dextromethorphan, diphenhydramine, doxepin, EDDP, fluoxetine, imipramine, ketamine, meperidine, methadone, mirtazapine, norfluoxetine, normeperidine, norpropoxyphene, nortriptyline, pheniramine, propoxyphene, propranolol, tramadol, trimipramine, venlafaxine and verapamil: Add 0.085 mL of the High Control Working Solution to 0.5 mL of Negative Control Blood.
- r. Qualitative Positive Control Blood:  
This is normally prepared in-house, but may be purchased from an appropriate vendor as needed. A Qualitative Positive Control Blood sample will be extracted and analyzed with every qualitative blood assay. The Qualitative Positive Control Blood may be prepared for one or more analytes, depending on which analytes are being confirmed in a given analytical run. Qualitative Positive Controls will typically be prepared fresh at any concentration above the assay's limit of detection from Control Working Solutions.
- s. Positive Control Urine:  
This is normally prepared in-house, but may be purchased from an appropriate vendor as needed. A Positive Control Urine sample will be extracted and analyzed with every urine assay. The Positive Control Urine may be prepared for one or more analytes, depending on which analytes are being confirmed in a given analytical run. Qualitative Positive Controls will typically be prepared fresh at any concentration above the assay's limit of detection from Control Working Solutions.
- t. Column Performance Mix: Dilute 0.010 mL of the Internal Standard Working Solution with 0.090 mL of Methanol:Water (10:90 v:v). Prepare fresh.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To a properly labeled 16 x 100 mm screw-top tube, add 0.5 mL of specimen, calibrator or control. Prepare case specimens and positive controls in duplicate for quantitative analysis. Smaller volumes may be analyzed if required to ensure that the sample is within the linear range of the procedure. A ten-fold dilution using 0.05 mL specimen and 0.45 mL deionized water is recommended. Case samples and positive controls do not need to be prepared in duplicate for qualitative analysis.
- b. Add 50  $\mu$ L of the Internal Standard Working Solution(s) to each sample.
- c. Add 0.2 mL of 4% sodium hydroxide to each sample and vortex briefly.
- d. Add 2 mL of hexane down the inside of each tube and extract for 20 minutes on a rotator. Centrifuge 10 minutes at a minimum of 3000 rpm. Use a wooden stick to break up any emulsions that develop, and spin again.
- e. Transfer organic (top) layer to a 12x75 mm culture tube.
- f. Evaporate the hexane to dryness under a gentle stream of nitrogen at 40°C. Do not overdry.
- g. Reconstitute each sample in 0.1 mL Methanol:Water (10:90 v:v).
- h. Analyze by LC/MS using the conditions below after verifying that the instrument is performing properly by analyzing the appropriate Column Performance Mix.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
A: Acetonitrile with 0.1% Formic Acid	total flow	0.3 mL/min		type	C-18 MS
	time (min)	%A	%B	length	15 cm
B: Water with 0.1% Formic Acid	0	10	90	internal diameter	3.0 mm
	5	10	90	particle size	3.5 μm
	20	90	10	temperature	30°C
	30	90	10		
	31	10	90		
	36	10	90		
	37	10	90		
	total time	37 min			

### 9.2 Mass Spectral Parameters

ionization mode	electrospray (+)
scan mode	full scan, centroid
scan range	200 - 500 AMU
resolution	15,000
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.	

## 10 Decision Criteria

### 10.1 Batch Acceptance Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All intended analytes should be present in the Positive Control. Each Quantitative Positive Control will quantitate within  $\pm 20\%$  of the target value. See the Guidelines for Toxicological Quantitations standard operating procedure (Tox 101) for more information.

## 10.2 Sample Acceptance Criteria

### 10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. Ion peaks are typically extracted at  $\pm 5$  mmu. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of an extracted Positive Control or extracted calibrator.

Approximate expected retention times are listed in Table 3 below for reference.

Table 3: Approximate expected retention times

Analyte	RT (min)	Analyte	RT (min)
doxylamine	8.03	doxepin	14.39
pheniramine	8.63	citalopram	14.47
ketamine	11.30	paroxetine	14.80
norfentanyl	11.52	desipramine	14.86
mirtazepine	11.53	cyclobenzaprine	14.98
metoprolol	12.06	EDDP	14.99
tramadol	12.14	imipramine	15.01
chlorpheniramine	12.74	nortriptyline	15.06
normeperidine	12.81	duloxetine	15.15
zolpidem	12.84	verapamil	15.17
meperidine	12.87	amitriptyline	15.21
brompheniramine	12.99	norfluoxetine	15.30
clozapine	13.02	trimipramine	15.37
venlafaxine	13.13	propoxyphene	15.38
bupropion	13.15	norsertraline	15.41
trazodone	13.38	fluoxetine	15.49
quetiapine	13.63	methadone	15.50
phencyclidine	13.69	chlorpromazine	15.56
propranolol	13.77	sertraline	15.57
dextromethorphan	14.02	clomipramine	15.76
fentanyl	14.07	thioridazine	16.20
nordoxepin	14.26	norpropoxyphene	19.10
diphenhydramine	14.36		

### 10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Note: nonsensical signal to noise values may result from high resolution mass spectral data. Further, the baseline signal for the peak of interest should be at least ten-fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

### 10.2.2 Mass Spectrometry

The M+1 for the analyte of interest should match those in Tables 4 and 5 within  $\pm 5$  mmu.

M+1 ions for the drugs that have been quantitatively validated are listed in Table 5. M+1 for drugs that have been validated qualitatively only are listed in Table 4 below.

Table 4: M + 1 ions for analytes

Analyte	M+1 (+ Br or Cl isotope, when applicable)
Brompheniramine	319.080, 321.078
Bupropion	240.115
Clozapine	327.137, 329.134
Metoprolol	268.191
Norsertaline	292.065, 294.062, 296.060
Quetiapine	384.174
Thioridazine	371.161

## 11 Calculations

Quantitation is performed by constructing a multi-point calibration curve based on the ratio of the area for the M+1 peak for each analyte to its internal standard. The chlorine isotope is added to the M+1 peak of both the analyte and the internal standard before ratioing, if applicable. Ion traces are drawn at a 5 mmu mass tolerance. 1/x weighting is used for all analytes. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

Table 5 lists the M+1 and internal standard (IS) for each quantitatively validated analyte in this procedure.

Analyte	M+1 (+ Cl isotope, when applicable)	IS	M + 1 for IS (+ Cl isotope)
Amitriptyline	278.190	Amitriptyline-d3	281.209
Chlorpheniramine	275.131 + 277.128	Chlorpheniramine-d6	281.169 + 283.166
Chlorpromazine	319.103 + 321.100	Chlorpromazine-d3	322.122 + 324.119
Citalopram	325.171	Citalopram-d6	331.209
Clomipramine	315.162 + 317.160	Clomipramine-d3	318.181 + 320.179
Cyclobenzaprine	276.175	Cyclobenzaprine-d3	279.194
Desipramine	267.186	Desipramine-d3	270.204
Dextromethorphan	272.201	Dextromethorphan-d3	275.220
Diphenhydramine	256.170	Diphenhydramine-d3	259.189
Doxepin	280.170	Doxepin-d3	283.188
Doxylamine	271.180	Doxylamine-d5	276.212
Duloxetine	298.128	Duloxetine-d3	301.145
EDDP	278.190	EDDP-d3	281.209
Fentanyl	337.227	Fentanyl-d5	342.259
Fluoxetine	310.141	Fluoxetine-d6	316.179
Imipramine	281.201	Imipramine-d3	284.220
Ketamine	238.099 + 240.097	Ketamine-d4	242.124 + 244.142
Meperidine	248.165	Meperidine-d4	252.190
Methadone	310.217	Methadone-d3	313.235
Mirtazapine	266.165	Norfentanyl-d5	238.196
Nordoxepin	266.154	Desipramine-d3	270.204
Norfentanyl	233.165	Norfentanyl-d5	238.196
Norfluoxetine	296.126	Norfluoxetine-d6	302.163
Normeperidine	234.149	Normeperidine-d4	238.174
Norpropoxyphene	326.211	Norpropoxyphene-d5	331.243
Nortriptyline	264.175	Nortriptyline-d3	267.194
Paroxetine	330.150	Paroxetine-d6	336.188
PCP	244.206	PCP-d5	249.237
Pheniramine	241.170	Pheniramine-d6	247.208
Propoxyphene	340.227	Propoxyphene-d5	345.258
Propranolol	260.165	Desipramine-d3	270.204
Sertraline	306.081+ 308.078 + 310.075	Setraline-d3	311.097
Tramadol	264.196	Tramadol-13C-d3	268.218
Trazodone	372.159, 374.156	Trazodone-d6	378.196, 380.193
Trimipramine	295.217	Trimipramine-d3	298.236
Venlafaxine	278.211	Venlafaxine-d6	284.249
Verapamil	455.290	Imipramine-d3	284.220
Zolpidem	308.176	Zolpidem-d6	314.215

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

a. Linear range using 1/x weighting and a linear calibration model:

Analyte	Range (ng/mL)	Analyte	Range (ng/mL)
Amitriptyline	50-1000	Mirtazapine	100-1000
Chlorpheniramine	50-1000	Nordoxepin	50-1000
Chlorpromazine	50-1000	Norfentanyl	10-200
Citalopram	50-1000	Norfluoxetine	50-1000
Clomipramine	50-1000	Normeperidine	50-1000
Cyclobenzaprine	10 - 200	Norpropoxyphene	50-1000
Desipramine	50-1000	Nortriptyline	50-1000
Dextromethorphan	50-1000	Paroxetine	10-200
Diphenhydramine	50-1000	PCP	10-200
Doxepin	50-1000	Pheniramine	50-1000
Doxylamine	50-750	Propoxyphene	50-1000
Duloxetine	50-1000	Propranolol	100-1000
EDDP	50-1000	Sertraline	50-1000
Fentanyl	10-200	Tramadol	50-1000
Fluoxetine	50-1000	Trazodone	50-1000
Imipramine	50-1000	Trimipramine	50-1000
Ketamine	50-1000	Venlafaxine	50-1000
Meperidine	50-1000	Verapamil	50-750
Methadone	50-1000	Zolpidem	10-200



b. Limit of Detection:

Analyte	LOD (ng/mL) Blood / Urine	Analyte	LOD (ng/mL) Blood / Urine
Amitriptyline	10 / 5	Metoprolol	10 / 5
Brompheniramine	1 / 1	Mirtazapine	10 / 5
Bupropion	5 / 5	Nordoxepin	25 / 5
Chlorpheniramine	10 / 5	Norfentanyl	5 / 5
Chlorpromazine	10 / 5	Norfluoxetine	25 / 5
Citalopram	10 / 5	Normeperidine	10 / 5
Clomipramine	10 / 5	Norpropoxyphene	10 / 5
Clozapine	10 / 5	Norsertaline	50 / 5
Cyclobenzaprine	1 / 1	Nortriptyline	25 / 5
Desipramine	10 / 5	Paroxetine	5 / 1
Dextromethorphan	10 / 5	PCP	1 / 5
Diphenhydramine	25 / 5	Pheniramine	10 / 10
Doxepin	10 / 5	Propoxyphene	10 / 5
Doxylamine	10 / 5	Propranolol	10 / 5
Duloxetine	25 / 10	Quetiapine	25 / 5
EDDP	10 / 5	Sertraline	10 / 5
Fentanyl	1 / 1	Thioridazine	10 / 10
Fluoxetine	10 / 5	Tramadol	10 / 5
Imipramine	10 / 5	Trazodone	10 / 5
Ketamine	10 / 5	Trimipramine	10 / 5
Meperidine	10 / 5	Venlafaxine	10 / 5
Methadone	10 / 5	Verapamil	10 / 5
		Zolpidem	1 / 1

c. Bias (n=15):

Analyte	Low	Medium	High
Amitriptyline	+2.72%	+3.96%	+3.68%
Chlorpheniramine	+2.03%	+5.49%	+4.59%
Chlorpromazine	+2.06%	+6.22%	+3.21%
Citalopram	-2.51%	+2.41%	+3.82%
Clomipramine	-1.69%	+1.06%	+1.88%
Cyclobenzaprine	-13.14%	-10.35%	-10.70%
Desipramine	-0.20%	+5.83%	+5.78%
Dextromethorphan	-6.34%	-4.28%	-2.97%
Diphenhydramine	+4.92%	+5.40%	+7.34%
Doxepin	+1.63%	+3.93%	+4.40%
Doxylamine	-10.27%	4.28%	-6.72%
Duloxetine	-11.34%	-7.25%	-7.51%
EDDP	-1.15%	+5.45% (n = 14)	+5.93%
Fentanyl	-1.74% (n = 12)	+2.13% (n = 12)	+0.84% (n = 12)

Analyte	Low	Medium	High
Fluoxetine	-0.27%	+5.19%	+4.52%
Imipramine	+0.17%	+6.32% (n = 14)	+5.38%
Ketamine	+0.56%	+5.68%	+2.08%
Meperidine	-2.07%	+1.85%	+2.61%
Methadone	-0.94%	+3.42% (n = 14)	+3.72%
Mirtazapine	+0.66%	+2.32%	+5.51% (n = 13)
Nordoxepin	-6.39%	-7.38%	-7.73%
Norfentanyl	-7.01% (n = 12)	-2.12% (n = 12)	-3.01% (n = 11)
Norfluoxetine	+2.54%	+6.43%	+7.14%
Normeperidine	-1.77%	-2.86%	-0.20%
Norpropoxyphene	-1.48%	+6.29%	+2.45%
Nortriptyline	+1.28%	+3.12%	+2.87%
Paroxetine	-9.06% (n = 12)	+0.41% (n = 12)	-3.54% (n = 11)
PCP	-3.14% (n = 11)	+0.67% (n = 12)	-1.52% (n = 12)
Pheniramine	-3.96%	+3.13% (n = 14)	+1.89%
Propoxyphene	-5.51%	-1.93%	-2.03%
Propranolol	-0.21% (n = 12)	-2.16% (n = 11)	+3.43 %
Sertraline	-17.81%	-12.52%	-9.18%
Tramadol	+4.02%	+7.81%	+4.99%
Trazodone	-8.94%	-6.27%	-6.53%
Trimipramine	-1.14%	+6.53%	+4.85%
Venlafaxine	-2.10%	-1.34%	-1.89%
Verapamil	-7.28%	-5.12%	-10.67%
Zolpidem	-0.57%	+0.51% (n = 12)	-0.83% (n = 14)

d. Repeatability (n=15):

Analyte	Low	Medium	High
Amitriptyline	1.31%	1.22%	2.30%
Chlorpheniramine	1.34%	1.69%	3.00%
Chlorpromazine	2.89%	0.62%	5.00%
Citalopram	1.58%	0.48%	3.73
Clomipramine	2.76%	1.80%	1.95%
Cyclobenzaprine	2.02%	1.44%	1.80%
Desipramine	5.68%	2.91% (n = 14)	3.07%
Dextromethorphan	1.18%	2.24%	0.61%
Diphenhydramine	1.66%	1.48%	1.81%
Doxepin	0.98%	0.71%	1.88%
Doxylamine	3.99%	7.37%	2.22%
Duloxetine	1.87%	1.45%	0.74%
EDDP	8.60%	3.74% (n = 14)	3.76%
Fentanyl	3.50% (n = 12)	1.15% (n = 12)	2.60% (n = 12)
Fluoxetine	1.14%	1.00%	1.46%

Analyte	Low	Medium	High
Imipramine	5.12%	3.07% (n = 14)	2.51%
Ketamine	1.58%	2.68%	1.88%
Meperidine	0.95%	1.73%	0.61%
Methadone	5.53%	3.29% (n = 14)	3.00%
Mirtazapine	19.81%	20.29%	7.93% (n = 13)
Nordoxepin	12.24%	4.85%	8.60%
Norfentanyl	2.25% (n=12)	2.94% (n=12)	3.04% (n=11)
Norfluoxetine	1.44%	2.23%	0.74%
Normeperidine	1.56%	0.50%	1.76%
Norpropoxyphene	6.33%	2.02%	2.51%
Nortriptyline	1.25%	0.87%	1.42%
Paroxetine	2.46% (n = 12)	1.79% (n = 12)	5.93% (n = 11)
PCP	3.99% (n = 11)	0.86% (n = 12)	2.31% (n = 12)
Pheniramine	7.64%	2.80% (n = 14)	2.57%
Propoxyphene	2.57%	2.28%	2.25%
Propranolol	16.84% (n = 12)	7.01% (n=11)	11.41%
Sertraline	1.56%	1.79%	1.59%
Tramadol	2.77%	2.19%	1.40%
Trazodone	1.58%	1.50%	1.02%
Trimipramine	5.41%	2.77% (n = 14)	2.47%
Venlafaxine	1.12%	1.00%	2.03%
Verapamil	17.63%	17.36%	18.13%
Zolpidem	3.06%	1.25% (n = 12)	2.22% (n = 14)

e. Intermediate Precision (n=15):

Analyte	Low	Medium	High
Amitriptyline	1.40%	1.92%	2.44%
Chlorpheniramine	2.75%	2.58%	3.11%
Chlorpromazine	3.06%	0.66%	5.00%
Citalopram	2.81%	0.95%	3.93%
Clomipramine	3.16%	2.21%	2.08%
Cyclobenzaprine	2.56%	1.78%	3.35%
Desipramine	6.41%	2.91%	3.07%
Dextromethorphan	2.46%	2.43%	0.78%
Diphenhydramine	3.38%	1.65%	1.81%
Doxepin	2.35%	1.81%	2.19%
Doxylamine	4.67%	8.26%	3.67%
Duloxetine	2.87%	1.45%	1.07%
EDDP	9.95%	3.74 % (n = 14)	3.88%
Fentanyl	5.43% (n = 12)	4.73 (n = 12)	2.60% (n = 12)
Fluoxetine	4.10%	1.69%	1.68%
Imipramine	5.22%	3.07% (n = 14)	2.62%

Analyte	Low	Medium	High
Ketamine	2.59%	2.68%	1.88%
Meperidine	3.19%	1.73%	1.31%
Methadone	5.76%	3.29% (n = 14)	3.08%
Mirtazapine	19.81%	20.29%	9.62%
Nordoxepin	13.70%	7.39%	8.92%
Norfentanyl	3.15% (n =12)	3.27% (n =12)	3.04% (n =11)
Norfluoxetine	5.26%	2.23%	1.00%
Normeperidine	1.93%	1.98%	2.48%
Norpropoxyphene	13.29%	5.62%	4.45%
Nortriptyline	1.90%	1.53%	1.81%
Paroxetine	3.66% (n =12)	4.20% (n =12)	5.93% (n =11)
PCP	5.72% (n = 11)	3.78% (n = 12)	2.42% (n = 12)
Pheniramine	8.02%	5.21% (n = 14)	4.52%
Propoxyphene	5.11%	2.87%	2.25%
Propranolol	16.84% (n =12)	9.52% (n =11)	12.59%
Sertraline	2.42%	2.60%	1.90%
Tramadol	5.30%	2.62%	1.41%
Trazodone	2.14%	1.85%	1.16%
Trimipramine	5.41%	2.77% (n = 14)	2.64%
Venlafaxine	2.30%	1.35%	2.03%
Verapamil	18.51%	17.36%	32.96%
Zolpidem	5.75%	2.98% (n = 12)	2.44% (n = 14)

- f. Interferences: The following drug pairs cannot be quantitated or identified if they are present in the same sample because they elute within 0.3 min and their exact masses are within 0.05 amu: amitriptyline and EDDP, methadone and propoxyphene, imipramine and EDDP, imipramine and amitriptyline, desipramine and nortriptyline, and nortriptyline and propoxyphene. Grossly decomposed or putrefied samples may affect both detection and quantitation limits.
- g. Carryover: For extracted negative control samples analyzed immediately following extracted 700 ng/ml positive control samples, no analyte showed signal greater than 2% of that seen in the positive control.
- h. Doxylamine elutes very early in the analysis time, so data should be interpreted with care due to possible sample related matrix effects.
- i. Processed sample stability: Six of eight compounds tested show no problems with processed sample stability after 8 days of refrigerated storage. However, thioridazine may degrade in prepared extracts, and negative results should be repeated if the extracted samples cannot be analyzed within the first 24 hours of extraction. Doxylamine controls will be monitored closely if the extracted samples cannot be analyzed within the first 24 hours of extraction.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Practices for Validating Chemical Procedures*; FBI Laboratory Operations Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
3	04/11/13	Added 8 new internal standards, adding doxylamine and trazodone to the list of quantitative analytes, and updating quantitations for dextromethorphan, duloxetine, citalopram, sertraline, venlafaxine and cyclobenzaprine. Subsequently, the following sections were updated: 2, 6c, 6q2, 6q3, 7.1, Table 4, Table 5, 14a, 14c, 14d, and 14e. A limitation for doxylamine was added in Section 14h. Added line for pipette documentation on bench sheet (Appendix 1).
4	10/01/14	In Sections 2 and 15, updated reference to Division validation procedure. In Sections 5 and 15, removed reference to Tox 103 and added preparation instructions that were not included to Section 5. In Sections 6.a-b., specified Lipomed as a second source for standard material. Removed Section 7 (Calibration), moved calibrator preparation information to Section 6, and renumbered subsequent sections. Added batch acceptance criteria in Section 10.1 and renumbered subsequent sections. Corrected a typo in Table 3. Deleted Section 10.3 since the information is covered elsewhere. Corrected a typo in Table 5. Added processed sample stability in Section 13.i. Reformatted Appendix 2 to include all pertinent instrumental parameters.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Quantitation and Confirmation of Alkaline Drugs  
Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.**

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## **Benzodiazepines and Metabolites from Blood and Urine by LC/MS (SRM)**

### **1 Introduction**

Benzodiazepines are one of the most commonly prescribed classes of drugs in the United States. They are also frequently abused. Therapeutic blood concentrations vary for benzodiazepines, depending on whether they are considered high dose benzodiazepines (e.g., diazepam) or low dose benzodiazepines (e.g., triazolam). Benzodiazepines are usually excreted into the urine as glucuronide metabolites, and may persist in the urine for days after administration due to elimination half lives that may exceed 24 hours.

### **2 Scope**

This procedure allows for the screening and confirmation of the following benzodiazepines in blood and urine: 7-aminoclonazepam, 7-aminoflunitrazepam,  $\alpha$ -hydroxyalprazolam,  $\alpha$ -hydroxymidazolam,  $\alpha$ -hydroxytriazolam, alprazolam, chlordiazepoxide, clonazepam, desalkylflurazepam, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, n-desmethylflunitrazepam, nordiazepam, oxazepam, temazepam, and triazolam. It also provides for quantitation of these compounds in blood. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

Biological specimens are qualitatively assayed and/or quantified for benzodiazepines and their metabolites. Specimens are mixed with deuterated internal standards. Proteins are precipitated from blood before extraction. Both blood and urine samples are extracted using solid phase extraction (SPE). Analysis of extracts is by liquid chromatography/tandem mass spectrometry in the single reaction monitoring mode (LC/MS(SRM)).

### **4 Specimens**

This procedure uses 0.2 mL of blood (in duplicate for quantitation<sup>1</sup>) or 0.4 mL of urine.

### **5 Equipment/Materials/Reagents**

- a. Routine laboratory supplies, including calibrated pipettes, disposable culture tubes, test tube racks, graduated cylinders, etc.

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<sup>1</sup> If case history, screening results, or other previous analysis indicates that the concentration of a benzodiazepine may be above the linear range of the method, the sample may be prediluted before extraction.

- b. Methanol (Optima Grade)
- c. Deionized water (18 MΩ)
- d. 1:1 Methanol:Water
- e. Zinc sulfate heptahydrate
- f. Zinc Sulfate (0.2 M): Combine 50 mL deionized water and 5.75 g of zinc sulfate heptahydrate in a 100 mL volumetric flask. Mix until dissolved. Bring to the mark with deionized water. Store in glass at room temperature. Stable for at least 6 months.
- g. Zinc Sulfate in Methanol: Combine 80 mL methanol and 20 mL zinc sulfate (0.2 M) in a volumetric flask and mix well. Mix well. Store in glass at room temperature. Stable for at least 2 months.
- h. Potassium dihydrogen phosphate
- i. Disodium hydrogen phosphate (anhydrous)
- j. Potassium Phosphate Buffer: Add 9.07 g potassium dihydrogen phosphate to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.
- k. Sodium Phosphate Buffer: Add 11.6 g disodium hydrogen phosphate (anhydrous) to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.
- l. Sorensen Buffer (pH 7.4): Add sodium phosphate buffer to the potassium phosphate buffer until the pH reads 7.4 with a pH meter. Store refrigerated in glass or plastic. Stable for at least three months.
- m. β-Glucuronidase - (>100,000 u/mL β-glucuronidase activity; from Red Abalone, *H. Rufescena*; available from Kura Biotech)
- n. Ammonium acetate (99.999% purity)
- o. Acetic acid, glacial (17 M, ACS grade)
- p. Ammonium Acetate Buffer (0.5 M; pH 5):  
Add 3.854 g ammonium acetate to a 100-mL volumetric flask containing about 75 mL deionized water. Mix well to dissolve. Add glacial acetic acid until pH registers between 4.5 and 5.5. Bring to volume with deionized water and mix well. Store refrigerated in glass or plastic. Stable at least three months.

- q. Vortexer
- r. Centrifuge
- s. Evaporator with nitrogen
- t. SPE manifold
- u. pH meter
- v. Oasis HLB 6 cc (500 mg) LP SPE cartridges
- w. Ammonium hydroxide, concentrated (15 M) (ACS grade)
- x. Methanol:Water:Ammonia (40:60:0.5):  
Combine 40 mL methanol, 60 mL deionized water and 0.5 mL ammonium hydroxide and mix well. Store at room temperature in glass. Prepare fresh daily.
- y. Methylene chloride (Optima grade)
- z. Isopropanol (HPLC grade)
- aa. Methylene Chloride:Isopropanol (75:25):  
Combine 75 mL methylene chloride and 25 mL isopropanol and mix well. Store at room temperature in glass. Stable for at least two months.
- bb. Water:Acetonitrile (90:10):  
Combine 90 mL deionized water and 10 mL acetonitrile (Optima grade) and mix well. Store at room temperature in glass. Stable for at least three months.
- cc. Centrifuge tube filters (0.45 micron, Nylon)
- dd. Ammonium formate
- ee. Formic acid
- ff. Mobile Phase A (5 mM Ammonium Formate with formic acid; pH~3.5): Add 0.3153 g ammonium formate to a 1 L volumetric flask. Add approximately 800 mL deionized water and mix well. Add 1 mL formic acid, and QS with deionized water. Store in glass at room temperature. Stable for at least one week.
- gg. Mobile Phase B (Acetonitrile with 0.1% Formic Acid): Combine 1 mL formic acid and 1000 mL acetonitrile and mix well. Store in glass at room temperature. Stable for at least two months.

- hh. ABI 5000 QTRAP Liquid Chromatograph/Mass Spectrometer equipped with Analyst software and a Phenomenex Kinetex XB-C18 (or equivalent) analytical column (150 mm x 2.1mm x 2.6  $\mu$ )

## 6 Standards and Controls

- a. Standard and Control Stock Solutions (1.0 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed or an equivalent supplier. The materials used to prepare the standard stock solutions will be from a different source than the materials used to prepare the control stock solutions. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam	clonazepam	n-desmethyflunitrazepam
7-aminoflunitrazepam	desalkylflurazepam	nordiazepam
$\alpha$ -hydroxyalprazolam	diazepam	oxazepam
$\alpha$ -hydroxymidazolam	flunitrazepam	temazepam
$\alpha$ -hydroxytriazolam	flurazepam	triazolam
alprazolam	lorazepam	
chlordiazepoxide	midazolam	

- b. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX) or an equivalent supplier. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam-d <sub>4</sub>	chlordiazepoxide-d <sub>5</sub>	midazolam-d <sub>4</sub>
7-aminoflunitrazepam-d <sub>7</sub>	clonazepam-d <sub>4</sub>	n-desmethyflunitrazepam-d <sub>4</sub>
$\alpha$ -hydroxyalprazolam-d <sub>5</sub>	desalkylflurazepam-d <sub>4</sub>	nordiazepam-d <sub>5</sub>
$\alpha$ -hydroxymidazolam-d <sub>4</sub>	diazepam-d <sub>5</sub>	oxazepam-d <sub>5</sub>
$\alpha$ -hydroxytriazolam-d <sub>4</sub>	flunitrazepam-d <sub>7</sub>	temazepam-d <sub>5</sub>
alprazolam-d <sub>5</sub>	lorazepam-d <sub>4</sub>	triazolam-d <sub>4</sub>
oxazepam glucuronide-d <sub>5</sub>		

- i. Internal Standard Intermediate Solution (5  $\mu$ g/mL):  
 Add 0.25 mL of each Internal Standard Stock Solution to a 5-mL volumetric flask and bring to the mark with methanol. Store in the freezer. Stable for at least 2 years.
- j. Internal Standard Working Solution (500 ng/mL):  
 Combine 0.1 mL of the Internal Standard Intermediate Solution (5  $\mu$ g/mL) and 0.9 mL methanol. Prepare fresh daily.

#### k. Calibration Scheme

This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101).

Preparation of High Calibration Solution			
Starting Solution	1	mg/mL	stock solution(s)
Starting Solution Aliquot	0.025	mL	
Diluent Volume	25	mL	methanol, volumetric flask
Resulting Concentration	1	µg/mL	storage: freezer; stability: ≥ 1 year

Preparation of Low Calibration Solution			
Starting Solution	1	µg/mL	High Calibrator Solution
Starting Solution Aliquot	0.5	mL	
Diluent Volume	10	mL	methanol, volumetric flask
Resulting Concentration	50	ng/mL	storage: freezer; stability: ≥ 1 year

Calibrator Level	Low Cal Spike (µL)	High Cal Spike (µL)	Resulting Concentration, ng/mL (in 0.2mL of blood)
1	10		2.5
2	20		5
3	100		25
4	200		50
5		20	100
6		30	150
7		50	250
8		75	375

#### l. Control Scheme

Negative Control Blood is purchased from Cliniqa or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

At least one Positive Control Blood Sample will be analyzed with each blood assay. For

quantitative analyses, both levels of Positive Control Blood Samples will be analyzed in duplicate.

Preparation of High Control Solution			
Starting Solution	1	mg/mL	stock solution(s)
Starting Solution Aliquot	0.025	mL	
Diluent Volume	25	mL	methanol, volumetric flask
Resulting Concentration	1	µg/mL	storage: freezer; stability: ≥ 1 year

Preparation of Low Control Solution			
Starting Solution	1	µg/mL	High Control Solution
Starting Solution Aliquot	0.5	mL	
Diluent Volume	10	mL	methanol, volumetric flask
Resulting Concentration	50	ng/mL	storage: freezer; stability: ≥ 1 year

Control Level	Low Control Spike (µL)	High Control Spike (µL)	Resulting Concentration, ng/mL (in 0.2mL of blood)
Negative	0	0	0
Low	20	0	5
High	0	50	250

- m. Hydrolysis Check Internal Standard Intermediate Solution (2.0 µg/mL d<sub>5</sub>-Oxazepam equivalent): To a 10-mL volumetric flask, add 0.322 mL of the d<sub>5</sub>-Oxazepam Glucuronide Stock Standard. Bring to volume with acetonitrile. Store frozen in glass. Stable for at least 6 months.
- n. Hydrolysis Check Internal Standard Working Solution (200 ng/mL d<sub>5</sub>-Oxazepam equivalent): Combine 0.1 mL of the Hydrolysis Check Internal Standard Intermediate Solution (2.0ug/mL) and 0.9 mL deionized water. Prepare fresh daily.
- o. Negative Control Urine:  
Prepared in-house or purchased from an appropriate vendor. Stable for 6 months when refrigerated. A Negative Control Urine sample will be extracted and analyzed with every urine assay.

p. Positive Control Urine Samples:

1. Low Positive Control Urine (1 ng/mL):  
Mix 0.020 mL of the Benzodiazepine Working Standard Control Solution (50 ng/mL) with 1.0 mL of Negative Control Urine. Mix well before withdrawing 0.4 mL for analysis. Prepare fresh.
2. High Positive Control Urine (10 ng/mL):  
Mix 0.010 mL of the Intermediate Standard Control Solution (1 µg/mL) with 1.0 mL of Negative Control Urine. Mix well before withdrawing 0.4 mL for analysis. Prepare fresh.
3. Hydrolysis Control Urine (10 ng/mL):  
Add 0.020 mL of the Hydrolysis Check Internal Standard to a 0.4 mL aliquot of the high positive control urine. (This results in a 10 ng/mL concentration of d5-oxazepam.) Prepare fresh.

At least one Positive Control Urine Sample will be analyzed with every urine assay.

- q. LC/MS Performance Standard (5 ng/mL): Add 5 µL of the Benzodiazepine Intermediate Standard Calibrator Solution (1 µg/mL) and 10 µL of the Internal Standard Working Solution to 1 mL of Water:Acetonitrile (90:10). Store in refrigerated autosampler tray for up to one week or prepare fresh daily.
- r. This procedure uses a multi-point calibration curve for the analyte(s) of interest following the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows the amount of the Benzodiazepine Working Standard Calibrator Solution to add to 0.2 mL of Negative Control Blood for calibrator preparation.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 8.1 Sample Preparation for Blood Specimens

- a. Prepare blood calibrators and controls as directed in Section 6 above. A second chemist will prepare the control solutions and positive control blood samples.
- b. Pipet 0.2 mL of each case blood sample into a properly labeled 12 x 75 mm test tube.

(Blood samples will be prepared in duplicate for quantitation and may have to be diluted for benzodiazepine concentrations that are above the linear range of the procedure.)

- c. Add 0.020 mL of the Internal Standard Working Solution (500 ng/mL) to each sample and vortex well.
- d. Add 2 mL zinc sulfate in methanol to each blood sample. Allow to sit for 1 minute, then vortex.
- e. Centrifuge samples for 5 minutes at 3000 rpm.
- f. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube.
- g. Concentrate samples to ~0.4 mL under nitrogen at 60°C.
- h. Add 5.5 mL of Sorenson buffer to each tube.
- i. Vortex and centrifuge samples for 1 minute at 3000 rpm.

## **8.2 Sample Preparation for Urine Specimens**

- a. Prepare urine controls as directed in Section 6 above.
- b. Pipet 0.4 mL of each case urine sample into a properly labeled 16 x 100 mm test tube.
- c. Add 0.010 mL of the Internal Standard Working Solution (500 ng/mL) to each sample (except the hydrolysis control sample) and vortex well.
- d. Add 0.6 mL Ammonium Acetate Buffer (0.5 M, pH 5) and 0.1 mL  $\beta$ -glucuronidase.
- e. Vortex, cap, and incubate 30 minutes at 68°C.
- f. Cool to room temperature.
- g. Add 2 mL zinc sulfate in methanol to each urine sample. Allow to sit for 1 minute, then vortex.
- h. Centrifuge samples for 5 minutes at 3000 rpm.
- i. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube.
- j. Concentrate samples to ~1.0 mL under nitrogen at 60°C.
- k. Add 5 mL of Sorenson buffer to each tube.



- l. Vortex and centrifuge samples for 1 minute at 3000 rpm.

### **8.3 Solid Phase Extraction (applicable to blood and urine samples)**

- a. Pre-rinse SPE extraction cartridge (Oasis HLB) by adding 2 mL of methanol.
- b. Condition cartridge with 3 mL of deionized water.
- c. Load sample on SPE cartridge.
- d. Wash cartridge with 2 mL of Methanol:Water:Ammonia (40:60:0.5).
- e. Dry cartridge at full vacuum for 15 minutes. (Use vacuum manifold; positive pressure source not shown to dry effectively.
- f. Elute with 5 mL Dichloromethane:Isopropanol (75:25) under gravity.
- g. Evaporate eluent to dryness at 60°C under nitrogen.
- h. Reconstitute blood extracts with 0.25 mL Water:Acetonitrile (90:10). Vortex.  
Reconstitute urine extracts with 0.1 mL of Water:Acetonitrile (90:10). Vortex.
- i. Filter samples through 0.45 micron filters.
- j. Analyze 5 µL of the LC/MS Performance Standard to verify that the instrument is operating properly and that retention times have not shifted outside of the analytes' multipole reaction monitoring (MRM) windows.
- k. Analyze extracts following the instrumental conditions in Section 9 below.

## **9 Instrumental Conditions**

Appendix 1 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### **9.1 Autosampler Parameters**

- a. Autosampler Temperature Setting: 14°C
- b. Injection volume = 5 µL for blood; 20 µL for urine.

## 9.2 Liquid Chromatograph Parameters

Column Oven Temp		23°C
Time (min)	% Mobile Phase A (Aqueous)	% Mobile Phase B (Organic)
0:01	90	10
12:00	60	40
21:00	60	40
25:00	0	100
28:00	90	10
40:00	90	10
Flow rate		0.2 mL/min

## 9.3 Mass Spectral Parameters

Scan Mode	Turbo Spray	Polarity	Positive
Resolution	Unit	Scan Type	MRM
Curtain Gas	Nitrogen (35)	Ionspray Voltage	3000
Source Temperature	670°C	Nebulizer Gas	Nitrogen (50)
Interface Heater	ON	Turbo Gas	Nitrogen (50)
Collision Gas	Nitrogen (Medium)	Entrance Potential	10

Q1 Mass	Q3 Mass	Time (min)*	Declustering Potential	Collision Energy	Collision Exit Potential
309.266	281.200	16.66	76	37	14
309.266 <sup>a</sup>	205.100	16.66	76	57	22
311.237	283.200	16.66	126	35	30
314.253	279.300	16.56	131	37	38
316.194 <sup>a</sup>	270.200	17.24	146	35	16
316.194	214.100	17.24	146	53	16
318.184	272.200	17.24	141	37	22
320.238	274.200	17.16	191	33	18
285.086 <sup>a</sup>	193.200	21.90	126	43	20
285.086	154.100	21.90	126	37	10
287.214	193.100	21.90	141	43	18
290.203	198.200	21.60	46	43	26
314.220 <sup>a</sup>	268.200	18.66	106	35	16
314.220	239.200	18.66	106	47	24
314.220	183.100	18.66	106	81	20

Q1 Mass	Q3 Mass	Time (min)*	Declustering Potential	Collision Energy	Collision Exit Potential
321.295	275.300	18.48	101	37	26
388.577 <sup>a</sup>	315.200	13.62	56	31	18
388.577	288.200	13.62	56	35	30
390.313	317.200	13.62	121	33	24
289.173 <sup>a</sup>	226.200	17.90	36	45	22
289.173	179.100	17.90	36	61	12
289.173	165.000	17.90	36	35	20
291.132	226.200	17.90	111	41	20
293.205	230.200	17.82	56	39	24
321.203 <sup>a</sup>	275.100	16.61	91	31	28
321.203	229.200	16.61	91	43	24
323.186	277.200	16.61	96	31	20
327.203	281.100	16.55	146	33	22
326.205	291.300	13.34	176	37	22
326.205 <sup>a</sup>	249.100	13.34	176	51	26
326.205	222.300	13.34	176	63	18
328.190	291.200	13.34	181	37	30
271.258	165.300	17.60	141	41	0
271.258 <sup>a</sup>	208.300	17.60	141	43	0
271.258	243.300	17.60	141	31	4
276.382	213.200	17.46	26	41	22
287.231 <sup>a</sup>	241.300	16.01	161	31	24
287.231	231.200	16.01	161	31	24
289.213	243.200	16.01	106	31	26
292.196	246.200	15.93	101	33	16
301.266 <sup>a</sup>	255.100	18.40	101	31	26
301.266	177.000	18.40	101	53	18
303.251	257.200	18.40	71	31	16
306.215	260.200	18.30	81	33	26
343.213	308.300	17.12	36	37	32
343.213 <sup>a</sup>	239.200	17.12	36	53	26
345.212	241.100	17.12	36	57	36
347.216	243.300	17.04	166	57	10
325.250 <sup>a</sup>	297.200	15.28	106	37	16
325.250	216.100	15.28	106	53	22
327.247	299.200	15.28	181	35	30
330.270	302.300	15.23	136	37	20
286.233 <sup>a</sup>	222.200	9.40	131	35	18
286.233	250.200	9.40	131	29	14

Q1 Mass	Q3 Mass	Time (min)*	Declustering Potential	Collision Energy	Collision Exit Potential
286.233	195.200	9.40	131	43	12
288.219	222.200	9.40	111	35	26
290.218	226.200	9.32	126	33	20
284.285 <sup>a</sup>	227.300	10.92	151	35	16
284.285	240.300	10.92	151	45	18
284.285	163.200	10.92	151	31	16
342.211 <sup>a</sup>	203.000	13.35	71	35	20
342.211	168.100	13.35	71	51	10
344.216	205.000	13.35	111	37	22
359.211	331.300	15.30	151	39	20
359.211 <sup>a</sup>	176.000	15.30	151	37	26
359.211	239.200	15.30	151	61	24
361.195	333.200	15.30	151	39	34
363.229	176.000	15.26	176	39	20
300.173	254.200	16.26	91	33	54
300.173 <sup>a</sup>	198.100	16.26	91	51	24
300.173	225.100	16.26	91	49	12
304.211	258.200	16.20	116	35	18
300.217 <sup>a</sup>	227.100	11.64	91	35	26
300.217	247.200	11.64	91	49	20
300.217	165.100	11.64	91	67	10
305.133	232.100	11.56	81	35	24
330.237	253.100	13.30	126	55	26
346.232	203.000	13.29	151	37	22
291.285	138.000	10.80	121	41	20

\*MRM Times may be adjusted over time due to changes in mobile phase and/or column performance. Small changes (less than 2 minutes) in these times are not considered modifications to the method and need not be recorded as modifications in case notes.

<sup>a</sup> This is the typical quant transition referred to in Section 11 below.

## 10 Decision Criteria

### 10.1 LC/MS Performance Standard Decision Criteria

Peaks should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. The chemist should ensure that the peaks entirely elute within their MRM windows, and adjust the MRM window times, if necessary.

## **10.2 Unknown Sample Decision Criteria**

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

### **10.2.1 Batch Acceptance**

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected in the LC/MS data. High and Low Positive Controls should fall within  $\pm 20\%$  of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for further guidance.

There should be a peak for d5-oxazepam in the Hydrolysis Check Positive Control Urine. This peak area should approximate the area of the oxazepam peak (within  $\pm 50\%$ ).

### **10.2.2 Unknown Sample Criteria**

Each of the Internal Standards should be detectable in the LC/MS data.

#### **10.2.2.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### **10.2.2.2 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

#### **10.2.2.3 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 10 when using the Analyst software. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

### 10.2.2.4 Mass Spectrometry

At least three independent MS/MS experiments are conducted for each analyte. (See Table 2 below.) Two ion ratios are calculated for each analyte. The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

**Table 2: MS/MS Transitions**

Analyte	Tran 1	Tran 2	Tran 3	Trans 4
Alprazolam	309.2→281.2	309.2→205.1	311.2→283.2	n/a
Clonazepam	316.1→270.2	316.1→214.1	318.1→272.2	n/a
Diazepam	285.0→193.2	285.0→154.1	287.2→193.1	n/a
Flunitrazepam	314.2→268.2	314.2→239.2	314.2→183.1	n/a
Flurazepam	388.5→315.2	388.5→288.2	390.3→317.2	n/a
Desalkylflurazepam	289.1→226.2	289.1→165.0	291.1→226.2	289.1→179.1
Lorazepam	321.2→275.1	321.2→229.2	323.1→277.2	n/a
Midazolam	326.2→291.3	326.2→249.1	326.2→222.3	328.1→291.2
Nordiazepam	271.2→165.3	271.2→208.3	271.2→243.3	n/a
Oxazepam	287.2→241.3	287.2→231.2	289.2→243.2	n/a
Temazepam	301.2→255.1	301.2→177.0	303.2→257.2	n/a
Triazolam	343.2→308.3	343.2→239.2	345.2→241.1	n/a
$\alpha$ -hydroxyalprazolam	325.2→297.2	325.2→216.1	327.2→299.2	n/a
7-aminoclonazepam	286.2→222.2	286.2→250.2	286.2→195.2	288.2→222.2
7-aminoflunitrazepam	284.2→227.3	284.2→240.3	284.2→163.2	n/a
$\alpha$ -hydroxymidazolam	342.2→203.0	342.2→268.1	344.2→205.0	n/a
$\alpha$ -hydroxytriazolam	359.2→331.3	359.2→176.0	359.2→239.2	361.2→333.2
desmethyflunitrazepam	300.1→254.2	300.1→198.1	300.1→225.1	n/a
chlordiazepoxide	300.2→227.1	300.2→247.2	300.2→165.1	n/a

## 11 Calculations

1/x<sup>2</sup> weighting is used for all calibration curves. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory Report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- a. Limit of Detection:
1. Blood: 1.25 ng/mL (or lower)
  2. Urine: 0.5 ng/mL (or lower)

- b. Limit of Quantitation: 2.5 ng/mL

- c. Accuracy (as % bias; n=15 for all values in table):

	Bias (%; at 5 ng/mL)	Bias (%; at 100 ng/mL)	Bias (%; at 250 ng/mL)
7-aminoclonazepam	-3.63	2.06	3.65
7-aminoflunitrazepam	2.71	5.89	1.32
$\alpha$ -OH midazolam	3.93	3.76	0.44
$\alpha$ -OH alprazolam	3.83	3.88	-1.22
diazepam	5.21	4.54	-2.80
clonazepam	0.39	3.69	1.41
alprazolam	6.84	6.45	-1.85
chlordiazepoxide	0.40	2.11	-2.69
flunitrazepam	1.23	2.09	-0.17
desalkylflurazepam	2.71	2.42	0.36
lorazepam	2.04	2.89	0.69
flurazepam	2.89	-1.79	-0.79
n-desmethyflunitrazepam	0.09	2.11	-0.78
midazolam	0.03	2.17	1.01
nordiazepam	2.29	4.72	-2.76
oxazepam	0.52	0.97	-1.00
temazepam	5.29	5.26	-2.21
triazolam	7.57	5.43	-3.56

$\alpha$ -OH triazolam	2.31	4.94	-0.14
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d. Precision (as both repeatability and intermediate precision; n=15 for all values in tables):

	Repeatability (%; at 5 ng/mL)	Repeatability (%; at 100 ng/mL)	Repeatability (%; at 250 ng/mL)
7-aminoclonazepam	7.20	9.71	8.96
7-aminoflunitrazepam	7.29	6.49	11.09
$\alpha$ -OH midazolam	5.45	3.22	4.26
$\alpha$ -OH alprazolam	4.95	2.78	4.22
diazepam	5.26	2.34	3.33
clonazepam	4.72	2.47	3.86
alprazolam	4.83	3.66	2.85
chlordiazepoxide	3.70	3.14	4.38
flunitrazepam	4.54	3.02	2.76
desalkylflurazepam	4.79	4.03	4.01
lorazepam	6.79	3.28	3.12
flurazepam	9.55	5.69	3.70
n-desmethyflunitrazepam	3.94	3.62	3.76
midazolam	4.31	3.17	3.75
nordiazepam	5.00	2.79	3.73
oxazepam	5.08	2.65	3.08
temazepam	4.32	2.70	2.96
triazolam	4.59	3.17	1.92
$\alpha$ -OH triazolam	4.67	3.37	4.00

	Intermediate Precision (%; at 5 ng/mL)	Intermediate Precision (%; at 100 ng/mL)	Intermediate Precision (%; at 250 ng/mL)
7-aminoclonazepam	10.29	9.71	9.17
7-aminoflunitrazepam	7.29	6.49	11.65
$\alpha$ -OH midazolam	6.45	5.25	5.74
$\alpha$ -OH alprazolam	5.72	5.04	5.37
diazepam	5.36	3.94	5.95
clonazepam	4.91	4.05	5.37
alprazolam	5.63	5.04	6.66
chlordiazepoxide	4.45	5.04	6.63
flunitrazepam	5.04	4.74	4.47
desalkylflurazepam	5.44	4.64	6.60
lorazepam	7.67	7.38	7.53
flurazepam	10.45	7.50	6.29
n-desmethyflunitrazepam	5.40	4.50	4.81
midazolam	5.29	5.09	6.33
nordiazepam	5.40	5.20	5.40
oxazepam	5.68	3.29	3.16
temazepam	5.52	5.84	5.45



triazolam	5.11	5.21	5.34
$\alpha$ -OH triazolam	4.91	5.83	6.22

- e. Cautionary Statement: Oxazepam may be unstable in methanol.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*, 9th ed.; Biomedical Publications, Seal Beach, California, 2011.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

*Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
5	02/09/18	Updated Scope language. Updated calibration/control scheme preparation in Section 6. Removed “reasonable degree of scientific certainty” language from Section 10.2.2.4. Updated approval lines. Removed TOX103 reference from Section 15. In Section 8.3e, specified vacuum manifold vs positive pressure. In section 6, reformatted the Calibration and Control scheme into a tabular format, and updated to a simpler preparation (also updated on the bench notes, which made a total of 3 pages from 2 pages).
6	04/01/19	Removed “subunit” (header, 15). Updated CUQA title in Reference Section and 12. Updated blood reconstitution volume to 0.25mL (8.3-h, Appendix). Made format/typo corrections on page 1 of Appendix. Updated enzyme to >100,000 (5-m) to account for product description.

### **Approval**

Redacted - Signatures on File

Toxicology  
Technical Leader:

Date: 03/28/2019

Chemistry Unit Chief:

Date: 03/28/2019

### **QA Approval**

Quality Manager:

Date: 03/28/2019

**Appendix 1: Abbreviated version of the Benzodiazepine Procedure for bench use (page 1-3)**

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## **Analysis of Opioids in Hair**

### **1 Introduction**

Opioids are a class of substances that include natural, semi-synthetic and synthetic alkaloidal agents derived from opium or substances which have morphine-like activity. Naturally occurring opioids such as morphine and codeine are typically referred to as opiates. Heroin (diacetylmorphine) is a semi-synthetic opioid that is synthesized by the acetylation of morphine. In humans, heroin is rapidly metabolized to 6-monoacetylmorphine (6-AM) and morphine. Other common opioids include hydromorphone, hydrocodone, oxycodone, oxycodone, methadone, meperidine, tramadol and fentanyl. These compounds may be found in the hair of individuals who have been exposed to the drugs.

### **2 Scope**

This procedure allows for the screening and confirmation of oxycodone in hair. It also allows for the screening, confirmation and quantitation of morphine, codeine, hydromorphone, hydrocodone, oxycodone, 6-AM, methadone, meperidine, tramadol and fentanyl in hair.

### **3 Principle**

Hair samples are decontaminated with methanol and methylene chloride washes before mechanical pulverization. The resulting hair powder is extracted overnight in methanol. Methanol extracts are taken to dryness, reconstituted in water, and extracted via solid phase extraction (SPE). Final extracts are analyzed by liquid chromatography-high resolution mass spectrometry (LC/HRMS). Positive sample extracts may be confirmed via liquid chromatography-tandem mass spectrometry (LC/MS/MS).

### **4 Specimens**

At least 40-mg of hair is needed for quantitative analysis.

### **5 Equipment/Materials/Reagents**

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Binary (or higher) liquid chromatograph coupled to an electrospray Orbitrap mass

spectrometer capable of at least 30000 resolution (and MS/MS for confirmatory analysis)

- b. Grace / Alltech Prevail Phenyl LC column: 150 x 2.1 mm, 5  $\mu$ m d<sub>p</sub>, with 7.5 x 2.1 mm guard column (or equivalent)
- c. Test tubes (16 x 125 mm screw-top, 16 x 100 mm and 12 x 75 mm culture, or comparable)
- d. Centrifuge
- e. Heating block
- f. Vortex mixer
- g. Solid phase extraction manifold (vacuum or positive pressure)
- h. CLEAN SCREEN DAU solid phase extraction (SPE) cartridges (200 mg x 10 mL)
- i. Evaporator with nitrogen
- j. Water (Optima grade and deionized)
- k. 0.1 M, pH 6 Phosphate buffer
- l. 1:1 Methanol:water
- m. Methanol (HPLC and Optima grades)
- n. 0.1 M Acetic acid
- o. Ammonium formate (99.995%+)
- p. Acetonitrile (Optima grade)
- q. 0.5  $\mu$ m PTFE membrane filter
- r. Methylene chloride (HPLC grade)
- s. Isopropanol (HPLC grade)
- t. Ammonium hydroxide (concentrated, reagent grade)
- u. Formic Acid (reagent grade)

- v. SPE elution solvent (78:20:2 methylene chloride:isopropanol:ammonia)
- w. Reconstitution solvent (5:95 methanol:water):  
Combine 5 mL water with 95 mL methanol (both Optima grade) and mix well. Store in glass at room temperature. Stable for 6 months.
- x. LC mobile phase #1 (95:5:0.05 10 mM ammonium formate : acetonitrile : formic acid):  
Dissolve 630 mg of ammonium formate in 1 L of Optima grade water. Remove 50 mL of this solution, save for LC Mobile Phase #2, and add 50 mL of acetonitrile. Mix well and vacuum filter through a 0.5  $\mu$ m PTFE membrane. Add 500  $\mu$ L formic acid and mix well. Store in glass at room temperature. Stable for 2 months.
- y. LC mobile phase #2 (5:95:0.05 10 mM ammonium formate : acetonitrile : formic acid):  
Add 25 mL of the aqueous formate solution from the preparation of LC mobile phase #1 to 475 mL of acetonitrile. Mix well and vacuum filter through a 0.5  $\mu$ m PTFE membrane. Add 250  $\mu$ L formic acid and mix well. Store in glass at room temperature. Stable for 2 months.
- z. Common laboratory supplies such as volumetric flasks, autosampler vials, pipette tips, etc.
- aa. Cryogrinder
- bb. Disposable centrifuge tubes
- cc. Disposable micro-stir bars

## 6 Standards and Controls

- a. d<sub>3</sub>-Morphine Stock Solution (100  $\mu$ g/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- b. d<sub>6</sub>-Codeine Stock Solution (100  $\mu$ g/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- c. d<sub>3</sub>-Oxymorphone Stock Solution (100  $\mu$ g/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.



- d. d<sub>6</sub>-Oxycodone Stock Solution (100 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- e. d<sub>3</sub>-Hydromorphone Stock Solution (100 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- f. d<sub>3</sub>-Hydrocodone Stock Solution (100 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- g. d<sub>5</sub>-Fentanyl Stock Solution (100 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- h. d<sub>3</sub>-6-MAM Stock Solution (100 µg/mL):  
An acetonitrile solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- i. 13C-d<sub>3</sub>-Tramadol Working Solution (2 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- j. d<sub>3</sub>-Methadone Stock Solution (100 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- k. d<sub>4</sub>-Meperidine Stock Solution (100 µg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- l. Morphine Stock Standard (0.1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- m. Codeine Stock Standard (0.1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- n. Hydromorphone Stock Standard (1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.

- o. Hydrocodone Stock Standard (1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- p. Oxymorphone Stock Standard (1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- q. Oxycodone Stock Standard (1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- r. 6-AM Stock Standard (0.1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- s. Tramadol Stock Standard (1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- t. Meperidine Stock Standard (1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- u. Fentanyl Stock Standard (0.1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- v. Methadone Stock Standard (0.1 mg/mL):  
A methanolic solution purchased from Cerilliant or other approved vendor. Stability and storage are determined by the manufacturer.
- w. Intermediate Mixed Opiate Stock Solution (50 PPM):  
Add 0.5 mL each of the Tramadol, Meperidine, Oxycodone, Oxymorphone, Hydrocodone and Hydromorphone Stock Solutions to a 10-mL volumetric flask and dilute to the mark with Optima Grade methanol. Store below 0°C in glass. Stable for at least one year.
- x. Intermediate Fentanyl Stock Solution (5 PPM):  
Add 0.5 mL of the Fentanyl Stock Solutions to a 10-mL volumetric flask and dilute to the mark with Optima Grade methanol. Store below 0°C in glass. Stable for at least one year.

- y. Working Control Solution (50/5<sup>1</sup> ng/mL):  
Add 0.050 mL of the Codeine, 6-AM, Morphine and Methadone Stock Solutions to a 100-mL volumetric flask. Add 0.100 mL each of the Intermediate Mixed Opiate Stock Solution and the Intermediate Fentanyl Stock Solution. Bring to the mark with Optima Grade methanol. Store below 0°C in glass. Stable for at least one year.
- z. Working Calibration Solution (50/5 ng/mL):  
Add 0.050 mL of the Codeine, 6-AM, Morphine and Methadone Stock Solutions to a 100-mL volumetric flask. Add 0.100 mL each of the Intermediate Mixed Opiate Stock Solution and the Intermediate Fentanyl Stock Solution. Bring to the mark with Optima Grade methanol. Store below 0°C in glass. Stable for at least one year.
- aa. Internal Standard Solution (150/20 ng/mL):  
Add 0.010 mL of the d<sub>5</sub>-Fentanyl Stock Solution and 0.075 mL of the remaining isotopically labeled Stock Solutions to a 50-mL volumetric flask. Bring to the mark with Optima Grade methanol. Store below 0°C in glass. Stable for at least two years.
- bb. Column Performance Evaluation Mix (0.25/0.025 µg/mL):  
Add 0.025 mL of the Codeine, 6-AM, Morphine and Methadone Stock Solutions to a 10-mL volumetric flask. Add 0.050 mL each of the Intermediate Mixed Opiate Stock Solution and the Intermediate Fentanyl Stock Solution. Bring to the mark with Optima Grade water. Store below 0°C in glass. Stable for at least one year.
- cc. Negative Control Hair: Obtained from drug-free donors. Stored in paper at room temperature. Negative Control Hair does not expire.
- dd. Negative Control Hair Powder: Obtained by washing Negative Control hair with methanol, methylene chloride, and methanol; drying the hair, and crushing under liquid nitrogen in a cryogrinder. Negative Control Hair Powder is stored in plastic at room temperature and does not expire.
- ee. Low Positive Control Hair Sample (300/30 pg/mg): Prepared by spiking 20 mg Negative Control Hair Powder with 0.120 mL of the working Control Solution. Prepared by a second analyst in duplicate the day of extraction.
- ff. High Positive Control Hair Sample (2400/240 pg/mg): Prepared by spiking 20 mg Negative Control Hair Powder with 0.960 mL of the working Control Solution. Prepared by a second analyst in duplicate the day of extraction.

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<sup>1</sup> Since the calibration range for fentanyl is approximately 10 times lower than that of the other components, it will be in a lower concentration in all stock solutions.

## 7 Calibration

This procedure may be used quantitatively via construction of a multi-point calibration curve for the analyte(s) of interest following the *Guideline for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows typical concentrations and volumes for calibrator preparation.

Table 1: Calibrator Preparation

Calibrator Level	pg/mg	µL Stock to add to 20 mg Negative Control Hair*
1**	100	40
2	200	80
3	500	200
4	1000	400
5	2000	800
6	3000	1200

\*Bring sample volume up to ~1.5 mL with HPLC Grade methanol.

\*\*This level is out of the linear range for fentanyl. All fentanyl calibrators are a factor of 10 lower.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Hair Preparation:

- Visually inspect hair and record observations.
- If segmental analysis is required, cut a portion of the hair sample into 2-cm segments.
- Accurately weigh 25-100 mg of each hair sample (or segment) into a properly labeled test tube (to the nearest 0.1 mg).
- Wash each hair sample with 1.5 mL HPLC Grade methanol by vortexing for approximately 1 minute. Discard this wash.

- e. Wash each hair sample with 1.5 mL methylene chloride by vortexing the sample for approximately 1 minute. Discard this wash.
- f. Wash each hair sample with 1.5 mL HPLC Grade methanol by vortexing for approximately 1 minute. Cap this wash and store refrigerated for later analysis, if necessary.
- g. Dry hair samples in a heating block at approximately 40°C to evaporate any remaining solvent.
- h. Cryogrind dry hair samples in the freezer mill using the settings in Section 10.5 of this procedure.
- i. Accurately weigh 20 mg of hair powder into a small vial (to the nearest 0.1 mg). Samples will be prepared in duplicate if specimen size allows. Smaller amounts may be weighed to account for high concentrations of analyte and/or limited specimen amount.
- j. Add a magnetic stir bar, 1.5 mL Optima Grade methanol, and 100 µL Internal Standard Solution to each vial.
- k. Similarly, prepare control and calibration samples as directed in Sections 6 and 7 above.
- l. Extract overnight (at least 12 hours) while stirring at approximately 40°C.
- m. Centrifuge samples at ~10000 rpm for 5 minutes.
- n. Remove methanol to a labeled 12 x 75 mm test tube.
- o. Evaporate to dryness under a gentle stream of nitrogen at approximately 40°C.
- p. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.

## **9.2 Solid Phase Extraction:**

- a. Add 4 mL of 0.1 M phosphate buffer to each sample and vortex.
- b. Pre-rinse one SPE extraction cartridge for each sample by adding 3 mL of methanol (HPLC Grade) at 1 mL/minute.
- c. Condition cartridges with 3 mL of deionized water followed by 1 mL of 0.1 M phosphate buffer at 1 mL/minute. Do not allow sorbent to dry.
- d. Load sample onto cartridge at 1-2 mL/minute. Do not allow sorbent to dry.

- e. Wash cartridge with 3 mL of deionized water, 1 mL of 0.1 M acetic acid, and 3 mL of methanol (HPLC grade) (each at 1-2 mL/minute).
- f. Dry cartridge under full vacuum for 3 minutes.
- g. Apply 3 mL of SPE Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm test tubes.
- h. Evaporate to dryness under nitrogen at approximately 40°C.
- i. Reconstitute the dry residue in 50 µL of Reconstitution Solvent (5:95 methanol:water) and analyze 10 µL portions by LC/HRMS with the conditions given in section 10. Be sure to analyze an injection of a solvent blank under the column wash conditions specified in Section 10.4 of this procedure at least every 15 analytical injections. Positive case samples may be reinjected (with appropriate controls) by LC/MSMS for confirmatory analysis.

### **9.3 Analysis of wash samples:**

- a. For samples in which an opiate is identified above the LLOQ of the method, add 100 µL Internal Standard Solution to each wash.
- b. Evaporate to dryness under a gentle stream of nitrogen at approximately 40°C.
- c. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.
- d. Extract using the procedure in Section 9.2 above. Analyze 10 µL portions by LC/HRMS with the conditions given in section 10.

## **10 Instrumental Conditions**

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 10.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
1: 5:95:0.05 10 mM formate : acetonitrile : formic acid	total flow	0.25 mL/min		type	Phenyl
	time (min)	%1	%2	length	15 cm
2: 95:5:0.05 10 mM formate : acetonitrile : formic acid	0	0	100	internal diameter	2.1 mm
	2	0	100	particle size	5 µm
	6	20	80	temperature	30°C
	10	20	80	guard length	7.5 mm
	11	60	40	guard ID	2.1 mm
	16	60	40		
	17	0	100		
	25	0	100		
	total time		25 min		

### 10.2 Mass Spectrometer Parameters – Screen and Quantitation

Mode: Electrospray; FTMS	Spray Voltage: +4 kV	Capillary Temperature: 225°C
Sheath Gas: 25 (arb units)	Aux Gas: 12 (arb units)	Sweep Gas: 0 (arb units)
Resolution: 30000	Scan Range: 200 - 370	
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		

### 10.3 Mass Spectrometer Parameters – Confirmation

Mode: Electrospray; FTMS	Spray Voltage: +4 kV	Capillary Temperature: 225°C
Sheath Gas: 25 (arb units)	Aux Gas: 12 (arb units)	Sweep Gas: 0 (arb units)
Set #1 (Fentanyl, Tramadol, Meperidine and Methadone)		5 scan events; all 30000 resolution
Scan Event #1	Scan Range: 200 - 370	CE = 30%
Scan Event #2	MS/MS 337.227	
Scan Event #3	MS/MS 264.196	
Scan Event #4	MS/MS 310.217	
Scan Event #5	MS/MS 248.165	
Set #2 (6-AM, Morphine and Codeine)		4 scan events; all 30000 resolution
Scan Event #1	Scan Range: 200 - 370	CE = 30%
Scan Event #2	MS/MS 286.144	
Scan Event #3	MS/MS 328.154	
Scan Event #4	MS/MS 300.159	
Set #3 (Oxycodone, Oxymorphone, Hydrocodone and		5 scan events; all 30000

Hydromorphone)		resolution
Scan Event #1	Scan Range: 200 - 370	
Scan Event #2	MS/MS 286.144	
Scan Event #3	MS/MS 302.139	
Scan Event #4	MS/MS 300.159	
Scan Event #5	MS/MS 316.154	
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		

**10.4 Column Washing** – At least once every 15 injections, the column should be washed under the following conditions to keep the analytical column in good working order.

Mobile Phase Compositions	Flow Parameters			Column Parameters	
1: 5:95:0.05 10 mM formate : acetonitrile : formic acid	total flow	0.25 mL/min		type	Phenyl
	time (min)	%1	%2	length	15 cm
2: 95:5:0.05 10 mM formate : acetonitrile : formic acid	0	0	100	internal diameter	2.1 mm
	1	0	100	particle size	5 µm
Mass Spectrometer	4	90	10	temperature	30°C
As above	14	90	10	guard length	7.5 mm
	17	0	100	guard ID	2.1 mm
	25	0	100		
	total time		25 min		

#### 10.5 Freezer/Mill Parameters

Cycles	1
Precool	9 min
Run time	8 min
Cool time	1 min
Rate	10 cps

### 11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control.



## **11.1 Chromatography**

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### **11.1.1 Retention Time**

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, calibrator, or Positive Control.

### **11.1.2 Signal-to-Noise**

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

## **11.2 Mass Spectrometry**

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## **11.3 Wash Decision Criteria**

If the final wash contains greater than ten times the amount of an opiate in a given hair sample, the sample's exterior is considered to be possibly contaminated, and will be reported as such.

## **12 Calculations**

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

The total amount of an opiate in a wash sample may be calculated against a new curve, or against the hair curve for that sample.

Example calculations for wash decision criteria:

Assume 20.0 mg of sample Q1 are washed per the SOP, and that the sample is subsequently determined to contain 500 pg morphine per milligram of hair. Then, the

hair sample contained 10000 pg total of morphine (20 x 500). The final wash must contain more than 1000 pg morphine (10000/10) for the Q1 sample to be reported as possibly contaminated.

### 13 Uncertainty of Measurement

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- precision of the balance used to weigh the hair powder
- precision of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

### 14 Limitations

a. Accuracy:

	% Bias at 300 pg/mg	% Bias at 1200 pg/mg	% Bias at 2400 pg/mg
6-AM	0.8%	-0.5%	-0.1%
Codeine	10%	13%	12%
Fentanyl*	0.4%	14%	17%
Hydrocodone	11%	12%	9%
Hydromorphone	11%	9%	5%
Meperidine	12%	11%	11%
Methadone	10%	10%	12%
Morphine	20%	20%	15%
Oxycodone	10%	10%	9%
Tramadol	12%	11%	12%

\*%Bias determined at 30, 120 and 240 pg/mg.

b. Calibration Model:

Linear from 100 – 3000 pg/mg using 1/x weighting for 6-AM, codeine, hydrocodone, hydromorphone, meperidine, methadone, morphine, oxycodone and tramadol.  
Linear from 20 – 300 pg/mg using 1/x weighting for fentanyl.

c. Ion Suppression / Enhancement:

	At Low Level (250 pg/mg)	At High Level (1750 pg/mg)
6-AM	-23%	+8%
Codeine	-20%	+18%
Fentanyl*	-78%	-72%
Hydrocodone	-28%	-29%
Hydromorphone	-21%	-16%
Meperidine	-59%	-54%
Methadone	-30%	+5%
Morphine	-16%	+50%
Oxycodone	-34%	-19%
Oxymorphone	-26%	-18%
Tramadol	-33%	-28%

\*Ion suppression / enhancement determined at 25 and 175 pg/mg.

d. Limit of Detection (LOD):

The LOD is administratively set at the LOQ.  
For oxymorphone, the LOD is 100 pg/mg.

e. Lower Limit of Quantitation (LLOQ):

The LLOQ is 20 pg/mg for fentanyl.  
The LLOQ is 100 pg/mg for 6-AM, codeine, hydrocodone, hydromorphone, meperidine, methadone, morphine, oxycodone and tramadol.

f. Intermediate Precision:

	at 300 pg/mg	at 1200 pg/mg	at 2400 pg/mg
6-AM	3.6%	4.8%	2.7%
Codeine	5.2%	5.6%	4.1%
Fentanyl*	11%	8.1%	10%
Hydrocodone	4.5%	4.6%	4.3%
Hydromorphone	3.9%	6.2%	4.9%
Meperidine	3.6%	3.6%	4.2%
Methadone	4.5%	3.3%	3.5%
Morphine	3.9%	2.8%	6.2%
Oxycodone	8.0%	7.7%	5.7%
Tramadol	3.5%	4.1%	3.2%

\*Intermediate Precision determined at 30, 120 and 240 pg/mg.

g. Processed Sample Stability:

All analytes are stable in processed sample extracts for at least one week except for methadone. Therefore, if samples are not analyzed within three days of extraction, negative methadone results should be interpreted with care.

h. Recovery:

	At Low Level (250 pg/mg)	At High Level (1750 pg/mg)
6-AM	37%	37%
Codeine	48%	53%
Fentanyl*	15%	15%
Hydrocodone	55%	56%
Hydromorphone	49%	50%
Meperidine	34%	35%
Methadone	10%	9%
Morphine	46%	59%
Oxycodone	53%	59%
Oxymorphone	53%	56%
Tramadol	60%	59%

\*Ion suppression / enhancement determined at 25 and 175 pg/mg.

i. Selectivity:

No known interferences.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Moffat, A.C., *Isolation and Identification of Drugs*, 2nd ed., Pharmaceutical Press: London, 1986.

Edinboro, L. E., Backer, R. C., Poklis, A., "Direct Analysis of Opiates in Urine by Liquid Chromatography-Tandem Mass Spectrometry", *Journal of Analytical Toxicology*, v. 29, pp. 704-

710, 2005.

Al-Asmari, A. I., Anderson, R. A., “Method for Quantification of Opioids and Their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry”, *Journal of Analytical Toxicology*, v. 31, pp. 394-408, 2007.

*FBI Laboratory Safety Manual.*

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit - Toxicology Subunit SOP Manual.

*Instrument Subunit SOP Manual*; FBI Laboratory Chemistry Unit.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit - Toxicology Subunit.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Rev. #	Issue Date	History
0	01/19/12	New document.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Opioids in Hair Procedure for bench use**

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**Appendix 2: Abbreviated version of the Opioids in Hair Instrumental Parameters**

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## **Analysis of Hair Samples for Sympathomimetic Amines**

### **1 Introduction**

Sympathomimetic amines (SMAs) are generally a class of synthetic phenethylamine-derived drugs often generically referred to as “amphetamines”. Almost all of these compounds show some degree of stimulant effects, but a wide variety of additional structure-dependent pharmacological effects can be seen in various compounds. These include pure stimulants (amphetamine and methamphetamine), decongestants (phenylpropanolamine and pseudoephedrine), anorexics (phentermine and fenfluramine), and hallucinogens (mescaline, one of the few relevant naturally occurring SMAs). Over the last few decades there has been particular interest in and concern over the widespread illicit use of various “designer” SMAs with combined stimulant and hallucinogenic properties. The “type specimen” of this class is 3,4-methylenedioxy-methamphetamine (MDMA or “ecstasy”), which was originally developed for possible use as an adjunct drug in psychotherapy, but now one of the most widely used illicit drugs in teenage and young adult populations. Chemists in clandestine drug laboratories have developed a wide array of related compounds, including thioalkyl- and halogen-containing analogues, in attempts to stay ahead of drug scheduling regulations.

### **2 Scope**

This procedure allows for screening, confirmation and quantitation of the following SMAs in hair samples: amphetamine, methamphetamine, ephedrine / pseudoephedrine, methylenedioxyethylamphetamine (MDEA), and methylenedioxymethamphetamine (MDMA). It also allows for the qualitative analysis of hair samples for methylenedioxyamphetamine (MDA).

### **3 Principle**

Hair samples are quantitatively assayed for SMAs. After washing, specimens are dried and cryoground into a powder. The resulting hair powder is mixed with an internal standard (normally a mixture of six deuterated SMAs) and extracted in methanol overnight. The methanol extracts are then taken to dryness and reconstituted in water. The aqueous extracts are adjusted to a basic pH, and extracted with hexane. The hexane is removed, acidified to prevent evaporation of volatile SMAs, and taken to dryness. The resulting residue is reconstituted in 10/90 methanol/water and analyzed by liquid chromatography with high resolution mass spectrometry (LC-FTMS). The extraction procedure is derived from work by Sadeghipour and Veuthey. The chromatographic and mass spectral procedures and parameters were developed in-house.

## 4 Specimens

This procedure uses 50 mg of hair if specimens are analyzed in duplicate.

## 5 Equipment/Materials/Reagents

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. 16x100 mm screw-top tubes with Teflon-lined caps
- b. 12x75 mm culture tubes with polypropylene snap-tops
- c. Acetonitrile (Optima grade or better)
- d. Formic Acid (Puriss grade or better)
- e. Hexane (UV grade or better)
- f. Hydrochloric acid (ACS grade or better)
- g. Methanol (Optima grade or better)
- h. Sodium hydroxide (ACS grade or better)
- i. Water (Deionized and Optima or better grade)
- j. 4% Sodium hydroxide  
Dissolve 2 g sodium hydroxide in 50 mL deionized water. Store in plastic at room temperature. Stable for at least 6 months.
- k. Methanol:Hydrochloric Acid (4:1 v:v)  
Mix 20 mL methanol with 5 mL hydrochloric acid. Store in glass at room temperature. Stable for at least 1 month.
- l. Methanol:Water (10:90 v:v)  
Mix 5 mL methanol with 45 mL water (both Optima grade or better). Store in glass at room temperature. Stable for at least 1 year.

- m. 0.1% Formic acid in acetonitrile  
Vacuum filter 500 mL acetonitrile through a 5  $\mu$ m PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.
- n. 0.1% Formic acid in water  
Vacuum filter 500 mL water (Optima grade or better) through a 5  $\mu$ m PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.
- o. Vortex mixer, Rotator, Centrifuge, Heating Block and Cryogrinder
- p. Evaporator with nitrogen
- q. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
- r. Liquid Chromatograph-Orbitrap Mass Spectrometer
- s. HPLC Column (Alltech Alltima C18, 2.1 x 150 mm, 5  $\mu$ m dp, with a 2.1 x 7.5 mm guard column; or equivalent)
- t. Methylene chloride (HPLC grade)
- u. Disposable magnetic stir bars
- v. Ultrafree-CL centrifuge filters (0.45  $\mu$ m PVDF)

## 6 Standards and Controls

- a. d<sub>3</sub>-Ephedrine Stock Solution (100  $\mu$ g/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- b. d<sub>5</sub>-Amphetamine Stock Solution (100  $\mu$ g/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- c. d<sub>5</sub>-Methamphetamine Stock Solution (100  $\mu$ g/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

- d. d<sub>5</sub>-MDA Stock Solution (100 µg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- e. d<sub>5</sub>-MDMA Stock Solution (100 µg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- f. d<sub>5</sub>-MDEA Stock Solution (100 µg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- g. Internal Standard Working Solution (2 µg/mL of each component):  
Combine 0.5 mL each of the d<sub>3</sub>-ephedrine, d<sub>5</sub>-amphetamine, d<sub>5</sub>-methamphetamine, d<sub>5</sub>-MDA, d<sub>5</sub>-MDMA, and d<sub>5</sub>-MDEA stock solutions in a 25 mL volumetric flask. Add 2 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 2 years.
- h. Hair Internal Standard Working Solution (25 ng/mL of each component):  
Add 625 µL of the Internal Standard Working Solution (2 µg/mL) to a 50 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0°C. Stable for at least 2 years.
- i. Ephedrine Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- j. MBDB (N-methylbenzodioxazolybutanamine, N-methyl-1-(3,4-methylenedioxy-phenyl)-2-butanamine) Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- k. Amine Mixture-6 (250 µg/mL each component):  
A methanol solution containing amphetamine, methamphetamine, phentermine, MDA, MDMA, and MDEA purchased from Cerilliant or another approved vendor. Stability and storage conditions are determined by the manufacturer.
- l. Column Performance Evaluation Mix (1 µg/mL each component):  
Combine 25 µL each of the MBDB and ephedrine stock solutions with 100 µL of the Amine Mixture-6 in a 25 mL volumetric flask. Add 2.4 mL methanol and bring to the mark with water (both Optima grade or better). Stable for at least 2 years. A 10 µL portion of

this solution is analyzed before each day's samples, in order to confirm acceptable instrument performance.

- m. Amphetamine Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- n. Methamphetamine Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- o. MDA Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- p. MDMA Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- q. MDEA Stock Solution (1 mg/mL):  
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.
- r. Control Working Solution (1 µg/mL each component):  
Mix 50 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 7 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 1 year.
- s. Hair Control Working Solution (125 ng/mL each component):  
Add 1.25 mL of the Control Working Solution (1 µg/mL) to a 10 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0°C. Stable for at least 1 year.
- t. Calibration Working Solution #1 (5 µg/mL each component):  
Mix 250 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 8.5 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 1 year.

- u. Calibration Working Solution #2 (0.5 µg/mL each component):  
Mix 25 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 9.9 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0°C. Stable for at least 1 year.
- v. Hair Calibration Working Solution #3 (125 ng/mL each component):  
Add 625 µL of the Calibration Working Solution #1 (5 µg/mL) to a 25 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0°C. Stable for at least 1 year.
- w. Hair Calibration Working Solution #4 (12.5 ng/mL each component):  
Add 625 µL of the Calibration Working Solution #2 (0.5 µg/mL) to a 25 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0°C. Stable for at least 1 year.
- x. Negative Control Hair:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Clinica, or prepared in-house from an appropriate blank specimen. Hair will be stored at room temperature, and does not expire. A Negative Control hair sample will be extracted and analyzed with every assay.
- y. Positive Control Hair:  
This is normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as needed. When prepared in house, it will be made fresh. Normally prepared at concentrations of 50 and 500 pg/mg by adding 10 and 100 µL of the Hair Control Working Solution to 25 mg of Negative Control Hair. Other levels may be used as circumstances dictate.

## 7 Calibration

This procedure may be used quantitatively via construction of a multi-point calibration curve for the analyte(s) of interest following the *Guideline for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows typical calibrators and preparation instructions for hair calibrators.

Table 1: Hair Calibrator Preparation

Cal Level (pg/mg)	Hair Amount (mg)	Hair Calibrator Working Solution #3 Volume (µL)	Hair Calibrator Working Solution #4 Volume (µL)
25	25	0	50
50	25	0	100
75	25	0	150
175	25	35	0
300	25	60	0
500	25	100	0
750	25	150	0
1000	25	200	0

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### *Preparation of Hair Samples:*

- Visually inspect hair and record observations.
- If segmental analysis is required, cut a portion of the hair sample into 2-cm segments.
- Accurately weigh 25-100 mg of each hair sample into a properly labeled test tube (to the nearest 0.1 mg).
- Wash each hair sample with 1.5 mL methanol by vortexing for approximately 1 minute. Discard this wash.
- Wash each hair sample with 1.5 mL methylene chloride by vortexing the sample for approximately 1 minute. Discard this wash.
- Wash each hair sample with 1.5 mL methanol by vortexing for approximately 1 minute. Save this final wash for later analysis, if necessary. Control washes need not be saved.



- g. Dry hair samples in a heating block at approximately 40°C to evaporate any remaining solvent.
- h. Cryogrind dry hair samples in the freezer mill using the settings in Section 10.3 of this procedure.
- i. Accurately weigh 25 mg of hair powder to a small vial (to the nearest 0.1 mg). Samples will be prepared in duplicate if specimen size allows. Smaller amounts may be weighed to account for high concentrations of analyte and/or limited specimen amount.
- j. Add a magnetic stir bar, 1.5 mL methanol, and 50 µL Hair Internal Standard Working Solution (25 ng/mL) to each vial.
- k. Extract overnight (at least 12 hours) with stirring at 37°C.
- l. Filter the methanol extract using an Ultrafree-CL 0.45 µm centrifuge filter by spinning at 3000 rpm for 5 minutes. Discard the stir bar and hair.
- m. Add 0.1 mL of 4:1 methanol:hydrochloric acid to filtrate and vortex briefly.
- n. Evaporate to dryness under a gentle stream of nitrogen at approximately 40°C.
- o. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.

*For Hair Extracts:*

- a. To a properly labeled 16x100 mm screw-top tube add 0.5 mL of water based hair extract.
- b. Add 0.2 mL of 4% sodium hydroxide to each sample and vortex briefly.
- c. Add 2 mL of hexane to each tube and extract for 20 minutes on a rotator. Centrifuge 10 minutes at a minimum of 3000 rpm. Use a wooden stick to break up any emulsions that develop.
- d. Transfer organic (top) layer to a 12x75 mm culture tube.
- e. Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.
- f. Evaporate the hexane to dryness under a gentle stream of nitrogen at approximately 40°C.
- g. Reconstitute the dried residue in 0.1 mL of 10:90 methanol:water.

- h. Analyze by LC-FTMS using the conditions given below (Sections 10.1 and 10.2).

*For Wash Samples:*

- a. For samples in which an SMA is identified above the LLOQ of the method, add 25  $\mu$ L Hair Internal Standard Working Solution (25 ng/mL) to each wash.
- b. Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.
- c. Evaporate to dryness under a gentle stream of nitrogen at approximately 40°C.
- d. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.
- e. Add 0.2 mL of 4% sodium hydroxide to each sample and vortex briefly.
- f. Add 2 mL of hexane to each tube and extract for 20 minutes on a rotator. Centrifuge 10 minutes at a minimum of 3000 rpm. Use a wooden stick to break up any emulsions that develop.
- g. Transfer organic (top) layer to a 12x75 mm culture tube.
- h. Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.
- i. Evaporate the hexane to dryness under a gentle stream of nitrogen at approximately 40°C.
- j. Reconstitute the dried residue in 0.1 mL of 10:90 methanol:water.
- k. Analyze by LC-FTMS using the conditions given below (Sections 10.1 and 10.2).

## **10 Instrumental Conditions**

Appendix 2 contains a checklist of method parameters that should be used to verify proper instrumental conditions prior to analysis of case samples.

### 10.1 Liquid Chromatograph Parameters (Shimadzu Prominence, or equivalent)

Mobile Phase Compositions	Flow Parameters			Column Parameters	
B: 0.1% formic acid in acetonitrile	total flow	0.3 mL/min		type	C18
	time (min)	%B	%C	length	15 cm
C: 0.1% formic acid in water	0	7.5	92.5	internal diameter	2.1 mm
	5	7.5	92.5	particle size	5 μm
	20	60	40	temperature	40°C
	23	60	40	guard length	7.5 mm
	28	7.5	92.5	guard ID	2.1 mm
	32	7.5	92.5		
	total time		32 min		

### 10.2 Mass Spectrometer Parameters Using FTMS (Thermo Orbitrap, or equivalent)

Source Parameters		
Mode: Electrospray	Spray Voltage: +5 kV	Capillary Temperature: 250°C
Sheath Gas: 25 (arb units)	Aux Gas: 10 (arb units)	Sweep Gas: 0 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Scan Range	100-350 m/z	
Resolution	30000	

### 10.3 Cryogrinder (Freezer/Mill) Parameters

Cycles	1
Precool	9 min
Run time	8 min
Cool time	1 min
Rate	10 cps

## 11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control.

## 11.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### 11.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, calibrator, or Positive Control.

### 11.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

## 11.2 Mass Spectrometry

The M+1 for the compound of interest should agree with the theoretical exact mass within 0.003 amu. See Table 4 below for theoretical exact masses.

Table 4: Theoretical Exact Masses (M+1)

Compound Name	Exact Mass (M+1)
Amphetamine	136.112
Methamphetamine	150.128
Ephedrine	166.123
MDA	180.102
MDMA	194.118
MDEA	208.133

## 11.3 Wash Decision Criteria

If the final wash contains greater than one tenth the amount of an SMA in a given hair sample, the sample's exterior is considered to be possibly contaminated, and will be reported as such.

## 12 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

For wash calculations, the total amount of an SMA may be calculated against a new curve, or against the hair curve for that sample.

Example calculations for wash decision criteria:

Assume 25.0 mg of sample Q1 are washed per the SOP, and that the sample is determined to contain 500 pg methamphetamine per milliliter of hair. Then, the hair sample contained 12500 pg total of methamphetamine (25 x 500). The final wash must contain more than 1250 pg methamphetamine (12500/10) for the Q1 sample to be reported as possibly contaminated.

## 13 Uncertainty of Measurement

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the balance used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 14 Limitations

### a. Method Performance Parameters:

Compound	LOD (pg/mg)	LLOQ (pg/mg)	Linear Range (pg/mg)	Accuracy (% bias at low and high controls)	Precision (% intermediate at low and high controls)
Amphetamine	21	25	25-1000	+3.81, +22.61	17.63, 17.54
Methamphetamine	14	25	25-1000	+1.72, +6.48	7.77, 8.79
(pseudo)Ephedrine	25	25	25-1000	-0.52, +9.99	11.76, 14.85
MDMA	14	25	25-1000	+5.66, +10.78	7.12, 5.39
MDEA	8	25	25-1000	-2.74, +5.33	9.14, 5.32
MDA	25	N/A; Qual Only			

### b. Interferences: None known.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

Sadeghipour, F. and Veuthey, J., *Journal of Chromatography A*, v. 787 (1997), pp. 137-143

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual.*

Rev.#	Issue Date	History
0	03/08/12	New document. SMA hair analysis was pulled out of Tox 420 into this free standing document.

**Approval**

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**Appendix 1: Abbreviated version of the SMA procedure for bench use. (Page 1 of 2)**

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**Appendix 1: Abbreviated version of the SMA procedure for bench use. (Page 2 of 2)**

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**Appendix 2: Instrumentation parameters checklist for the SMA procedure.**

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## Synthetic Cannabinoids from Blood

### 1 Introduction

Synthetic cannabinoids began appearing widely in the United States in 2010. This group of compounds has been termed synthetic cannabinoids because they bind to CB<sub>1</sub> and CB<sub>2</sub> receptors in the brain. The synthetic cannabinoids are typically sprayed onto herbs which are sold as incense. The incense is then smoked. Common street names for these compounds include Spice and K2. Effects of the synthetic cannabinoids mimic those of marijuana, and include euphoria, incoordination and hallucinations. In the spring of 2011, the DEA controlled 5 synthetic cannabinoids as schedule I controlled substances. These compounds are: CP 47, 497; CP 47, 497-C8; JWH-018; JWH-073 and JWH-200. HU-210 is considered controlled by the DEA because of its structural similarity to  $\Delta^9$ -THC.

### 2 Scope

This procedure allows for the identification of sixteen synthetic cannabinoids in blood samples. (JWH-015, JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210, JWH-250, JWH-251, JWH-398, RCS-4, RCS-8, AM-694, AM-2201 and HU210/211)

### 3 Principle

Blood samples are extracted via liquid/liquid extraction. Extracts are taken to dryness, reconstituted and analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) using high resolution and data dependent scanning.

### 4 Specimens

This procedure uses 1-mL of blood for a screen, and a second 1-mL portion of blood for confirmatory analysis.

### 5 Equipment/Materials/Reagents

- a. Volumetric glassware
- b. Adjustable Pipettes with disposable tips
- c. Centrifuge

- d. Vortex Mixer
- e. Rotating Mixer
- f. Evaporator with nitrogen
- g. Disposable glassware including culture tubes, screw-cap test tubes and autosampler vials
- h. Liquid chromatograph/mass spectrometer (capable of high resolution MS and data dependent MS/MS)
- i. Waters Xterra C18 MS LC Column: 150 x 3.0 mm, 3.5  $\mu$ m d<sub>p</sub> (or equivalent) with a 2.1 x 7.5 mm C18 guard column (or equivalent)
- j. Acetonitrile (Optima Grade)
- k. Water (Optima Grade)
- l. Formic Acid (98%, or better)
- m. Diethyl Ether (High purity grade)
- n. 200 Proof Ethanol sparged with nitrogen for at least 15 minutes
- o. Sodium Carbonate (98%, or better)
- p. Sodium Bicarbonate (98%, or better)
- q. Sodium Sulfate (98%, or better)
- r. 3.0 M Sodium Carbonate Buffer, pH 10:  
Combine 23.8 g sodium carbonate and 18.9 g sodium bicarbonate in a graduated cylinder and bring to the 150-mL mark with deionized water. Mix well. Store in glass or plastic at room temperature. Stable for at least two months.
- s. LC Mobile Phase 1 (0.1% Formic Acid in Water):  
Add 0.5 mL formic acid to 500 mL water (Optima grade or better). Store in glass at room temperature. Stable for 2 months.
- t. LC Mobile Phase 2 (0.1% Formic Acid in Acetonitrile):  
Add 0.5 mL formic acid to 500 mL. Store in glass at room temperature. Stable for 2 months.

## 6 Standards and Controls

- a. The following are purchased from Cerilliant or another approved vendor as 0.1 mg/mL solutions. Storage conditions and stability are determined by the manufacturer.
- JWH-018
  - HU-210
- b. The following are purchased from Cayman Chemical or another approved vendor as 5.0 mg samples. They are diluted with 1 mL of sparged ethanol to yield 5.0 mg/mL solutions. Diluted standards are stored below 0°C in glass and are stable for at least 2 years.
- JWH-015
  - JWH-019
  - JWH-073
  - JWH-081
  - JWH-200
  - JWH-250
  - JWH-251
  - JWH-122
  - AM-694
  - AM-2201
- c. The following are purchased from Cayman Chemical or another approved vendor as 1.0 mg samples. They are diluted with 1 mL of sparged ethanol to yield 1.0 mg/mL solutions. Diluted standards are stored below 0°C in glass and are stable for at least 2 years.
- JWH-398
  - RCS-4
  - RCS-8
  - JWH-210
- d. Positive Control Working Solution and Column Check Mix (1 µg/mL each HU-210, JWH-018, JWH-073 and JWH-200):  
Combine 0.25 mL each of the JWH-018 and HU-210 solutions (0.1 mg/mL) in a 25 mL volumetric flask. Add 0.005 mL each of the JWH-073 and JWH-200 solutions (5 mg/mL). Bring to the mark with sparged ethanol. Store below 0°C in glass. Stable for at least 2 years.
- e. JWH-018-d<sub>9</sub> Solution (5.0 mg/mL):  
Purchased from Cayman Chemical or another approved vendor. Storage and stability determined by the manufacturer.

- f. Internal Standard Working Solution (1  $\mu\text{g/mL}$ ):  
Add 0.010 mL of the JWH-018- $\text{d}_9$  Solution (5.0 mg/mL) to a 50 mL volumetric flask. Bring to the mark with sparged ethanol. Store below 0°C in glass. Stable for at least 2 years.
- g. Negative Control Blood:  
Purchase from Cliniqa or another approved vendor. Storage conditions and stability determined by manufacturer. Alternatively, obtain in house and store refrigerated or frozen for at least 2 years.
- h. Positive Control Blood (25 ng/mL HU-210, JWH-018, JWH-073 and JWH-200):  
Prepare on the day of extraction by adding 0.025 mL of the Positive Control Working Solution (1  $\mu\text{g/mL}$ ) to 1 mL of Negative Control Blood.  
Note: Positive Controls may be prepared for other target compounds if suspected or requested in a particular analysis.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Add 1 mL of control or questioned blood specimen to properly labeled screw cap test tubes.
- b. Add 25  $\mu\text{L}$  of the Internal Standard Working Solution (1.0  $\mu\text{g/mL}$  JWH-018- $\text{d}_9$ ).
- c. Add 1 mL of 3.0 M Carbonate Buffer to each tube. Vortex.
- d. Add 5 mL of diethyl ether to each tube.
- e. Extract via rotation for 20 minutes.
- f. Centrifuge at ~3000 rpm for at least 15 minutes.
- g. Transfer diethyl ether layer to a clean test tube.
- h. Evaporate to dryness at  $\leq 40^\circ\text{C}$ .

- i. Reconstitute with 100  $\mu$ L of sparged ethanol. Vortex.
- j. Verify that the instrument is operating properly by analyzing 10  $\mu$ L of the column testmix using the instrumental conditions that follow. Analyze 10  $\mu$ L of each extract by LC/MS/MS(ESI).

## 9 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Parameters			
initial composition	92.5% LC mobile phase #1	7.5% LC mobile phase #2	
initial hold	5 minutes		
composition at 20 minutes	40% LC mobile phase #1	60% LC mobile phase #2	
composition at 30 minutes	30% LC mobile phase #1	70% LC mobile phase #2	
hold	5 minutes		
composition at 40 minutes	10% LC mobile phase #1	90% LC mobile phase #2	
hold	10 minutes		
composition at 51 minutes	92.5 % mobile phase #1	7.5% mobile phase #2	
final hold	10 minutes	flowrate	0.3 mL/min
column temperature	35° C	runtime	60 minutes

Column Parameters	
type	Waters Xterra C18 MS, or equivalent
length	15 cm
internal diameter	3.0 mm
particle size	3.5 $\mu$ m
guard column	C18 (2.1 x 7.5 mm)



## 9.2 Mass Spectral Parameters – LC/MS/MS

Scan Event #1		Scan Event #2	
ionization mode	electrospray (+)	ionization mode	electrospray (+)
scan mode	FTMS res=7500 centroid	scan mode	FTMS res=7500 centroid MS/MS DDS
scan range	250 – 650 m/z	precursor ions Note: others may be included, but these are required.	most intense of m/z 320.20, 322.18, 328.17, 336.20, 342.19, 356.20, 360.178, 370.21, 372.20, 376.15, 376.23, 385.19, 387.29, 436.05
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.		reject mass (optional)	351.24
		isolation width	2.0
		collision energy	35%
		scan range	software control

## 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control.

### 10.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or an extracted Positive Control.

### 10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

## 10.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted positive control within a reasonable degree of scientific certainty. Exact mass and product ions for each analyte are listed in Table 1 below. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

**Table 1: Target Analytes**

Compound	Empirical Formula	Exact Mass (M+1 adduct)	Product Ions	Approx RT (minutes)
JWH-015	C <sub>23</sub> H <sub>21</sub> NO	328.1695	155, 200	33.11
JWH-018	C <sub>24</sub> H <sub>23</sub> NO	342.1852	155, 214	38.82
JWH-019	C <sub>25</sub> H <sub>25</sub> NO	356.2008	155, 228	41.83
JWH-073	C <sub>23</sub> H <sub>21</sub> NO	328.1695	155, 200	35.13
JWH-081	C <sub>25</sub> H <sub>25</sub> NO <sub>2</sub>	372.1958	185, 214	40.12
JWH-122	C <sub>25</sub> H <sub>25</sub> NO	356.2008	169, 214	41.40
JWH-200	C <sub>25</sub> H <sub>24</sub> N <sub>2</sub> O <sub>2</sub>	385.1910	155, 298	18.65
JWH-210	C <sub>26</sub> H <sub>27</sub> NO	370.2165	183, 214	43.09
JWH-250	C <sub>22</sub> H <sub>25</sub> NO <sub>2</sub>	336.1958	121, 188	34.33
JWH-251	C <sub>22</sub> H <sub>25</sub> NO	320.2008	105, 188, 214	36.58
JWH-398	C <sub>24</sub> H <sub>22</sub> ClNO	376.1462	189, 214	43.02
RCS-4	C <sub>21</sub> H <sub>23</sub> NO <sub>2</sub>	322.1801	135, 214	33.26
RCS-8	C <sub>25</sub> H <sub>29</sub> NO <sub>2</sub>	376.2271	121, 228	41.66
AM-694	C <sub>20</sub> H <sub>19</sub> FINO	436.0568	230, 309	30.10
AM-2201	C <sub>24</sub> H <sub>22</sub> FNO	360.1758	155, 232	31.52
HU-210	C <sub>25</sub> H <sub>38</sub> O <sub>3</sub>	387.2893	201, 243, 261	41.37
JWH-073 methyl butyl analog*	C <sub>24</sub> H <sub>23</sub> NO	342.1852	155, 214	38.38
JWH-018-d <sub>9</sub>	C <sub>24</sub> H <sub>15</sub> d <sub>9</sub> NO	351.2417		40.21

\*This is not a target analyte, but is listed as an isomer of JWH-018.

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

- a. Limits of Detection: 5 ng/mL for HU210/211; 1 ng/mL for all other target analytes.
- b. Interferences: HU210 and HU211 cannot be differentiated by this method.
- c. JWH-018 and JWH-073 3-methyl butyl analog have the same accurate mass and tandem mass spectra, and a retention time that differs by only 20 seconds. Care should be taken when identifying these analytes.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual.*

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Rev. #	Issue Date	History
0	04/06/12	New document.
1	06/13/12	Added Table 1 to Section 11.2. Added 14 c.
2	10/01/14	Clarified wording in Section 4. In Sections 5 and 15, removed references to Tox 103. In Section 5.1, clarified grade of formic acid. In 6.d and 8.j, specified a column check mix to verify instrumental performance before analysis. Deleted Section 7 and renumbered remaining sections. Changed title of newly numbered Section 12. Corrected a typo in Table 1. Separated instrumental conditions into a separate sheet (Appendix 2).

Approval

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Synthetic Cannabinoids from Blood Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Synthetic Cannabinoids from Blood Instrumental  
Conditions for bench use.**

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## Analysis of Cannabinoids from Biological Specimens by LC/MS/MS

### 1 Introduction

Marijuana, obtained from the *Cannabis sativa* plant, is a commonly abused illicit drug. It is typically dried and smoked.  $\Delta^9$ -Tetrahydrocannabinol (THC) is the primary psychoactive component of marijuana. 11-hydroxy-  $\Delta^9$ -tetrahydrocannabinol (THC-OH) is a major active metabolite of THC. 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THC-COOH) is a major inactive metabolite of THC.

### 2 Scope

This procedure is used to screen, confirm and quantitate THC and its primary metabolites in blood specimens. It can also be used to qualitatively screen and identify THC-COOH in urine specimens. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

Biological specimens are commonly assayed for the presence of THC, THC-OH, and THC-COOH. Specimens are mixed with an internal standard solution containing the deuterated analogs of the analytes of interest. Blood specimens are prepared via protein precipitation using acetonitrile. Urine specimens are prepared by hydrolysis with a strong base, followed by neutralization with acid. Prepared samples are extracted using solid phase extraction (SPE). Extracts are analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) in the multiple reaction mode (MRM).

### 4 Specimens

This procedure uses a biological fluid such as: blood, serum, plasma, or urine. Typically, 0.5 mL of blood or urine is used. Dilution of samples due to limited specimen volume or suspicion of high drug and metabolite concentrations is acceptable.

### 5 Equipment/Materials/Reagents

- a. Disposable test tubes (silanized glass)
- b. Calibrated pipettors with disposable tips



- c. Vortexer
- d. Heating block
- e. Heated evaporator with nitrogen
- f. Glacial acetic acid (ACS grade)
- g. Sodium acetate trihydrate (reagent grade)
- h. 0.1 M Sodium Acetate Buffer (pH 7.0): To a 250-mL volumetric flask, add 3.4 g sodium acetate trihydrate and 200 mL deionized water. Mix well and adjust to  $6.5 < \text{pH} < 7.5$  by slow addition of 1 N hydrochloric acid. Bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.
- i. Potassium hydroxide (reagent grade)
- j. 11.8 N Potassium Hydroxide (Hydrolysis Reagent) (KOH): To a 100-mL Nalgene volumetric flask add 66 g potassium hydroxide and 50 mL deionized water. Mix well to dissolve and bring to volume with deionized water. Store at room temperature in Nalgene container. Stable 1 year.
- k. Centrifuge
- l. Acetonitrile (Optima grade)
- m. Autosampler vials
- n. CEREX PolyChrom THC solid phase extraction columns (3 or 6 cc)
- o. Ammonium Hydroxide (Reagent grade)
- p. Water:Acetonitrile:Ammonia (90:10:1): Combine 90 mL deionized water, 10 mL acetonitrile and 1 mL ammonium hydroxide and mix well. Prepare fresh.
- q. Hexane (HPLC grade)
- r. Ethyl Acetate (Optima grade)
- s. Acetic Acid (ACS grade)
- t. Hexane:Ethyl Acetate:Acetic Acid (88:10:2): Combine 88 mL hexane, 10 mL ethyl acetate and 2 mL acetic acid and mix well. Prepare fresh.

- u. LC Mobile Phase A (0.1% Formic Acid in Water): Combine 500 mL deionized water and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two weeks.
- v. LC Mobile Phase B (0.1% Formic Acid in Acetonitrile): Combine 500 mL acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two weeks.
- w. ABI 5000 QTRAP Mass Spectrometer equipped with a Shimadzu LC System
- x. Xterra MS C18 (5cm x 3mm x 5 $\mu$ m) analytical column
- y. Positive pressure solid phase extraction manifold
- z. Water (deionized and Optima grade)
- aa. Methanol (Optima grade)
- bb. Formic Acid (Optima LC/MS grade)

## 6 Standards and Controls

- a. d<sub>3</sub>-THC Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- b. d<sub>3</sub>-THC-OH Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- c. d<sub>3</sub>-THC-COOH Stock Standard (0.1 mg/mL):  
Purchased from Cerilliant International. Storage conditions and stability determined by manufacturer.
- d. THC Internal Standard (IS) Working Solution (d<sub>3</sub>-THC, d<sub>3</sub>-THC-OH and d<sub>3</sub>-THC-COOH – 0.25/1.25  $\mu$ g/mL): In a 10-mL volumetric flask, combine 0.025 mL each of the d<sub>3</sub>-THC and d<sub>3</sub>-THC-OH stock standards. Add 0.125 mL of the d<sub>3</sub>-THC-COOH stock standard. Dilute to the mark with methanol. Mix well. Store below 0°C. Stable for at least 1 year.
- e. THC Stock Standard (1.0 mg/mL):  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.

- f. THC-OH Working Solution (Soln) (0.1 mg/mL):  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- g. THC-COOH Working Soln (0.1 mg/mL):  
Purchased from Cerilliant International and Lipomed. Storage conditions and stability determined by manufacturer.
- h. THC Working Soln (0.1 mg/mL):  
Combine 0.1 mL of the THC Stock Standard and 0.9 mL methanol and mix well. Store refrigerated in a crimped vial. Prepare fresh.
- i. Cannabinoid High Calibrator (Cal) Soln (0.25/1.25 µg/mL):  
To a 10-mL volumetric flask, add 25 µL the THC and THC-OH Cerilliant Working Solutions and 125 µL of the Cerilliant THC-COOH Working Solution. Dilute to the mark with acetonitrile. Store below 0°C in glass. Stable for at least six months.
- j. Cannabinoid Low Cal Soln (0.025/0.125 µg/mL):  
To a 10-mL volumetric flask, add 1.0 mL of the Cannabinoid High Calibrator Solution. Dilute to the mark with acetonitrile. Store below 0°C in glass. Stable for at least six months.
- k. Cannabinoid High Control (Ctl) Soln (0.5/2.5 µg/mL):  
To a 10-mL volumetric flask, add 50 µL the THC and THC-OH Lipomed Working Solutions and 250 µL of the Lipomed THC-COOH Working Solution. Dilute to the mark with acetonitrile. Store below 0°C in glass. Stable for at least six months.
- l. Cannabinoid Low Ctl Soln (0.05/0.25 µg/mL):  
To a 10-mL volumetric flask, add 1.0 mL of the Cannabinoid High Control Solution. Dilute to the mark with acetonitrile. Store below 0°C in glass. Stable for at least six months.
- m. Negative Control Urine:  
Synthetic urine (Surine) may be purchased from Dyna-Tek, Inc., Lenexa, KS; alternatively, blank urine may be obtained in-house. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control Urine Sample is extracted and analyzed with every urine assay.
- n. Negative Control Blood:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or prepared in-house from an appropriate blank specimen. Store frozen, refrigerated or obtain fresh. Stability determined by manufacturer. In-house Negative Control Blood is stable for at least 2 years when frozen. A Negative Control Blood sample is extracted and analyzed with every blood assay.

- o. System Suitability Mixture (0.05/0.25 µg/mL):  
Mix 0.1 mL of the High Ctl Solution in an autosampler vial with 0.45 mL water and 0.45 mL acetonitrile. Store refrigerated or in cooled autosampler tray. Stable for at least one week.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the chemist performing the procedure.

- a. Add 0.5 mL of blood and/or urine to a labeled test tube. For quantitation, case samples and positive control are typically analyzed in duplicate.
- b. For blood quantitation, prepare calibration samples in labeled test tubes:  
Calibrator 1/5 ng/mL: Add 20 µL Low Cal Solution to 0.5 mL Negative Control blood.  
Calibrator 2/10 ng/mL: Add 40 µL Low Cal Solution to 0.5 mL Negative Control blood.  
Calibrator 5/25 ng/mL: Add 100 µL Low Cal Solution to 0.5 mL Negative Control blood.  
Calibrator 10/50 ng/mL: Add 20 µL High Cal Solution to 0.5 mL Negative Control blood.  
Calibrator 20/100 ng/mL: Add 40 µL High Cal Solution to 0.5 mL Negative Control blood.  
Calibrator 40/200 ng/mL: Add 80 µL High Cal Solution to 0.5 mL Negative Control blood.
- c. Prepare positive control blood samples:  
Low Ctl (3/15 ng/mL): Add 30 µL Low Ctl Solution to 0.5 mL Negative Control blood.  
High Ctl (33/165 ng/mL): Add 33 µL High Ctl Solution to 0.5 mL Negative Control blood.
- d.. Prepare positive control urine samples:  
Low Control (20 ng/mL): Add 40 µL Low Ctl Solution to 0.5 mL Negative Control Urine.  
High Control (200 ng/mL): Add 40 µL High Ctl Solution to 0.5 mL Negative Control Urine.
- e.. Add 20 µL of the THC IS Working Solution to each sample, vortex and allow samples to

stand for 15 minutes.

- f. *For blood samples:* Add 2.0 mL cold acetonitrile drop-wise to each blood sample while vortexing. Vortex for ~30s and centrifuge at approximately 3000 rpm for 3 minutes. Remove the supernatant and transfer to a labeled test tube. Add 4 mL deionized water and vortex.

*For urine samples:* Add 0.075 mL 11.8 N potassium hydroxide to each urine sample. Place on a heating block at approximately 60°C for approximately 15 minutes. Remove from heating block and cool for approximately 5 minutes. Add 0.075 mL glacial acetic acid and vortex. Add 5.0 mL 0.1 M sodium acetate buffer and vortex. Verify pH to be 4.5 - 6.5. If not, adjust with more glacial acetic acid or 11.8 N potassium hydroxide.

- g. Load samples onto CEREX THC solid phase extraction columns and push through with positive pressure.
- h. Wash each column with 1.0 mL Water:Acetonitrile:Ammonia (90:10:1).
- i. Dry each column for 15 minutes under full pressure.
- j. Elute with 2.0 mL Ethyl Acetate by gravity.
- k. Dry each column for 10 minutes at 40°C.
- l. Elute with 2.0 mL Hexane:Ethyl Acetate:Acetic Acid (88:10:2) by gravity into the same tube as above.
- m. Evaporate to dryness at 40°C.
- n.. To the evaporated sample, add 0.050 mL acetonitrile, vortex, and transfer to labeled autosampler vial.
- o. To the same evaporated sample, add 0.050 mL water, vortex and transfer to the same autosampler vial.
- p. Add 0.050 mL deionized water to each sample before analysis by LC/MS/MS.
- q. Verify that instrument is operating properly by analyzing the System Suitability Mixture and verifying that all three analytes are present.

## 9 Instrumental Conditions

Following are the operating parameters for the instruments used in this procedure. Appendix 2

contains an abbreviated version of instrumental parameters used in this procedure. This checklist may be used by the examiner or chemist performing the procedure.

### 9.1 Autosampler Procedures

Autosampler Temperature Setting: 15°C

Injection Volume: 10 µL

### 9.2 Liquid Chromatograph Parameters (25°C)

Time (min)	Total Flow (mL/min)	%A (0.1% Formic Acid in Water)	%B (0.1% Formic Acid in Acetonitrile)
0.01	0.5	60	40
7.00	0.5	0	100
10.00	0.5	0	100
12.00	0.5	60	40

### 9.3 Mass Spectrometer Parameters

Scan Mode	Turbo Spray	Polarity	Positive
Resolution	Unit	Scan Type	MRM
Curtain Gas	Nitrogen (35)	Ionspray Voltage	4000
Source Temperature	670°C	Nebulizer Gas	Nitrogen (50)
Entrance Potential	10	Turbo Gas	Nitrogen (50)

Q1 Mass	Q3 Mass	Dwell Time (ms)	Declustering Potential	Collision Energy	Collision Exit Potential
315.30 <sup>a</sup>	193.20 <sup>a</sup>	25	101	33	20
315.30	123.10	25	101	47	18
315.30	259.20	25	101	29	26
315.30	135.10	25	101	29	16
318.339 <sup>a</sup>	196.153 <sup>a</sup>	25	76	33	18
318.339	123.017	25	76	47	20
331.305 <sup>a</sup>	193.10 <sup>a</sup>	25	81	35	20
331.305	313.30	25	81	21	34
331.305	201.052	25	81	33	14
331.305	105.052	25	81	49	14
334.333 <sup>a</sup>	196.140 <sup>a</sup>	25	91	37	20
334.333	316.225	25	91	35	32
345.297 <sup>a,b</sup>	299.289 <sup>a</sup>	25	116	21	24
345.297	193.103	25	116	37	20
345.297	327.249	25	116	23	34

Q1 Mass	Q3 Mass	Dwell Time (ms)	Declustering Potential	Collision Energy	Collision Exit Potential
345.297	119.144	25	116	37	12
348.313 <sup>a,b</sup>	196.115 <sup>a</sup>	25	101	37	20
348.313	302.268	25	101	29	32

<sup>a</sup>This is the typical quant transition referred to in Section 11 below.

<sup>b</sup>Only the 345 and 348 transitions are routinely collected for the analysis of urine samples.

## 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, all of the below should be met in order to identify THC or related compounds within a biological specimen:

### 10.1 Chromatography

All four ion transition peaks for the analyte of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. For low concentrations of analyte (less than 5 ng/mL), there may only be three strong transitions. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

#### 10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

### 10.2 Mass Spectrometry

Four independent MS/MS experiments are conducted for each analyte. (See Table 2 below.) At least two ion ratios are calculated for each analyte; the ion ratios should compare favorably to ions ratios from a reference standard or an extracted positive control. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

**Table 2: MS/MS Transitions**

Analyte	Tran 1	Tran 2	Tran 3	Tran 4
THC	315.3→193.2	315.3→123.1	315.3→259.2	315.3→135.1
THC-OH	331.30→193.1	331.30→313.3	331.30→201.2	331.30→105.1
THC-COOH	345.297→299.4	345.297→193.4	345.297→119.2	345.297→327.2

## 11 Calculations

The ratio of one transition for each analyte to its corresponding internal standard should be graphed against concentration as a  $1/x^2$  weighted linear equation. Typical quantitative transitions for THC-d<sub>3</sub>, THC, THC-OH-d<sub>3</sub>, THC-OH, THC-COOH-d<sub>3</sub>, and THC-COOH are noted in Sections 9.3 and 10.2 above.

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices for calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.



### 13 Limitations

	THC	THC-COOH		THC-OH
	Blood	Blood	Urine	Blood
<b>Limits of Detection (ng/mL):</b>	0.5	2.5	5	1.0

	THC Blood	THC-COOH Blood	THC-OH Blood
<b>Linear Range (ng/mL):</b>	1-40	5-200	1-40
<b>Bias (at Low Conc):</b>	-4.11%	-6.32%	5.14%
<b>Bias (at Medium Conc):</b>	-5.28%	-5.37%	4.58%
<b>Bias (at High Conc):</b>	-5.17%	-6.98%	5.95%
<b>Repeatability (at Low Conc):</b>	7.05%	6.93%	7.21%
<b>Repeatability (at Medium Conc):</b>	5.30%	5.33%	3.70%
<b>Repeatability (at High Conc):</b>	3.91%	3.64%	4.02%
<b>Intermediate Precision (at Low Conc):</b>	7.54%	7.91%	8.31%
<b>Intermediate Precision (at Medium Conc):</b>	7.78%	7.22%	5.38%
<b>Intermediate Precision (at High Conc):</b>	5.42%	6.30%	6.48%

### 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

### 15 References

*FBI Laboratory Safety Manual.*

*Guidelines for Toxicological Quantitations (Tox 101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.*

*Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.*

*Chemistry Unit Procedures for Estimating Measurement Uncertainty (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.*

Rev. #	Issue Date	History
4	04/01/19	Updated scope statement (2). Removed 'subunit' (header, Section 15). Updated CUQA-13 title (Sections 12, 15). Added vortex time specificity and equilibrium time (8-f, Appendix). Removed evaporation step, and clarified procedure. (8-g, Appendix). 5-a: specified silanized glassware. Added 5-z (water), 5-aa (methanol), and formic acid (5-bb). Specified Optima grade for 5-r. In 8-d, removed second chemist requirement. Updated mass table (Section 9.3). Made several wording changes to account for qualitative use.
5	08/05/19	Updated scope statement, procedure and bench notes to allow SOP to be also used for screening and blood controls as single aliquots for screening. Removed immunoassay screen from specimen section.

**Approval**

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Acting Toxicology  
Technical Leader:

Date: 08/05/2019

Chemistry Unit Chief:

Date: 08/05/2019

**QA Approval**

Quality Manager:

Date: 08/05/2019

**Appendix 1: Abbreviated version of the Cannabinoid Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.**

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## **Analysis of Salicylic Acid and/or Acetaminophen in Blood by LC/MS**

### **1 Introduction**

Aspirin, or acetylsalicylic acid, is one of the most common over-the-counter drugs that is taken for its analgesic, anti-inflammatory, antipyretic and/or anticoagulant effects. Acetylsalicylic acid is rapidly metabolized in the body to salicylic acid, with a half-life of ~15 minutes. Blood concentrations of less than 100 micrograms per milliliter ( $\mu\text{g/mL}$ ) of salicylic acid are usually considered to be therapeutic. In cases of potential aspirin overdose, blood concentrations of salicylic acid may reach several hundred  $\mu\text{g/mL}$ .

Acetaminophen is another common over-the-counter drug. It is taken for its analgesic and antipyretic effects; it is also available in combination with many other prescribed analgesics such as codeine and hydrocodone. Acetaminophen overdose can cause liver toxicity. A single blood concentration of acetaminophen may be of limited use when trying to determine acetaminophen toxicity, unless the time of ingestion is known.

### **2 Scope**

This procedure is used to analyze salicylic acid and acetaminophen in blood specimens qualitatively or quantitatively. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

Blood samples are extracted with acetonitrile using isotopically labeled salicylic acid and acetaminophen as internal standards, filtered, and analyzed by liquid chromatography/mass spectrometry (LC/MS). Salicylic acid is analyzed in negative ion mode, and acetaminophen is analyzed in positive ion mode.

### **4 Specimens**

This procedure uses 0.1 mL blood per replicate to analyze a specimen. (Quantitative analysis is typically prepared with duplicate samples.)

### **5 Equipment/Materials/Reagents**

- a. Acetonitrile (Optima grade)
- b. Methanol (HPLC grade)

- c. Centrifuge tubes
- d. Autosampler vials with inserts and caps
- e. Vortexer
- f. 0.45  $\mu\text{m}$  centrifuge filters
- g. Centrifuge
- h. Calibrated pipettors with disposable tips
- i. Volumetric glassware
- j. Liquid Chromatograph equipped with a mass spectrometer and a Xterra Phenyl LC column: 150 x 2.1 mm. 5  $\mu\text{m}$   $d_p$ , with 7.5 x 2.1 mm guard column (or equivalent)
- k. Water (Optima grade)
- l. Formic Acid (Puriss grade or better)
- m. 0.1% Formic acid in acetonitrile:  
Combine 500 mL acetonitrile and 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.
- n. 0.1% Formic acid in water:  
Combine 500 mL water and 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

## 6 Standards and Controls

- a. Salicylic Acid Calibration Stock (2.0 mg/mL):  
Add 23.3 mg of sodium salicylate (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Add approximately 1 mL methanol and swirl to completely dissolve the salicylic acid. Bring to the mark with acetonitrile and mix well. Store refrigerated in glass or plastic. Stable at least two years.
- b. Salicylic Acid Control Stock (2.0 mg/mL):  
Add 23.3 mg of sodium salicylate (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Add approximately 1 mL methanol and swirl to completely dissolve the salicylic acid. Bring to the mark with acetonitrile and mix well. Store refrigerated in glass or plastic. Stable at least two years.

- c. Acetaminophen Calibration Stock (1.0 mg/mL):<sup>1</sup>  
Add 10 mg of acetaminophen (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass or plastic. Stable at least two years.
- d. Acetaminophen Control Stock (1.0 mg/mL):<sup>1</sup>  
Add 10 mg of acetaminophen (purchased from Sigma-Aldrich or another approved vendor) to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well to dissolve. Store refrigerated in glass or plastic. Stable at least two years.
- e. Negative Control Blood:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqua, or prepared in-house from an appropriate blank specimen. Store refrigerated, frozen, or obtain fresh. Stability determined by manufacturer. A Negative Control Blood sample is extracted and analyzed with every assay.
- f. Positive Control Blood:  
Prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101). For in-house prepared controls, recommended control concentrations are 30 and 320 µg/mL for acetaminophen and 60 and 640 µg/mL for salicylic acid. Prepared fresh on the day of extraction as described in Section 6 Table 2.
- g. Salicylic Acid-d<sub>4</sub> (100 µg/mL):  
Purchased from Cerilliant or another approved vendor. Storage and stability determined by the manufacturer.
- h. Acetaminophen-d<sub>4</sub> (100 µg/mL):  
Purchased from Cerilliant or another approved vendor. Storage and stability determined by the manufacturer.
- i. LC Column Check Mix (5 µg/mL each salicylic acid and acetaminophen):  
Combine 25 µL of the Salicylic Acid Calibration or Control Stock (2.0 mg/mL) and 50 µL of the Acetaminophen Calibration or Control Stock (1.0 mg/mL) in a 10-mL volumetric flask. Bring to the mark with acetonitrile. Store refrigerated in glass or plastic. Stable for at least 2 years.

This procedure may be used quantitatively via construction of a multi-point calibration curve for the analyte(s) of interest following the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101). Table 1 shows the concentrations and volumes used for preparation of calibration samples.

<sup>1</sup> Alternatively, Standard Stock Solutions (1 mg/mL) may be purchased from Cerilliant (typically used for calibrators), Lipomed (typically used for controls) or another approved supplier.

Table 1: Typical Calibrator Preparation (0.1 mL of each Calibrator Level is added to 0.1 mL Negative Control Blood as described in Section 9.)

Calibrator Level (µg/mL)	Volume of Salicylic Acid Calibration Stock (µL)*	Volume of Acetaminophen Calibration Stock (µL)*	Volume of Acetonitrile (µL)
800/400	400	400	200
600/300	300	300	400
400/200	200	200	600
200/100	100	100	800
100/50	Dilute 0.5 mL Cal 200/100 with 0.5 mL acetonitrile		
50/25	Dilute 0.5 mL Cal 100/50 with 0.5 mL acetonitrile		
20/10	Dilute 0.1 mL Cal 200/100 with 0.9 mL acetonitrile		

\*If only one analyte is quantitated, add acetonitrile to compensate for the stock volume missing. If alternate concentrations of calibrator solutions are used, adjust volumes accordingly.

Table 2: Typical Control Preparation (0.1 mL of each Control Level is added to 0.1 mL Negative Control Blood as described in Section 9.)

Control Level (µg/mL)	Volume of Salicylic Acid Control Stock (µL)*	Volume of Acetaminophen Control Stock (µL)*	Volume of Acetonitrile (µL)
640/320	320	320	360
60/30	Dilute 0.375 mL 640/320 Control with 3.625 mL acetonitrile		

\*If only one analyte is calibrated, add acetonitrile to compensate for the stock volume missing. If alternate concentrations of control solutions are used, adjust volumes accordingly.

For qualitative analysis, analyze a negative control and at least one positive control or calibrator listed in Tables 1-2.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- Measure 100 µL of each case sample into properly labeled centrifuge tubes. Prepare case samples in duplicate for quantitations.



- b. Measure 100 µL Negative Control Blood into 12 labeled centrifuge tubes for the calibrators, Positive Controls (in duplicate), and a Negative Control.
- c. Add 100 µL acetonitrile to each case sample and the Negative Control.
- d. Add 100 µL of each Calibrator and Control (prepared as directed in Section 6) to the calibrator and positive control samples, respectively.
- e. Add 50 µL each Salicylic Acid-d<sub>4</sub> and Acetaminophen-d<sub>4</sub> to all samples.
- f. Vortex tubes well. Centrifuge at 10000 rpm for 10 minutes.
- g. Filter the top layer through 0.45 µm centrifuge filters by centrifuging at 10000 rpm for 5 minutes.
- h. Transfer extracts to labeled autosampler vials. Add 50 µL of water to each ALS vial. Analyze 10 µL by LC/MS using the conditions in Section 10 after verifying that the results of the column check mix are acceptable. (Note: salicylic acid is analyzed in negative ion mode and acetaminophen is analyzed in positive ion mode.)

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters		Column
A: 0.1% Formic acid in water	Flow	0.2 mL/min	Phenyl (Xterra)
C: 0.1% Formic acid in acetonitrile		0.1 mL/min	Oven set at 40°
Total Run time: 7 minutes	Autosampler Temp: 15°C		

## 9.2 Mass Spectrometer Parameters – Salicylic Acid

Source Parameters		
Mode: Electrospray, Negative	Spray Voltage: -5 kV	Capillary Temperature: 275°C
Sheath Gas: 27 (arb units)	Aux Gas: 6 (arb units)	Sweep Gas: 3 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Event #1	full scan m/z 110 - 300, unit mass resolution	
Event #2	MSMS 137→50-150	
	1.5 amu isolation width	45% collision energy
Event #3	MSMS 141→50-150	
	1.5 amu isolation width	45% collision energy

## 9.3 Mass Spectrometer Parameters – Acetaminophen

Source Parameters		
Mode: Electrospray, Positive	Spray Voltage: +5 kV	Capillary Temperature: 275°C
Sheath Gas: 34 (arb units)	Aux Gas: 3 (arb units)	Sweep Gas: 3 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Event #1	full scan m/z 110 - 300, unit mass resolution	
Event #2	MSMS 152→50-160	
	1.5 amu isolation width	45% collision energy
Event #3	MSMS 156→50-160	
	1.5 amu isolation width	45% collision energy

## 10 Decision Criteria

### 10.1 Column Check Mix Performance Criteria

The performance of the LC/MS is demonstrated each day samples are analyzed. The LC Column Check Mix effectively evaluates system suitability. Depending upon the MS parameters used, the salicylic acid or acetaminophen peak should be present with reasonable peak shape.

### 10.2 Analyte Performance Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

### 10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. The peak shape of salicylic acid is known to tail. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following criteria should be met.

#### 10.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

#### 10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

### 10.2.2 MS Spectra

For salicylic acid (fragments of 137), the only peaks present in the MS/MS spectrum above 10% should be  $m/z$  93 and the precursor. Due to the stability of salicylic acid, the precursor ion may be the base peak.

For acetaminophen (fragments of 152), the base peak in the MS/MS spectrum should be  $m/z$  110 with no other fragment more than 15% of the base peak intensity. Additionally, there should be a chromatographically detectable trace for  $m/z$  134.

### 10.3 Reporting Cut-offs for College of American Pathologists (CAP) T Series and FTC Series:

See Quality Control for Toxicology Examinations (TOX101) for guidance on estimating the amount of an analyte in a specimen. When analyzing CAP T-Series or FTC specimens, if all decision criteria for an analyte of interest are met, but the concentration of acetaminophen is estimated to be below 5  $\mu\text{g/mL}$  and/or the concentration of salicylic acid is estimated to be below 10  $\mu\text{g/mL}$  in two independent analyses, the analyte will not be reported. Note: the second analysis may be a repeat of this procedure or via another validated procedure. A Positive Control at the Cut-off Level is recommended for the second analysis.

## 11 Calculations

Analyte	Molecular Ion	Internal Standard Molecular Ion	Calibration Weighting
Salicylic acid	137	141	1/x
Acetaminophen	152	156	unweighted

See the *Quality Control for Toxicology Examinations* standard operating procedure (TOX101) for acceptable practices for calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Measurement Uncertainty* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

## 13 Limitations

- Linearity: 20-800 µg/mL for salicylic acid  
10-400 µg/mL for acetaminophen
- Limit of Detection: 10 µg/mL for salicylic acid  
5 µg/mL for acetaminophen
- Bias, Repeatability, and Intermediate Precision (n=15 per level):

Salicylic Acid	Bias	Repeatability	Intermediate Precision
60 µg/mL	-1.04%	4.07%	5.48%
300 µg/mL	-0.12%	3.98%	4.60%
640 µg/mL	+3.19%	2.62%	4.90%

Acetaminophen	Bias	Repeatability	Intermediate Precision
30 µg/mL	-4.24%	2.31%	3.41%
150 µg/mL	-1.28%	2.37%	2.37%
320 µg/mL	-2.61%	1.65%	2.63%

- d. Matrix Effects: Average matrix effects were 0.8% for salicylic acid and -42.8% for acetaminophen.
- e. Interferences: None known.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

*Quality Control for Toxicology Examinations* (TOX101); FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

*Chemistry Unit Procedures for Estimating Measurement Uncertainty* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Rev. #	Issue Date	History
1	10/09/18	Updated SOP title to “analysis” (vs. quantitation). Section 1: added “overdose” for clarification. Updated Section 2 scope language to reflect current staff requirements as well as include qualitative analysis. Section 4 and 8a: updated to account for one replicate. Section 6: updated to account for qualitative examinations, and updated TOX101 reference; moved some information to Section 11 Calculations. Updated 8f to include addition of 0.050 mL of water to improve peak shape. Updated 9.2 to enable sweep gas to reduce adduct formation. Removed “Calibration” section and renumbered sections. Updated 12 title phrase to “Measurement Uncertainty”, and updated title of CUQA 13. Updated Section 15 TOX101 and CUQA 13 titles.
2	11/15/19	In Sections 6 c and d, added footnote to allow purchase of stock standards. In Sections 6 and 8 revised to see Section 6 table 1-2, not Section 7. In Tables 1 and 2, added language to adjust volumes for alternate concentrations. Added 10.3 reporting cut-offs for proficiency testing. Revised bench sheet calibrator and control section.

**Approval**

Redacted - Signatures on File

Acting Toxicology  
Technical Lead:

Date: 11/14/2019

Chemistry Unit Chief:

Date: 11/14/2019

**Appendix 1: Abbreviated version of the Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use.**

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## **Benzodiazepines, Ketamine, and Zolpidem in Hair**

### **1 Introduction**

Drugs in the bloodstream can be incorporated into the keratin matrix of hair through the root. This makes hair a suitable forensic toxicology specimen for determining a history of drug exposure. Because of the nature of hair as a forensic toxicology specimen, the window of detection after drug exposure is wider than that of blood or urine.

Benzodiazepines are one of the classes of drugs most commonly associated with drug-facilitated sexual assault (DFSA). When hair is the only suitable specimen for toxicological screening in a suspected DFSA case, benzodiazepines are a logical class of drugs to target in the absence of circumstantial evidence pointing to a specific drug. Ketamine and zolpidem are also commonly associated with DFSA.

### **2 Scope**

This procedure addresses sample preparation and analysis of hair specimens to screen for the presence of 13 benzodiazepines (7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, clonazepam, diazepam, flunitrazepam, flurazepam, lorazepam, midazolam, nordiazepam, oxazepam, temazepam, and triazolam), ketamine and zolpidem. Detection limits of this procedure are below levels associated with chronic exposure to these drugs. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### **3 Principle**

Samples are segmented (if applicable), pulverized and extracted with methanol. The methanol extracts are taken to dryness, reconstituted in buffer with and without deionized water, and extracted using one of two solid phase extraction (SPE) procedures. Final extracts are analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) in the multiple reaction monitoring (MRM) mode. To confirm any positive findings, a second aliquot of the hair sample may be extracted and analyzed; additional confirmation is not needed if two or more hair segments test positive.

### **4 Specimens**

This screening procedure requires approximately 50 mg of hair growth that corresponds to the 1-2 month time frame surrounding the incident in question. It can also be used on bulk hair specimens to screen for exposure over time.

## 5 Equipment/Materials/Reagents

- a. Scissors
- b. Ruler
- c. Analytical Balance
- d. Retsch Mixer Mill Cryo Mill (or equivalent)
- e. Liquid Nitrogen
- f. Grinder Vials, Small, Medium and Large
- g. 2 mL Polypropylene Centrifuge Tubes with Caps
- h. Micro Stir Bars
- i. Methanol (HPLC grade and Optima grade)
- j. Vortex Mixer
- k. Heating/Stirring Block
- l. Microcentrifuge
- m. 12 x 75 mm test tubes
- n. Water (Deionized and Optima grade)
- o. Evaporator with nitrogen
- p. CLEAN SCREEN DAU SPE Cartridges (200 mg /10 mL) and/or Oasis HLB 6 cc (500 mg) LP SPE cartridges.
- q. Vacuum Manifold or Positive Pressure Manifold
- r. Sodium phosphate dibasic heptahydrate (ACS grade,  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ )
- s. Sodium phosphate, monobasic monohydrate (ACS grade,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )
- t. 100 mM Phosphate Buffer (pH 6.0):  
To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to

dissolve. Verify  $5.8 < \text{pH} < 6.1$ . Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.

- u. Acetonitrile (HPLC and Optima grades)
- v. 20% Acetonitrile/80% 0.1 M Phosphate Buffer:  
Combine 80 mL 0.1 M phosphate buffer (pH 6) with 20 mL HPLC grade acetonitrile and mix well. Store in glass at room temperature. Stable 1 month.
- w. Ethyl acetate (HPLC grade)
- x. Ammonium hydroxide, concentrated (15 M) (ACS grade)
- y. Ethyl Acetate with 2% Ammonium Hydroxide:  
Combine 49 mL ethyl acetate with 1 mL concentrated ammonium hydroxide and mix well. Store in glass at room temperature. To be prepared fresh.
- z. Hexane (Pesticide grade)
- aa. Formic Acid (98%)
- bb. Ammonium formate
- cc. Centrifuge tube filters (0.45 micron, Nylon)
- dd. Mobile Phase #1 (5 mM Ammonium Formate with 0.1% formic acid; pH~3.5):  
Add 0.3153 g ammonium formate to a 1-L volumetric flask. Add approximately 800 mL deionized water and mix well. Add 1 mL formic acid, and QS with deionized water. Store in glass at room temperature. Stable for at least one week.
- ee. Mobile Phase #2 (0.1% Formic Acid in Acetonitrile): Combine 500 mL Optima grade acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two months.
- ff. ABI 6500 Liquid Chromatograph/Mass Spectrometer equipped with Analyst software and a 150 mm x 2.1 mm x 2.6  $\mu$  Phenomenex Kinetex XB-C18 (or equivalent) analytical column
- gg. Routine laboratory supplies, including disposable glass pipets, autosampler vials with caps, spatulas, graduated cylinders, test tube racks, pH paper etc.
- hh. Methylene Chloride (HPLC grade and Optima grade)
- ii. Potassium dihydrogen phosphate (HPLC grade)

- jj. Disodium hydrogen phosphate (anhydrous, ACS grade)
- kk. Potassium Phosphate Buffer: Add 9.07 g potassium dihydrogen phosphate to a 1-L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.
- ll. Sodium Phosphate Buffer: Add 11.6 g disodium hydrogen phosphate to a 1-L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.
- mm. Sorensen Buffer (pH 7.4): Add sodium phosphate buffer to the potassium phosphate buffer until the pH reads 7.4 with a pH meter. Store refrigerated in glass or plastic. Stable for at least three months.
- nn. Methanol:Water:Ammonia (40:60:0.5):  
Combine 40 mL HPLC grade methanol, 60 mL deionized water and 0.5 mL ammonium hydroxide and mix well. Store at room temperature in glass. Prepare fresh daily.
- oo. Isopropanol (HPLC grade)
- pp. Methylene Chloride:Isopropanol (75:25):  
Combine 75 mL Optima grade methylene chloride and 25 mL isopropanol and mix well. Store at room temperature in glass. Stable for at least two months.
- qq. Reconstitution Solvent (90:10:0.1% Water:Acetonitrile:Formic Acid): Combine 45 mL Optima grade water, 5 mL acetonitrile and 0.05 mL formic acid and mix well. Store in amber glass at room temperature. Stable for at least two months.

## 6 Standards and Controls

- a. Standard Stock Solutions (1.0 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed or an equivalent supplier. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

7-aminoclonazepam	flunitrazepam	nordiazepam
7-aminoflunitrazepam	flurazepam	oxazepam
alprazolam	ketamine	temazepam
clonazepam	lorazepam	triazolam
diazepam	midazolam	zolpidem

- b. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX) or an equivalent supplier. Solutions may be in methanol or acetonitrile, and will be stored according to the manufacturer's recommendations.

Stability is determined by the manufacturer.

7-aminoclonazepam-d <sub>4</sub>	flunitrazepam-d <sub>7</sub>	nordiazepam-d <sub>5</sub>
7-aminoflunitrazepam-d <sub>7</sub>	ketamine-d <sub>4</sub>	oxazepam-d <sub>5</sub>
alprazolam-d <sub>5</sub>	lorazepam-d <sub>4</sub>	temazepam-d <sub>5</sub>
clonazepam-d <sub>4</sub>	midazolam-d <sub>4</sub>	triazolam-d <sub>4</sub>
diazepam-d <sub>5</sub>		zolpidem-d <sub>6</sub>

- c. Intermediate Internal Standard Mixture (0.5 µg/mL):  
Combine 0.05 mL of each Internal Standard Stock Solution to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well. Store in the refrigerator or in the freezer in glass. Stable for six months.
- d. Internal Standard Solution (1 ng/mL):  
Add 0.02 mL of the Intermediate Internal Standard Mixture to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well. Store in the refrigerator or in the freezer in glass. Stable for six months.
- e. Intermediate Positive Control Mixture #1 (5 µg/mL):  
Combine 0.05 mL of each of the Stock Standard Solutions in a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well. Store in the refrigerator or in the freezer in glass. Stable for six months.
- f. Intermediate Positive Control Mixture #2 (100 ng/mL):  
Add 0.2 mL of the Intermediate Positive Control Mixture #1 to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well. Store refrigerated in glass. Stable for six months.
- g. Positive Control Solution (1 ng/mL):  
Add 0.1 mL of the Intermediate Positive Control Mixture #2 to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well. Store refrigerated in glass. Stable for six months.
- h. Negative Control Hair:  
Obtain in house. Store at room temperature. Stable indefinitely. A Negative Control Hair sample will be extracted and analyzed with every analysis.

Negative Control Hair samples may be washed and pulverized in bulk and stored at room temperature in plastic centrifuge tubes or centrifuge vials for use as needed. Negative Control Hair may also be weighed, washed, and pulverized along with case specimens.

- i. Positive Control Hair:
  1. Low Positive Control Hair (1 pg/mg): Combine 25 mg of pulverized Negative Control Hair and 25 µL of the Positive Control Solution in a clean

centrifuge tube.

2. High Positive Control Hair (5 pg/mg): Combine 25 mg of pulverized Negative Control Hair and 125  $\mu$ L of the Positive Control Solution in a clean centrifuge tube.

At least one Positive Control will be extracted and analyzed with every assay.

- j. Intermediate System Suitability Testmix (100 ng/mL):  
Add 0.2 mL of the Intermediate Positive Control Mixture #1 (5  $\mu$ g/mL) and 2 mL of the Intermediate Internal Standard Mixture (0.5  $\mu$ g/mL) to a 10-mL volumetric flask. Bring to the mark with acetonitrile and mix well. Store in the refrigerator or in the freezer in glass. Stable for at least one year.
- k. System Suitability Testmix (10 ng/mL):  
On the day of analysis, add 100  $\mu$ L of Intermediate System Suitability Testmix (100 ng/mL) to an autosampler vial. Dilute with 810  $\mu$ L of aqueous reconstitution solvent (5-qq) and 90  $\mu$ L of organic reconstitution solvent (5-ee).

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### *Hair Preparation:*

- a. Make note of approximate length of questioned hair sample, color, curvature, condition, and any other pertinent observations. Typically, 25 mg of hair per segment is used for this analysis. If the weight used is other than  $25 \pm 1$  mg, record the actual weight used.
- b. Thoroughly clean scissors with methanol. Cut a portion or portions of the hair sample into 1-2 cm segments. The length of the segments may be 1 or 2 cm, depending on the width of the hair sample provided. (Typically, in suspected DFSA cases, the segment corresponding to the incident in question will be analyzed, along with a 1-2 cm segment bracketing the time frame of the incident. This determination is made assuming average head hair growth of 1 cm/month.). Weigh hair segment portions into 2mL polypropylene centrifuge tubes ( $25 \pm 1$  mg).

- c. Wash the sample twice with 2 mL of HPLC grade methylene chloride for two minutes each. Discard methylene chloride. Dry hair samples at room temperature, in a heating block, or in an oven to remove any excess methylene chloride.
- d. Prepare Negative and Positive Control Hair Samples similarly.
- e. Grind the hair sample(s) in the cooled Freezer/Mill using the settings provided in this document.
- f. Prepare Positive Controls by adding 25 and 125  $\mu$ L Positive Control Solution to the Low and High Controls, respectively. Add 50  $\mu$ L of the Internal Standard Solution, 1.5 mL methanol and a micro stir bar to each tube. Cap and vortex mix for 15-30 seconds. Incubate at 40°C overnight (for at least 12 hours) while stirring.
- g. Centrifuge the centrifuge tube and contents at 10,000 RPM for 5-10 minutes.
- h. Remove as much of the methanol as possible from each tube and transfer to a labeled centrifuge tube. A small amount of hair powder may be transferred to the centrifuge tube; this is acceptable.
- i. Centrifuge the centrifuge tube and contents at 10,000 RPM for 5-10 minutes.
- j. Remove as much of the methanol as possible from each tube and transfer to a labeled 12 x 75 mm culture.
- k. Take the methanol to approximately 0.1 mL under nitrogen at 35°C.
- l. Proceed to one of the two solid phase extractions below. (For general benzodiazepine screening, proceed to Option #1. If zolpidem or ketamine is suspected, proceed to Option #2. See Section 13 for details concerning limits of detection for target analytes.)

*Solid Phase Extraction Option #1 (UCT cartridges):*

- a. Add 2 mL deionized water to each tube and vortex well. Add 1 mL phosphate buffer and vortex well.
- b. Pre-rinse SPE extraction cartridge (UCT) by adding 3 mL of methanol at 1-2 mL/minute.
- c. Condition cartridge with 3 mL of deionized water followed by 2 mL of 100 mM phosphate buffer. Do not allow sorbent to dry.
- d. Load sample on SPE cartridge at 1-2 mL/minute. Do not allow sorbent to dry.
- e. Wash cartridge with 2 mL of deionized water and 2 mL of 20% Acetonitrile/80% 0.1 M

Phosphate Buffer.

- f. Dry cartridge for 1 minute under full vacuum.
- g. Wash cartridge with 2 mL of hexane.
- h. Dry cartridge for 1 minute under full vacuum.
- i. Rinse cartridge with 2 mL of deionized water.
- j. Dry cartridge for 1 minute under full vacuum.
- k. Elute with 2.5 mL of Ethyl Acetate with 2% Ammonium Hydroxide at 0.5 mL/minute.
- l. Evaporate eluent at 35°C to dryness. Do not over dry.
- m. Reconstitute extracts with 100 µL of Reconstitution Solvent. Vortex well. |  
Optional: for optimal reconstitution volume yield, centrifuge for 1 minute at 1000 RPM.
- n. Filter through 0.45 micron filters at 10,000 rpm for 2 minutes. Transfer extracts to autosampler vials.
- o. Verify that the LC/MS/MS is performing properly by analyzing a solvent blank followed by 5 µL of the System Suitability Testmix.
- p. Inject 20 µL of each extract into the LC/MS/MS using the Instrumental Parameters in Section 10.

*Solid Phase Extraction Option #2 (Waters cartridges):*

- a. Add 5.5 mL of Sorenson buffer to each tube and vortex well.
- b. Vortex and centrifuge samples for 1 minute at 3000 rpm.
- c. Pre-rinse SPE cartridge (Waters) by adding 2 mL of methanol.
- d. Condition cartridge with 3 mL of deionized water.
- e. Load sample on SPE cartridge.
- f. Wash cartridge with 2 mL of Methanol:Water:Ammonia (40:60:0.5).
- g. Dry cartridge at full vacuum for 15 minutes.



- h. Elute with 5 mL Methylene Chloride:Isopropanol (75:25) under gravity.
- i. Evaporate eluent to dryness below 50°C under nitrogen. Do not over dry.
- j. Reconstitute extracts with 100 µL of Reconstitution Solvent. Vortex well.  
Optional: for optimal reconstitution volume yield, centrifuge for 1 minute at 1000 RPM.
- k. Filter through 0.45 micron filters at 10,000 rpm for 2 minutes. Transfer extracts to autosampler vials.
- l. Verify that the LC/MS/MS is performing properly by analyzing a solvent blank followed by 5 µL of the System Suitability Testmix.
- m. Inject 20 µL of each extract into the LC/MS/MS using the Instrumental Parameters in Section 10 after confirming that the instrument is operating properly.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Freezer/Mill Settings

	Frequency	Time
Precool:	5 Hz	Auto
Grind:	25 Hz	7-12 minutes
Cycles	1	

### 9.2 Autosampler Parameters

- a. Autosampler Temperature Setting: 15°C
- b. Injection volume = 20 µL

### 9.3 Liquid Chromatograph Parameters

LC Column Temp: 28°C

Time (min)	% Mobile Phase #1	% Mobile Phase #2
0:01	90	10
2:00	90	10
12:00	60	40
21:00	60	40
25:00	0	100
28:00	90	10
40:00	90	10
Flow rate		0.2 mL/min

### 9.4 Mass Spectral Parameters

<b>Scan Mode</b>	Turbo Spray	<b>Polarity</b>	Positive
<b>Resolution</b>	Unit	<b>Scan Type</b>	MRM
<b>Curtain Gas</b>	Nitrogen (30)	<b>Ionspray Voltage</b>	4000
<b>Source Temperature</b>	700°C	<b>Nebulizer Gas</b>	Nitrogen (75)
<b>Entrance Potential</b>	10	<b>Turbo Gas</b>	Nitrogen (75)
<b>Dwell Time</b>	15 ms	<b>Pause Time</b>	5 ms
<b>Collision Gas</b>	Nitrogen (10)		

Q1 Mass	Q3 Mass	Declustering Potential	Collision Energy	Collision Exit Potential
285.935	222.069	1	33	12
285.935	250.021	1	29	14
285.935	121.059	1	37	14
289.942	226.059	71	35	14
283.966	135.049	116	37	16
283.966	226.997	116	35	12
283.966	240.045	116	45	14
290.995	138.110	106	37	6
308.930	280.993	96	37	16
308.930	205.022	96	55	12
308.930	273.993	96	35	16

Q1 Mass	Q3 Mass	Declustering Potential	Collision Energy	Collision Exit Potential
313.937	286.025	71	37	18
315.867	269.952	41	35	18
315.867	214.055	41	49	12
315.867	240.990	41	47	20
319.902	274.022	106	35	18
284.939	193.044	106	43	12
284.939	154.027	106	37	8
284.939	222.073	106	37	14
289.945	198.078	106	45	10
313.910	268.042	106	35	16
313.910	239.041	106	47	12
313.910	183.050	106	67	16
320.967	275.067	110	37	16
388.045	314.992	86	31	28
388.045	317.009	86	27	30
388.045	225.028	86	55	20
320.860	274.934	41	31	16
320.860	229.004	41	41	12
320.860	194.046	41	57	18
326.891	280.965	61	31	24
325.935	291.024	96	37	18
325.935	223.006	96	51	12
325.935	249.026	96	49	14
329.958	295.068	166	37	18
270.929	140.012	121	37	16
270.929	208.054	121	39	12
270.929	165.008	121	39	18
275.953	213.081	101	39	14
286.920	240.990	71	31	14
288.920	242.990	71	31	14
286.920	104.011	71	43	12
291.936	246.006	1	33	14
300.942	254.998	66	31	14
300.942	177.032	66	53	10
300.942	193.061	66	45	10
305.858	260.028	71	31	14
342.921	307.997	26	37	26
342.921	314.938	26	39	20
342.921	239.004	26	57	20

Q1 Mass	Q3 Mass	Declustering Potential	Collision Energy	Collision Exit Potential
346.951	312.010	56	37	28
237.986	125.009	46	37	14
237.986	207.005	46	19	14
237.986	179.021	46	23	10
242.006	118.045	31	75	14
308.016	235.074	76	35	18
308.016	263.051	76	35	18
308.016	219.036	76	73	18
314.031	235.059	121	49	14

## 10 Decision Criteria

### 10.1 System Suitability Decision Criteria

All 15 analytes should be present in the System Suitability sample. The retention times should be within 5% of the retention times of the last analysis of the System Suitability Testmix, but mobile phase composition and column age may lead to small changes in retention time.

### 10.2 Batch Decision Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All intended analytes should be present in the Positive Control.

### 10.3 Sample Acceptance Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, the criteria in Sections 10.3.1 and 10.3.2 should be met in order to consider the hair specimen positive for a particular analyte.

#### 10.3.1 Chromatography

All three ion transition peaks for the analyte of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### 10.3.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute) obtained from injection of an extracted Positive Control.

### 10.3.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio using the Analyst software should exceed 10. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control Hair Sample or a solvent blank injected just prior to that sample.

### 10.3.2 Mass Spectrometry

Three independent MS/MS experiments are conducted for each analyte. (See Table 2 below.) Two ion ratios are calculated for each analyte. The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra standard operating procedure (Tox 104) for further guidance.

**Table 2: MS/MS Transitions**

Analyte	Precursor Ion	Product Ion 1	Product Ion 2	Product Ion 3
7-Aminoclonazepam	285.9	222.0	250.0	121.0
7-Aminoflunitrazepam	283.9	135.0	226.9	240.0
Alprazolam	308.9	280.9	205.0	273.9
Clonazepam	315.8	269.9	214.0	240.9
Diazepam	284.9	193.0	154.0	222.0
Flunitrazepam	313.9	268.0	239.0	183.0
Flurazepam	388.0	314.9	317.0	225.0
Lorazepam	320.8	274.9	229.0	194.0
Midazolam	325.9	291.0	223.0	249.0
Nordiazepam	270.9	140.0	208.0	165.0
Oxazepam	286.9 / 288.9	240.9	242.9	104.0
Temazepam	300.9	254.9	177.0	193.0
Trizolam	342.9	307.9	314.9	239.0
Ketamine	237.9	125.0	207.0	179.0
Zolpidem	308.0	235.0	263.0	219.0

### 10.4 Confirmation

When a drug is indicated in a hair sample following this screening procedure, the sample should be re-extracted and re-analyzed targeting the drug(s) in question. Identifying the drug in two separate aliquots on the screen also constitutes a confirmation.

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

a. Limits of Detection (LOD):

Analyte	LOD using UCT SPE (pg/mg)	LOD using Waters SPE (pg/mg)
7-Aminoclonazepam	1	1
7-Aminoflunitrazepam	1	1
Alprazolam	0.25	0.5
Clonazepam	0.5	0.75
Diazepam	0.5	0.25
Flunitrazepam	0.25	0.75
Flurazepam	1	1
Lorazepam	0.75	1
Midazolam	0.5	1
Nordiazepam	0.25	0.5
Oxazepam	1	1
Temazepam	0.25	0.5
Trizolam	0.25	0.25
Ketamine	1	1
Zolpidem*	0.5	0.5

\*Peak shape for zolpidem is much better with the Waters extraction than with the UCT extraction.

Note: LODs vary widely from sample to sample. The LODs reported here are conservative estimates based on validation experiments performed on different lots of blank hair spiked with analytes. The LOD for an individual sample may be lower than what is listed here.

b. Interferences: none known.

## 14 Precautionary Statements

Research on hair testing following single dosing of drugs is limited. Reports will include a statement that negative results do not exclude the possibility that the individual in question was

exposed to a single dose of a drug tested for in this procedure.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 16 References

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

*Extraction and Analysis of Drugs in Hair* (Tox 208); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

Instrument Operation and Support SOP Manual.

FBI Laboratory Safety Manual.

Negrusz, A., et al. Deposition of 7-Aminoflunitrazepam and Flunitrazepam in Hair After a Single Dose of Rohypnol®. *J Forensic Sci* 2001; 46(5): 1143-1151.

Rev. #	Issue Date	History
0	09/08/14	New document that replaces Tox 211, 213 and 214
1	04/01/19	Updated Scope language. Removed “reasonable degree of scientific certainty” language from Section 10.3.2. To 8-a, added language about typical weight used, in 8-b clarified the order of of weighting, removed 8-d, and renumbered. Removed all instances of “subunit” (header and 16). Updated 6-k for clarity and to match initial mobile phase conditions. Added Oasis cartridges to 5-p. Simplified reconstitution solvent in 5-ee and 5-qq. Reduced stability in 6-c through 6-g. 6-h: clarified possible prep of Negative Control Hair. In Section 8-m (UCT), 8-j (Waters), and bench notes (both pages) updated the reconstitution solvent scheme. Updated wording in Section 14.

**Approval**

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Toxicology  
 Technical Leader:

Date: 03/28/2019

Chemistry Unit Chief:

Date: 03/28/2019

**QA Approval**

Quality Manager:

Date: 03/28/2019



**Appendix 1: Abbreviated version of the Benzodiazepine Hair Procedure for bench use.**

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**Appendix 1: Abbreviated version of the Benzodiazepine Hair Procedure for bench use.  
(continued)**

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**Appendix 1: Abbreviated version of the Benzodiazepine Hair Procedure for bench use.**  
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**Appendix 2: Abbreviated version of the Instrumental Parameters for the Benzodiazepine  
Hair Procedure.**

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## **Solid Phase Extraction of Lysergides from Biological Samples**

### **1 Introduction**

Lysergic acid diethylamide (LSD) is one of the most potent hallucinogenic drugs. Detection of LSD in biological samples can be an analytical challenge due to the low doses consumed and extensive metabolism. LSD's major metabolite 2-oxo-3-hydroxy LSD (OH-LSD) is detectable in urine at 16-43 times the concentration of LSD; to date OH-LSD has never been detected in blood.

### **2 Scope**

This procedure qualitatively detects LSD in blood and urine. It also qualitatively detects its primary metabolite, OH-LSD, in both blood and urine. It is derived from "LC-MS Analysis of 2-oxo-3-hydroxy LSD from Urine Using a Speedisk<sup>®</sup> Positive-Pressure Processor with Cerex<sup>®</sup> PolyChrom<sup>™</sup> CLIN II Columns" and "Liquid Chromatography-Electrospray Ionization Mass Spectrometry for the Detection of Lysergide and a Major Metabolite, 2-Oxo-3-Hydroxy-LSD, in Urine and Blood". The solid phase extraction (SPE) scheme is followed essentially intact. The analysis of LSD and its major metabolite OH-LSD are performed by liquid chromatography-electrospray ionization-high resolution mass spectrometry.

### **3 Principle**

Blood and urine specimens can be selectively assayed for OH-LSD and LSD by this method. Following the addition of internal standard ( $d_3$ -LSD), blood and/or urine samples are buffered and extracted with Cerex<sup>®</sup> PolyChrom<sup>™</sup> CLIN II solid phase columns. LSD and OH-LSD are collected in the same fraction and taken to dryness. Once dry, the eluent is reconstituted in 20% acetonitrile. Samples are analyzed by liquid chromatography-electrospray ionization-high resolution mass spectrometry (LC-ESI-FT-MS/MS) for both LSD and OH-LSD.

### **4 Specimens**

This procedure uses 2 mL of blood or urine.

### **5 Equipment/Materials/Reagents**

- a. 16x100 mm screw-top tubes with caps

- b. 12x75 mm and 16x100 mm culture tubes with snap-tops
- c. Routine laboratory supplies and equipment, including disposable pipets, graduated cylinders, volumetric flasks, pH paper, vortex mixer, centrifuge, etc.
- d. Positive pressure solid-phase extraction manifold with nitrogen supply
- e. CereX<sup>®</sup> PolyChrom<sup>™</sup> CLIN II solid phase columns (50mg) 6mL
- f. Evaporator with nitrogen supply
- g. Liquid chromatography Fourier transform mass spectrometry system (LTQ Orbitrap-XL<sup>®</sup>, or equivalent)
- h. HPLC column (Grace Alltima<sup>®</sup> C18, 150x2.1 mm, 5 µm d<sub>p</sub>, or equivalent with pre-filter)
- i. Acetonitrile (Optima grade)
- j. Methanol (HPLC and Optima grades)
- k. Water (deionized and Optima grade)
- l. Ethyl Acetate (HPLC grade)
- m. Concentrated Hydrochloric Acid (HCl) (certified ACS grade)
- n. Concentrated Ammonium Hydroxide (certified ACS grade)
- o. Concentrated Formic Acid (Puriss grade)
- p. Concentrated Acetic Acid (certified ACS grade)
- q. Ammonium Formate (99.99+ %)
- r. Sodium Phosphate, Monobasic, Monohydrate (certified ACS grade)
- s. Sodium Phosphate, Dibasic, Heptahydrate (certified ACS grade)
- t. Anhydrous Potassium Carbonate (certified ACS grade)
- u. Anhydrous Potassium Bicarbonate (certified ACS grade)

- v. Phosphate Buffer (0.1 M, pH 6):  
To a 1-L volumetric flask, add 900 mL DI (deionized) water. Add 12.2 g sodium phosphate, monobasic, monohydrate and 3.2 g sodium phosphate, dibasic, heptahydrate and mix well to dissolve. Verify pH ~6 and dilute to the mark with DI water. Store refrigerated in glass. Stable for 2 months.
- w. Carbonate Buffer (0.27 M, pH 9):  
To a 250-mL volumetric flask, add 200 mL DI water. Add 2.5 g anhydrous potassium carbonate and 5 g anhydrous potassium bicarbonate and mix well to dissolve. Adjust pH to ~9 with concentrated acetic acid or concentrated ammonium hydroxide and dilute to the mark with DI water. Store refrigerated in glass. Stable for 1 month.
- x. 0.1 M Hydrochloric Acid:  
In a 100-mL graduated cylinder, dilute 0.8 mL of concentrated hydrochloric acid to 96 mL with DI water and mix well. Store in glass at room temperature. Stable for 6 months.
- y. Elution Solvent (25:1 ethyl acetate:ammonium hydroxide):  
Measure 75 mL of ethyl acetate into a 100-mL graduated cylinder. Add 3 mL of concentrated ammonium hydroxide and mix well. Prepare fresh daily.
- z. Reconstitution Solvent (20:80 acetonitrile:water):  
Combine 4 mL acetonitrile with 16 mL water (Optima grade) and mix well. Store in glass at room temperature. Stable for 6 months.
- aa. LC Mobile Phase #1 (20 mM ammonium formate, pH 4.4):  
Add 1.26 g ammonium formate to 1 L of water (Optima grade). Mix well to dissolve, and filter. Add 70  $\mu$ L concentrated formic acid and verify pH ~4.4, adjusting with concentrated formic acid or concentrated ammonium hydroxide as necessary. Store in glass at room temperature. Stable for 1 month.

## 6 Standards and Controls

- a. **d<sub>3</sub>-LSD Stock Standard (100 µg/mL):**  
Purchased from Cerilliant or another approved vendor. Stability and storage determined by manufacturer.
- b. **d<sub>3</sub>-LSD Working Internal Standard (100 ng/mL):**  
To a 25-mL volumetric flask, add 25 µL of the d<sub>3</sub>-LSD Stock Standard. Dilute to the mark with acetonitrile and mix well. Store below 0°C in glass. Stable for at least 1 year.
- c. **LSD Stock Standard (1 mg/ml):**  
Purchased from Cerilliant or another approved vendor. Stability and storage determined by manufacturer.
- d. **LSD Intermediate Working Solution (2 µg/ml):**  
To a 10-mL volumetric flask, add 20 µL of the LSD Stock Standard. Dilute to the mark with acetonitrile and mix well. Store below 0°C in glass. Stable for at least 1 year.
- e. **OH-LSD Stock Standard (100 µg/ml):**  
Purchased from Cerilliant or another approved vendor. Stability and storage determined by manufacturer.
- f. **OH-LSD Intermediate Working Solution (2 µg/ml):**  
To a 10-mL volumetric flask, add 200 µL of the OH-LSD Stock Standard. Dilute to the mark with acetonitrile and mix well. Store below 0°C in glass. Stable for at least 1 year.
- g. **Lysergide Control Working Solution (20 ng/ml LSD; 40 ng/ml OH-LSD):**  
To a 25-mL volumetric flask, add 250 µL of the LSD intermediate Working Solution and 500 µL of the OH-LSD Intermediate Working Solution. Dilute to the mark with acetonitrile and mix well. Store below 0°C in glass. Stable for at least 1 year.
- h. **Lysergic Acid Methypropylamide (LAMPA) Stock Standard (1 mg/ml):**  
Purchased from Cerilliant or another approved vendor. Stability and storage determined by manufacturer.
- i. **LAMPA Intermediate Working Solution (2 µg/ml):**  
To a 10-mL volumetric flask, add 20 µL of the LAMPA Stock Standard. Dilute to the mark with acetonitrile and mix well. Store below 0°C in glass. Stable for at least 1 year.
- j. **iso-LSD Stock Standard (100 µg/ml):**  
Purchased from Cerilliant or another approved vendor. Stability and storage determined by manufacturer.



- k. iso-LSD Intermediate Working Solution (2 µg/ml):  
To a 10-mL volumetric flask, add 200 µL of the iso-LSD Stock Standard. Dilute to the mark with acetonitrile and mix well. Store below 0°C in glass. Stable for at least 1 year.
- l. Lysergide Testmix Stock Solution (25 ng/ml each component):  
To a 10-mL volumetric flask, add 125 µL each of the OH-LSD, LAMPA, and iso-LSD Intermediate Working Solutions. Dilute to the mark with acetonitrile and mix Well. Store below 0°C in glass. Stable for at least 1 year.
- m. LC-MS Performance Standard (5 ng/ml per component):  
Combine 30 µL of the Lysergide Testmix Stock Solution with 120 µL Optima-grade water and mix well. Prepare fresh daily. A 10 µL portion of this standard is analyzed before each day's samples, in order to confirm acceptable instrument performance.
- n. Negative Control Blood and/or Urine:  
Purchased from Cliniq, UTAK, Dynatek, or another approved vendor or prepared in-house from an appropriate blank specimen. Stability and storage determined by manufacturer. A negative control will be analyzed with every assay. When possible, the negative control will be matrix matched.
- o. Positive Control Blood and/or Urine (0.5 ng/ml LSD; 1 ng/ml OH-LSD):  
These are normally prepared in-house, but may be purchased from an approved vendor as needed. Normally prepared the day of extraction by adding 50 µL of Lysergide Control Working Solution to 2 mL of Negative Control Blood or Urine.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To properly labeled 16 x 100 mm screw-cap tubes add 2 mL of specimen or control.
- b. Add 20 µL of Internal Standard Working Solution and vortex briefly.
- c. Add 2 mL of 0.1 M, pH 6 Phosphate Buffer and vortex for 30 seconds.
- d. For blood and turbid urine specimens:

Centrifuge for 15 min at 3500 rpm. Decant supernatant into a 16 x 100 mm culture tube.

For non-turbid urine specimens, skip to step e.

- e. Place Cerex<sup>®</sup> PolyChrom<sup>™</sup> CLIN II solid phase columns (50 mg) 6 mL onto SPE positive pressure manifold.
- f. Load sample on SPE cartridges.
- g. Push sample through column using low range setting (setting 0-2).
- h. Wash column with 1 mL Potassium Carbonate Buffer ( $K_2CO_3/KHCO_3$ ) (pH 9) (setting 2-3).
- i. Wash column with 2 mL 0.1M HCl (setting 2-3).
- j. Wash column with 1 mL Methanol (setting 2-3).
- k. Wash with 3 mL Ethyl Acetate (setting 2-3).
- l. Elute with 4 mL Elution Solvent into 12 x 75 mm test tubes by gravity.
- m. Evaporate to dryness under nitrogen at 55°C.
- n. Reconstitute the residue in 100  $\mu$ L Reconstitution Solvent.
- o. Analyze 10  $\mu$ L of the extract by LC-ESI-FT-MS/MS using the instrumental conditions given in section 9, after verifying that the instrument is in proper working order.

## 9 Instrumental Conditions

Appendix 2 contains a checklist of method parameters to be used to verify proper instrument conditions prior to the analysis of case samples.

### 9.1 Liquid Chromatograph Parameters

Column Parameters								
Phase	Alltima® C18	Length		150 mm		Diameter		2.1 mm
Particle Size	5 µm	Temperature		35°C		Flow Rate		0.3 mL/min
Mobile Phase A:		Methanol (Optima)		Mobile Phase B:		LC Mobile Phase #1		
Gradient Program    All Ramps Are Linear								
Time (min)	0	3	10	19	20	25	26	31
%A	20	20	35	48	90	90	20	20
%B	80	80	65	52	10	10	80	80

### 9.2 Mass Spectrometer Parameters

Number of Segments: 4		Ionization Mode: Electrospray Positive Ion	
Segment #1 (0 - 6 min); 1 event			
Full Scan MS	Range: 200-400 m/z	FTMS 15000 resolution	
Segment #2 (6 - 13 min); 2 events			
#1: Full Scan MS	Range: 200-400 m/z	FTMS 15000 resolution	
#2: Full Scan MS/MS	Precursor: m/z 356.2	Range: 100 - 380 m/z	
FTMS 7500 resolution	Isolation Width: 2.0 Da	Collision: CID @ 20	
Segment #3 (13 - 20 min); 2 events			
#1: Full Scan MS	Range: 200-400 m/z	FTMS 15000 resolution	
#2: Full Scan MS/MS	Precursor: m/z 324.2	Range: 85 - 350 m/z	
FTMS 7500 resolution	Isolation Width: 2.0 Da	Collision: CID @ 40	
Segment #4 (20 - 31 min); 1 event			
Full Scan MS	Range: 200-400 m/z	FTMS 15000 resolution	

## 10 Decision Criteria

### 10.1 LC-MS Performance Standard Decision Criteria

#### 10.1.1 Chromatography

In order for the LC to be considered in good operating condition, full MS molecular ion traces for each analyte in the performance standard should generate reasonably symmetric shaped chromatographic peaks. m/z 356.196 is traced for OH-LSD and m/z 324.206 for iso-LSD and LAMPA. The retention times of the 3 analytes should be within  $\pm 5\%$  of the previous run of the performance standard. LAMPA and iso-LSD should be resolved with at least a 50% drop to baseline when displayed in an extracted ion chromatogram (EIC) with a  $\pm 0.005$  Da tolerance.

#### 10.1.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the three analytes in the performance standard should be present. The following ions should be present for each analyte. Each observed mass should be within  $\pm 0.005$  Da of the theoretical value.

OH-LSD:	MS – 356.196	MS/MS – 338.185 (base peak), 265.096, 237.102
Iso-LSD:	MS – 324.206	MS/MS – 281.164 (base peak), 251.117, 223.122, 208.075
LAMPA:	MS – 324.206	MS/MS – 281.164, 251.117, 223.122 (base peak), 208.075

### 10.2 Batch Acceptance Criteria

d<sub>3</sub>-LSD (m/z 327.226) must be detectable in an EIC for every extracted sample. None of the targeted lysergides (LSD, OH-LSD, LAMPA, iso-LSD) may be detected in the Negative Control Sample(s). Both LSD and OH-LSD must be detectable, based upon criteria given in 10.1.2 and using the mass spectral parameters of LAMPA for LSD, in the Positive Control Sample(s).

### 10.3 Analyte Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, all of the below should be met in order to identify LSD or OH-LSD within a biological specimen:

#### 10.3.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### 10.3.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or positive control.

### 10.3.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

### 10.3.2 Mass Spectrometry

The MS/MS fragmentation spectra should meet the criteria given in Toxicology SOP 104, *Guidelines for Comparison of Mass Spectra*, with comparison to a contemporaneously analyzed reference standard or positive control.

- a. LSD: (fragments of  $m/z$  324.206) The base peak should be  $m/z$  223.122.  $m/z$  281.164 and either 251.117 or 208.075 will be used for the calculation of ion ratios.
- b. OH-LSD: (fragments of  $m/z$  356.196) The base peak should be  $m/z$  338.185.  $m/z$  265.096 and 237.102 will be used for the calculation of ion ratios.

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

- a. Limit of Detection: LSD: at least 150 pg/mL in blood and urine  
OH-LSD: at least 300 pg/mL in blood and urine
- b. Interferences: Grossly decomposed or putrefied samples may adversely affect limits of detection. Iso-LSD is an isomer of LSD, and is not chromatographically resolved from

LSD. The two compounds yield the same MS/MS fragment ions, but with radically different ion ratios. The presence of significant levels of iso-LSD in a sample will adversely affect the limit of detection for LSD.

- c. LSD is known to be subject to photodegradation. Standards and processed samples should be stored in darkness whenever possible.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Johansen SS, Jensen JL, “Liquid chromatography-tandem mass spectrometry determination of LSD, Iso-LSD, and the main metabolite 2-oxo-3-hydroxy-LSD in forensic samples and application in a forensic case,” *J Chrom B* 825:21-28, (2005).

Sklerov JH, Magluilo J Jr, Shannon KK, et al, “Liquid chromatography –electrospray ionization Mass Spectrometry for the Detection of Lysergide and Major Metabolite, 2-oxo-3-hydroxy-LSD, in Urine and Blood,” *J Anal Tox* 24(7):543-9, (2000).

Horn CK, Klette, KL, Stout PR, LC-MS Analysis of 2-oxo-3-hydroxy-LSD from Urine using a Speedisk® Positive Pressure Processor with Cerex® Polychrom™ Clin II Columns,” *J Anal Tox* 27: 459-463, (2003).

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Moffat, A.C., *Isolation and Identification of Drugs*, 2nd ed., Pharmaceutical Press: London, 1986.

Winek, C. *Drug and Chemical Blood-Level Data*, 1994.

*Performance Monitoring Protocol (QA/QC) for the Thermo LTQ Orbitrap XL LC/MS (ESI)* (Inst 207): FBI Laboratory Chemistry Unit – Instrument Operations and Support Subunit Standard Operating Procedures Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104): FBI Laboratory Chemistry Unit – Toxicology Subunit Standard Operating Procedures Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
0	11/20/14	New document that replaces Tox 412-2.

**Approval**

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**Appendix 1: Abbreviated version of the LSD procedure for bench use.**

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**Appendix 2: Instrument parameters checklist for the LSD procedure.**

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## **Analysis of Low Level Opioids in Hair by UPLC/HRMS**

### **1 Introduction**

Opioids are a class of substances that include natural, semi-synthetic, and synthetic alkaloidal agents derived from opium or substances which have morphine-like activity. Naturally occurring opioids such as morphine and codeine are typically referred to as opiates. Heroin (diacetylmorphine) is a semi-synthetic opioid that is synthesized by the acetylation of morphine. In humans, heroin is rapidly metabolized to 6-monoacetylmorphine (6-AM) and morphine. Other common opioids include hydromorphone, hydrocodone, oxycodone, oxycodone, methadone, meperidine, and tramadol. Buprenorphine and fentanyl are two potent synthetic opioids which are usually given at a much lower dose than other opioids. These compounds and their metabolites may be found in the hair of individuals who have been exposed to the drugs.

### **2 Scope**

This procedure allows for the screening and confirmation of morphine, codeine, hydromorphone, hydrocodone, oxycodone, 6-AM, methadone, meperidine, tramadol, methadone, fentanyl, buprenorphine, and norbuprenorphine at low levels in hair.

### **3 Principle**

Hair samples are decontaminated with methylene chloride, water, and methanol washes before cryogrinding. The resulting hair powder is digested using a proteinase based solution. The resulting digest solution is cleaned up using solid phase extraction (SPE). Final extracts are analyzed by ultra performance liquid chromatography-high resolution mass spectrometry (UPLC/HRMS). Positive sample extracts are confirmed via UPLC/HRMS with tandem mass spectrometry.

### **4 Specimens**

20 milligrams of hair is preferred for each analysis. Lower amounts of hair may be used with an increase in limit of detection

### **5 Equipment/Materials/Reagents**

Specified items may be substituted with an equivalent material/product if necessary.

## 5.1 Hair Weighing, Decontamination, and Cryogrinding

Material / Equipment	Grade/Type	Supplier	Product/Part No.
Eppendorf vials, 2.0 mL, snap-cap	Polypropylene, curved/conical bottom	Fisher Scientific	05-408-138
Methylene chloride	HPLC/Optima	Varies	n/a
Water	Millipore/18mΩ	In-house	n/a
Methanol	HPLC/Optima	Varies	n/a
Laboratory balance	0.1 mg resolution	Varies	n/a
3.0 mm grinding balls	stainless steel	Retsch	22.445.0011
Cryogrinder	programmable	Retsch	MM200

## 5.2 Digestion

Material / Equipment	Grade/Type	Supplier	Product/Part No.
potassium phosphate, dibasic	ACS	Fisher	P-288-500
potassium phosphate, monobasic	ACS	Fisher	P-286-1
1.0 M potassium phosphate, dibasic	Weigh 87 g of potassium phosphate, dibasic to a 500 mL volumetric flask. Dilute to the mark with deionized water. Mix well and store refrigerated in glass. Stable for 3 months.		
1.0 M potassium phosphate, monobasic	Weigh 68 g of potassium phosphate, monobasic to a 500 mL volumetric flask. Dilute to the mark with deionized water. Mix well and store refrigerated in glass. Stable for 3 months.		
0.1 M potassium phosphate buffer, pH 8	Combine 94 mL of the 1.0 M potassium phosphate, dibasic solution and 6 mL of the 1.0 M potassium phosphate, monobasic solution. Dilute to 1 L with deionized water. Mix well and store refrigerated in glass. Stable for 3 months.		
Proteinase-K enzyme	Tritirachium album, ≥ 30 units/mg protein	Sigma-Aldrich	P-6556-100MG
Proteinase-K enzyme working solution, 40 mg/mL	Weigh 40 mg of proteinase-K into a tared Eppendorf snap-cap vial. Add 1mL of 0.1M potassium phosphate buffer, pH 8. Vortex and store refrigerated. Stable for 3 days. 1 mL of working solution is sufficient for 20 digests (0.050 mL per digest).		
Urea	Electrophoresis grade	Sigma-Aldrich	U-6504
Dithiothreitol (DTT)	≥ 98% (TLC)	Sigma-Aldrich	D0632
Calcium chloride, 1.0 M	Fluka/volumetric	Sigma-Aldrich	21114-1L

Digestion Solution	To a 15 mL conical tube, add 308 mg of DTT, 900 mg of urea and 0.050 mL of 1.0M calcium chloride. Dilute to 10 mL with 0.1M potassium phosphate buffer, pH 8. Vortex and store in heating block at 55°C until use. Stable for one day. Makes 10 mL of solution, which is sufficient for 20 digests (0.5 mL per digest).		
Thermomixer	programmable, capable of 55°C and 1000 rpm	Eppendorf	Model 5355, R
5% potassium phosphate, monobasic	Weigh 25 g potassium phosphate (monobasic) and dilute to 500 mL with deionized water. Mix well. Store refrigerated, stable for 3 months.		

### 5.3 Extraction

Material / Equipment	Grade/Type	Supplier	Product/Part No.
Positive pressure manifold	multi-sample, capable of 25 psi	SPEWare	289-0004
Concentrator	multiple sample, capable of 40°C	SPEWare	279-0050
SPE Column, Polychrom Clin II	SCX strong cation exchange, 35 mg, 3 mL capacity	SPEWare/Cerex	650-353
Potassium bicarbonate	ACS	Fisher	P9144-500
Potassium carbonate	ACS	Fisher	P208-500
Potassium carbonate buffer, pH 9	Dissolve 20 g of potassium bicarbonate and 10 g of potassium carbonate in 500 mL of deionized water. Adjust the pH to 9 and then dilute to 1 L. Store at ambient temperature. Stable for at least 3 months.		
Hydrochloric acid	ACS	Fisher	A144-500
0.1 M hydrochloric acid	Aliquot 800 µL of hydrochloric acid and dilute to 96 mL with deionized water. Stable for at least 3 months. Store in glass at room temperature.		
Ethyl acetate	ACS	Fisher	E195-1
Ammonium hydroxide	ACS	Fisher	A6695-500
Elution Solvent (98% ethyl acetate with 2% ammonia)	To 40 mL of ethyl acetate, add 0.8 mL of ammonium hydroxide. Mix well. Prepare fresh, just prior to elution.		
0.2µ centrifugal filtration vial, Spin-X	0.2µ, nylon	Fisher	CLS8169
Reconstitution solvent	95:5 water:acetonitrile	Combine 5 mL acetonitrile with 95 mL water (both Optima grade) and 0.1 mL	

	with 0.1% formic acid	formic acid and mix well. Store in glass at room temperature. Stable for 3 months.
--	-----------------------	--

#### 5.4 Liquid Chromatography/Mass Spectrometry

Material / Equipment	Grade/Type	Supplier	Product/Part No.
UPLC Binary Pump System	Acquity-I class	Waters	
Mass Spectrometer	Hybrid quadrupole-Orbitrap	Thermo Scientific	Q-Exactive
LC Column	BEH UPLC C18: 2.1x100 mm, 1.7u	Waters	186002352
Acetonitrile	HPLC/Optima	Fisher	A996-4
Water	HPLC/Optima	Fisher	W7-4
Formic Acid	reagent	Sigma/Fluka	94318
Mobile Phase A, Aqueous / Weak Wash	In a 500 mL graduated cylinder, add 250 mL of Optima grade water. Add 0.5 mL of formic acid. Dilute to 500 mL and mix well. Stable for 5 days. To prevent microbial growth discard after 5 days.		
Mobile Phase B, Organic / Strong Wash	In a 500 mL volumetric cylinder, add 250 mL of Optima grade acetonitrile. Add 0.5 mL of formic acid. Dilute to 500 mL and mix well. Stable for at least 3 months.		
Seal Wash, Solvent A2	Methanol:Water 1:1. Combine equal volumes of each solvent. Store at ambient temperature. Stable for at least 6 months.		
Solvent B2	Acetonitrile		

#### 5.5 Miscellaneous Laboratory Supplies

Material / Equipment	Grade/Type	Supplier	Product/Part No.
Pipettes	20µL to 1 mL range	varies	n/a
Heating block	n/a	Varies	n/a
Falcon 15mL conical tube	n/a	Fisher-Scientific	14-959-49D
Aluminum weighing dish	fluted sides, tab	Fisher-Scientific	08-732-100
Centrifuge	micro-vial size, 10,000 rpm capable	varies	n/a
Centrifuge	12x75 mm size, 1500 rpm capable	varies	n/a

Glass tubes, 12 x 75 mm	n/a	Fisher Scientific	14-961-26
Autosampler vials with inserts	9 mm with insert	Wheaton	09-1200-101
Autosampler caps	9 mm, Blue S/T	Wheaton	09-0034B

## 6 Standards and Controls

### 6.1 Internal Standards (Cerilliant, or other approved vendor. Stability determined by manufacturer)

Analyte	Concentration (mg/mL)	Solvent	Product No.	Aliquot for Stock (mL)
d <sub>3</sub> -Morphine	0.1	methanol	M-003	0.250
d <sub>6</sub> -Codeine	0.1	methanol	C-040	0.250
d <sub>3</sub> -Oxymorphone	0.1	methanol	O-003	0.250
d <sub>6</sub> -Oxycodone	0.1	methanol	O-005	0.250
d <sub>3</sub> -Hydromorphone	0.1	methanol	H-006	0.250
d <sub>3</sub> -Hydrocodone	0.1	methanol	H-005	0.250
d <sub>3</sub> -Tramadol-13C	0.1	methanol	T-029	0.250
d <sub>3</sub> -Methadone	0.1	methanol	M-008	0.250
d <sub>4</sub> -Meperidine	0.1	methanol	M-036	0.250
d <sub>4</sub> -Buprenorphine	0.1	methanol	B-901	0.025
d <sub>3</sub> -Norbuprenorphine	0.1	methanol	N-920	0.025
d <sub>5</sub> -Fentanyl	0.1	methanol	F-001	0.025
d <sub>3</sub> -6-AM	0.1	acetonitrile	A-010	0.250

#### 6.1.1 Stock Internal Standard Solution (methanol, 0.005/0.0005 mg/mL)

Aliquot the standards as indicated in section 6.1 (except d<sub>3</sub>-6-AM) into a 5 mL volumetric flask. Dilute to the mark with Optima grade methanol. Store below 0°C in glass. Stable for at least one year.

#### 6.1.2 Stock Internal Standard Solution, d<sub>3</sub>-6-AM (acetonitrile, 0.005 mg/mL)

Aliquot the d<sub>3</sub>-6-AM standard as indicated in section 6.1 into a 5 mL volumetric flask. Dilute to the mark with Optima Grade acetonitrile. Store below 0°C in glass. Stable for at least one year.

#### 6.1.3 Working Internal Standard Solution (aqueous, 0.05/0.005 µg/mL)

Aliquot 0.050 mL of both of the stock internal standard solutions to a partially filled 5

mL volumetric flask. Bring to the mark with HPLC grade water. Store refrigerated in glass. Stable for approximately one week.

## 6.2 Controls (Cerilliant, Lipomed or other approved vendor. Stability determined by manufacturer)

Analyte	Concentration (mg/mL)	Solvent	Product No.	Aliquot for Stock (mL)
Morphine	1	methanol	M-030	0.250
Codeine	1	methanol	C-006	0.250
Oxymorphone	1	methanol	O-004	0.250
Oxycodone	1	methanol	O-002	0.250
Hydromorphone	1	methanol	H-004	0.250
Hydrocodone	1	methanol	H-003	0.250
Tramadol	1	methanol	T-027	0.250
Methadone	1	methanol	M-019	0.250
Meperidine	1	methanol	M-035	0.250
Buprenorphine	1	methanol	B-902	0.025
Norbuprenorphine	1	methanol	N-912	0.025
Fentanyl	1	methanol	F-002	0.025
6-AM	1	acetonitrile	A-009	0.250

### 6.2.1 Stock Control Solution (methanol, 0.01/0.001 mg/mL)

Aliquot the standards as indicated in section 6.2 (except 6-AM) into a 25 mL volumetric flask. Dilute to the mark with Optima grade methanol. Store below 0°C in glass. Stable for at least one year.

### 6.2.2 Stock Control Solution, 6-AM (acetonitrile, 0.01 mg/mL)

Aliquot the 6-AM standard as indicated in section 6.1 into a 5 mL volumetric flask. Dilute to the mark with Optima grade acetonitrile. Store below 0°C in glass. Stable for at least one year.

### 6.2.3 Working Control Solution (high, aqueous, 80/8 ng/mL)

Aliquot 0.080 mL of both of the stock standard solutions to a partially filled 10 mL volumetric flask. Dilute to the mark with HPLC grade water. Store refrigerated in glass. Stable for approximately one week.

### 6.2.4 Working Control Solution (low, aqueous, 4/0.4 ng/mL)

Aliquot 0.50 mL of the Working Standard Solution (High) to a partially filled 10 mL

volumetric flask. Bring to the mark with HPLC grade water. Store refrigerated in glass. Stable for approximately one week.

**6.3** Column Performance Evaluation Mix (2/0.2 ng/mL):

Aliquot 0.05 mL of the Working Standard Solution (low, aqueous) to an autosampler vial. Aliquot 0.05 mL of reconstitution solvent and mix. Stable for approximately one week.

**6.4** Negative Control Hair: Obtained from drug-free donors. Stored in paper or plastic at room temperature. Negative Control Hair does not expire.

**6.5** Negative Control Hair Powder: Obtained by washing Negative Control hair with methylene chloride, water, and methanol; drying the hair, and pulverizing in a cryogrinder. Negative Control Hair Powder is stored in plastic at room temperature and does not expire.

**6.6** Low Positive Control Hair Sample (5/0.5 pg/mg): Prepared by spiking 20 mg Negative Control Hair Powder with 0.025 mL of the Working Standard Control Solution (Low, Aqueous).

**6.7** High Positive Control Hair Sample (100/10 pg/mg): Prepared by spiking 20 mg Negative Control Hair Powder with 0.025 mL of the Working Standard Control Solution (High, Aqueous).

## **7 Sampling**

Not applicable.

## **8 Procedure**

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### **8.1 Hair Weighing, Decontamination, and Cryogrinding:**

- a. Visually inspect hair and record observations.
- b. If segmental analysis is required, cut a portion of the hair sample into 2-cm segments.
- c. Weigh approximately 20 mg of each hair sample (or segment) into a properly labeled 2.0 mL Eppendorf tube. Record weight to the nearest 0.1 mg.



- d. Wash each hair sample with 1.0 mL methylene chloride by vortexing for approximately 1 minute. Discard this wash.
- e. Wash each hair sample with 1.0 mL water (Millipore) by vortexing the sample for approximately 1 minute. Discard this wash.
- f. Wash each hair sample with 1.0 mL HPLC Grade methanol by vortexing for approximately 1 minute. This wash may be capped and stored refrigerated for later analysis, if necessary.
- g. Dry hair samples in a heating block or sample concentrator at approximately 40°C to evaporate any remaining solvent. Dry for 30 minutes or until samples are dry.
- h. Cryogrind dry hair samples using the settings in Section 9.5 of this procedure. Tap tubes to loosen any cryoground hair from the cap before proceeding.
- i. Similarly, prepare negative and positive control samples as directed in Section 6.
- j. Open tubes, and add 0.5 mL Digestion Solution, 0.050 mL Proteinase-K Working Solution, and 0.020 mL Internal Standard Working Solution to each Eppendorf tube. Recap and vortex briefly.
- k. Thermomix for 60 minutes at 55°C and 750 rpm (may be extended if necessary).
- l. Add 1 mL of 5% potassium phosphate monobasic, continue thermomixing at 55°C and 750 rpm for five minutes.
- m. Centrifuge samples at ~10,000 rpm for 5 minutes, decant supernatant to 12 x 75 mm glass tube.
- n. Add an additional 1 mL of 5% potassium phosphate, monobasic.

## **8.2 Solid Phase Extraction:**

- a. Condition SPE extraction cartridges: 3 mL of methanol, followed by 1 mL of deionized water at 1-2 mL/minute.
- b. Load sample onto cartridge at 1-2 mL/minute.
- c. Wash cartridge with 1 mL of potassium carbonate buffer, 1 mL of water, 1 mL of 0.1 M hydrochloric acid, and 1 mL of methanol (each at 1-2 mL/minute).

- d. Dry cartridge at 25 psi for 5 minutes.
- e. Apply 2 mL of Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm test tubes.
- f. Evaporate to dryness under nitrogen at 40°C.
- g. Reconstitute the dry residue in 75 µL of Reconstitution Solvent. Centrifuge the 12 x 75 mm tubes for 1 min at 1500 rpm to consolidate the solvent.
- h. Transfer the solvent to a 0.2µ filtration vial. Centrifuge at 10,000 rpm for 5 minutes.
- i. Transfer the eluent to an autosampler vial with insert.
- j. Analyze 10 µL portions by UPLC/HRMS with the conditions given in section 9. Analysis may include full scan, SIM, or tandem mass spectrometry modes depending upon the case scenario. Positive case samples may be reinjected (with appropriate controls) by tandem UPLC/HRMS for confirmatory analysis.

### **8.3 Analysis of wash samples (optional):**

- a. For samples in which an opiate is identified above the LOD of the method, add 20 µL Internal Standard Solution to each wash.
- b. Evaporate to dryness under a gentle stream of nitrogen at approximately 40°C.
- c. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.
- d. Extract using the procedure in Section 8.2 above. Analyze 10 µL portions by LC/HRMS with the conditions given in section 9.

## **9 Instrumental Conditions**

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

## 9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
A: 0.1% formic acid in water	total flow	0.40 mL/min		type	C18
	time (min)	% A	% B	length	100 mm
B: 0.1% formic acid in acetonitrile	0	98	2	internal diameter	2.1 mm
	0.5	98	2	particle size	1.7 µm
	5	50	50	temperature	50°C
	5.5	10	90	Autosampler Temp: 10°C Seal Wash: 5.00 min ACQ-SM: Weak Wash Vol: 600 µL Strong Wash Vol: 200 µL	
	7.5	10	90		
	7.75	98	2		
	9.75	98	2		
	total time		9.75 min		

## 9.2 Mass Spectrometer Parameters – Full Scan and SIM

Parameter	Full Scan	tSIM	Parameter	Value
Runtime	0 to 9.75 min	0 to 9.75 min	Mode	ESI
Polarity	positive	positive	Spray Voltage	+3.5 kV
In-Source CID	0.0 eV	0.0 eV	Capillary Temperature	375°C
Inclusion	-	on	Heater Temperature	350°C
Microscans	1	1	Sheath Gas	35
Resolution	35,000	35,000	Aux Gas	1
AGC Target	1e6	2e4	Sweep Gas	0
Maximum IT	50 ms	100 ms	S-Lens RF Level	70
MSX Count	-	1	For Inclusion List Values see Section 9.4	
Isolation Window	-	1.5 m/z		
Scan Ranges	1	-		
Scan Range	100-650 m/z	100-650 m/z		

## 9.3 Mass Spectrometer Parameters – Full Scan and Tandem MS

Parameter	Full Scan	tMS2	Parameter	Value
Runtime	0 to 9.75 min	0 to 9.75 min	Mode	ESI
Polarity	positive	positive	Spray Voltage	+3.5 kV
In-Source CID	0.0 eV	0.0 eV	Capillary Temperature	375°C
Inclusion	-	on	Heater Temperature	350°C
Microscans	1	1	Sheath Gas	35

Resolution	17,500	17,500	Aux Gas	1
AGC Target	1e6	2e4	Sweep Gas	0
Maximum IT	50 ms	100 ms	S- Lens RF Level	70
MSX Count	-	1	For Inclusion List Values see Section 9.4	
Isolation Window	-	1.5 m/z		
Scan Ranges	1	-		
Scan Range	100-650 m/z	100-650 m/z		

#### 9.4 Mass Spectrometer Parameters – Inclusion List for tSIM and tMS2

Mass (m/z)	Polarity	Start (min)	End (min)	NCE (%)	CS (z)	Name
286.14445	Positive	1.40	1.62	55	1	morphine
302.13935	Positive	1.60	1.74	50	1	oxymorphone
286.14445	Positive	1.70	1.88	55	1	hydromorphone
300.16007	Positive	2.11	2.28	55	1	codeine
316.15500	Positive	2.28	2.45	42	1	oxycodone
328.15500	Positive	2.30	2.50	55	1	6-AM
300.16007	Positive	2.40	2.59	55	1	hydrocodone
264.19648	Positive	3.05	3.24	10	1	tramadol
248.16517	Positive	3.30	3.45	45	1	meperidine
414.26456	Positive	3.50	3.65	62	1	norbuprenorphine
337.22809	Positive	3.88	4.02	35	1	fentanyl
256.17025	Positive	4.04	4.12	70	1	diphenhydramine*
468.31150	Positive	4.16	4.30	62	1	buprenorphine
310.21720	Positive	4.68	4.78	30	1	methadone

*\*inclusion optional*

#### 9.5 Cryomill Parameters

Cycles	1
Precool	Auto
Run time	6.5 min
Rate	25 hz

#### 10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control.

## 10.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

### 10.1.1 Retention Time

The retention time of the peak should be within  $\pm 2\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or Positive Control.

### 10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

## 10.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

## 11 Calculations

Not applicable.

## 12 Measurement Uncertainty

Not applicable.

## 13 Limitations

### a. Limit of Detection (LOD):

The LOD is administratively set at 10 pg/mg for all drugs except fentanyl, buprenorphine, and norbuprenorphine, which each have an LOD of 1 pg/mg. Lower amounts may be

reported if the decision criteria in Section 10 are met.

b. Processed Sample Stability:

All analytes are stable in processed sample extracts for at least four days except for buprenorphine and norbuprenorphine. After three days norbuprenorphine partially converts to buprenorphine.

c. Selectivity:

A low level interferent for tramadol is observed in some samples which coelutes and has a similar parent mass (264.195). In order to establish tramadol as detected in a sample, there must also be a) tandem mass spectrometry that passes Tox 104 criteria, b) have a detectable isotope trace (265.199) with a  $17 \pm 2$  % ratio, and c) have an estimated concentration of greater than 1 pg/mg. No other interferences were observed.

d. Diphenhydramine and other commonly detected drugs:

Diphenhydramine and other common over-the-counter/prescription drugs may be detected by this method. In order to report such a drug as detected in a case sample, that drug must not be detected in the Negative Control Hair used for the batch.

## 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 15 References

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Kumar, P., et al., "Targeted analysis with benchtop quadrupole-orbitrap hybrid mass spectrometer: Application to determination of synthetic hormones in animal urine", *Analytica Chimica Acta*, 780 (2013), pp. 65-73.

Hill, V., et al., "Multiple Aspects of Hair Analysis for Opiates: Methodology, Clinical and Workplace Populations, Codeine, and Poppy Seed Ingestion", *Journal of Analytical Toxicology*, Vol. 29, pp. 696-703, October 2005.

Baumgartner, W., Hill, V., "Sample Preparation Techniques", *Forensic Science International*, 63

(1993), pp. 121-135.

Dioumaeva, I., “LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood Using Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 Column”, Application Note, 2013.

*FBI Laboratory Safety Manual.*

*Instrument Subunit SOP Manual*; FBI Laboratory Chemistry Unit.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Rev. #	Issue Date	History
0	12/19/14	New document.

**Approval**

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**Appendix 1: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS  
Materials Preparation and Procedure for bench use (page 1)**

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**Appendix 1: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS  
Materials Preparation and Procedure for bench use, continued (page 2)**

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**Appendix 1: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS  
Materials Preparation and Procedure for bench use, continued (page 3)**

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**Appendix 2: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS  
Instrumental Parameters**

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## **Quantitation and Confirmation of Gabapentin**

### **1 Introduction**

Gabapentin is a drug used for its anticonvulsant and analgesic properties, however, the exact mechanism of action for gabapentin is not understood. Structurally, gabapentin resembles GABA ( $\gamma$ -amino butyric acid) but does not interact or interfere with GABA<sub>A</sub> or GABA<sub>B</sub> neurotransmitter sites within the body; furthermore, gabapentin crosses the blood-brain barrier unlike GABA. Gabapentin is not protein bound to any significant extent and is eliminated as the parent drug with a half-life of approximately five to seven hours.

### **2 Scope**

This procedure allows for the quantitation and confirmation of gabapentin in blood. It may also be used for the confirmation of gabapentin in urine with proper validation.

### **3 Principle**

Specimens are mixed with a deuterated analog internal standard, prepared for solid phase extraction using dilution, pH adjustment, and centrifugation. The specimens are extracted using Clean Screen<sup>®</sup> DAU solid phase extraction (SPE) cartridges and eluted from the cartridges using a solvent mixture. The eluent is then taken to dryness and reconstituted in a methanol water mixture and analyzed by liquid chromatography – electrospray – mass spectrometry (LC-ESI-MS).

### **4 Specimens**

This procedure is validated for whole blood. Typically, 2 x 0.5 mL samples are analyzed; however, samples suspected to be above the procedure's linear range may be diluted before extraction.

### **5 Equipment/Materials/Reagents**

- a. 16 x 100 mm screw-top tubes with caps
- b. 12 x 75 mm culture tubes with polypropylene snap-tops
- c. Methanol (Optima grade or better)
- d. Formic Acid (Puriss grade or better)

- e. 0.1 M Sodium Phosphate Buffer (pH 6.0):  
To a 500 mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve, verify that the pH is between 5.8 and 6.1; fill to the mark with deionized water. Store refrigerated in glass. Solution is stable for two months.
- f. 0.1 M Hydrochloric Acid (0.1 M HCl):  
To a 100 mL graduated cylinder, add 80 mL deionized water and 0.8 mL concentrated hydrochloric acid. Bring to 96 mL with deionized water and mix well; store at room temperature in glass. Solution is stable for six months.
- g. Water (Deionized, 18 MΩ)
- h. Centrifuge
- i. Evaporator w/ Nitrogen
- j. Vortex mixer
- k. Methanol:Water (10:90 v:v):  
To a 50 mL graduated cylinder, add 5 mL methanol (Optima grade) and 45 mL water (Optima grade), mix well; store at room temperature in glass. Solution is stable for one year.
- l. Elution Solvent [Methylene Chloride/Isopropanol/Ammonium Hydroxide (78:20:2)]:  
To a 100 mL graduated cylinder, add 78 mL methylene chloride, 20 mL isopropanol, and 2 mL ammonium hydroxide, mix well. Solution is to be made and used on the same day.
- m. Clean Screen DAU<sup>®</sup> SPE cartridges (regular flow) - 200 milligrams
- n. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
- o. Liquid Chromatograph - Mass Spectrometer capable of 15,000 resolution.
- p. HPLC Column (Xterra C-18 MS, 3.0 x 150 mm, 3.5 μm dp; or equivalent)
- q. Mobile Phase A (0.1% Formic Acid in Acetonitrile):  
To a 500 mL graduated cylinder, add 500 mL acetonitrile (Optima grade) and 0.5 mL formic acid, mix well; store at room temperature in glass. Solution is stable for two months.
- r. Mobile Phase B (0.1% Formic Acid in Water)  
To a 500 mL graduated cylinder, add 500 mL water (Optima grade) and 0.5 mL formic acid,

mix well; store at room temperature in glass. Solution is stable for two months.

## 6 Standards and Controls

- a. **d<sub>10</sub>-Gabapentin Stock Standard (1.00 µg/mL):**  
 Purchased from Cerilliant International or equivalent manufacturer. Storage conditions and stability determined by manufacturer.
- b. **Gabapentin Stock Standard (1.0 mg/mL):**  
 Purchased from Cerilliant International and Lipomed or equivalent manufacturers. Storage conditions and stability determined by manufacturer.
- c. **Internal Standard Working Solution (1.00 µg/mL):**  
 The Cerilliant, or equivalent manufacturer, deuterated stock solution is used as received. Store in glass at or below 0°C after opening; stable for at least six months.
- d. **High Calibration Working Solution (1.0 mg/mL):**  
 Purchased from Cerilliant International or equivalent manufacturer. Storage conditions and stability determined by manufacturer.
- e. **Low Calibration Working Solution (1.00 µg/mL):**  
 Add 0.5 mL of the 1.0 mg/mL Standard Stock Solution in a 5-mL volumetric flask and bring to the mark with Methanol. Store in glass at or below 0°C; stable for at least six months.

Table 1: Calibrator Preparation for Gabapentin (Add these amounts to 0.5 mL Negative Control Blood)

Calibrator Level (µg/mL)	High Cal WS (1 mg/mL) Volume (µL)	Low Cal WS (100 µg/mL) Volume (µL)
5	-	25
10	-	50
20	-	100
30	-	150
46	23	-
60	30	-
80	40	-
100	50	-

- f. **High Control Working Solution (1.0 mg/mL):**  
 The Lipomed, or equivalent manufacturer, stock solution is used as received. Store in glass at or below 0°C after opening; stable for at least six months after opening.

- g. Low Control Working Solution (100 µg/mL):  
Add 0.5 mL of the 1.0 mg/mL Standard Stock Solution in a 5 mL volumetric flask and bring to the mark with Methanol. Store in glass at or below 0°C; stable for at least six months.
- h. Negative Control Blood:  
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or obtained in-house from a drug-free donor. Store refrigerated or frozen. Stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.
- i. Quantitative Positive Control Blood:  
This is normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as needed. A Quantitative Positive Control Blood sample will be extracted in duplicate with every quantitative assay. The Quantitative Positive Controls will typically be prepared fresh from Control Working Solutions as described below:
  - 1. Low Control (15 µg/mL):  
Add 75 µL of the Low Control Working Solution to 0.5 mL of Negative Control Blood.
  - 2. High Control (80 µg/mL):  
Add 40 µL of the High Control Working Solution to 0.5 mL of Negative Control Blood.
- j. Qualitative Positive Control Blood:  
This is normally prepared in-house, but may be purchased from an appropriate vendor as needed. A Qualitative Positive Control Blood sample will be extracted and analyzed with every qualitative blood assay. The Qualitative Positive Control will typically be prepared fresh at any concentration above the assay's limit of detection from Control Working Solutions.
- k. Column Performance Mix: Dilute 0.010 mL of the Internal Standard Working Solution with 0.090 mL of Methanol:Water (10:90 v:v). Prepare fresh.

## 7 Sampling

Not applicable.



## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. To a properly labeled 16 x 100 mm screw-top tube, add 0.5 mL of specimen, calibrator or control. Prepare case specimens and positive controls in duplicate for quantitative analysis. Smaller volumes may be analyzed if required to ensure that the sample is within the linear range of the procedure. Case samples and positive controls do not need to be prepared in duplicate for qualitative analysis.
- b. Add 30  $\mu$ L of the Internal Standard Working Solution to each sample.
- c. Bring all samples to approximately 5 mL with deionized water and vortex.
- d. Add 2 mL of 0.1 M phosphate buffer to each sample and vortex. Allow to stand for 5 minutes.
- e. Verify the pH of each sample is  $6.0 \pm 0.5$ .
- f. Centrifuge for 10 minutes at 3500 rpm.
- g. Prepare appropriately labeled SPE cartridges by conditioning each cartridge with 3 mL of methanol, 3 mL of deionized water, and 1 mL of 0.1 M phosphate buffer.
- h. Load the samples onto the appropriate SPE cartridges.
- i. Rinse the column with 2 mL of deionized water, 2 mL of 0.1 M HCl solution, and 3 mL of methanol.
- j. Dry the cartridges under full vacuum for 90 seconds.
- k. Apply 3 mL of freshly prepared Elution Solvent and collect eluent into appropriately labeled 12 x 75 mm test tubes.
- l. Evaporate the eluent to dryness under nitrogen at 40°C.
- m. Reconstitute each sample in 200  $\mu$ L Methanol:Water (10:90 v:v). Transfer 100  $\mu$ L to an appropriately labeled autosampler vial.
- n. Analyze 10  $\mu$ L of each sample by LC/MS using the conditions below after verifying that the instrument is performing properly by analyzing the Column Performance Mix.

## 9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

### 9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions		Flow Parameters			Column Parameters	
A: Acetonitrile with 0.1% Formic Acid		total flow	0.3 mL/min		type	C18-MS
		time (min)	%A	%B	length	150 mm
B: Water with 0.1% Formic Acid		0	10	90	internal diameter	3.0 mm
		5	10	90	particle size	3.5 μm
		20	90	10	temperature	30°C
Autosampler Parameters		30	90	10		
injection volume	10 μL	31	10	90		
temperature	15°C	37	10	90		
		total time	37 min			

### 9.2 Mass Spectral Parameters

ionization mode	electrospray (+)
scan mode	full scan, centroid
scan range	85 - 400 AMU
resolution	15,000
All source parameters are set through the instrument tuning process. See the Instrument Operations and Support Subunit SOP Manual for details.	

## 10 Decision Criteria

### 10.1 Batch Acceptance Criteria

No gabapentin should be detected in the Negative Control.

Gabapentin should be present in the Positive Control. Each Quantitative Positive Control will quantitate within  $\pm 20\%$  of the target value. See the Guidelines for Toxicological Quantitations standard operating procedure (Tox 101) for more information.

## 10.2 Sample Acceptance Criteria

### 10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. Ion peaks are typically extracted at  $\pm 5$  mmu. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 10.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time (relative or absolute, as appropriate) obtained from injection of an extracted Positive Control or extracted calibrator.

#### 10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Note: nonsensical signal to noise values may result from high resolution mass spectral data. Further, the baseline signal for the peak of interest should be at least ten-fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

### 10.2.2 Mass Spectrometry

The M+1 for gabapentin in each sample should be  $172.133 \pm 5$  mmu.

The M+1 for d<sub>10</sub>-gabapentin in each sample should be  $182.196 \pm 5$  mmu.

## 11 Calculations

Quantitation is performed by constructing a multi-point calibration curve based on the ratio of the area for the M+1 peak for the analyte to the internal standard. Ion traces are drawn at a 5 mmu mass tolerance. 1/x weighting is used. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

## 12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the pipette used to deliver the sample
- accuracy of the pipette used to deliver the calibrators

- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

### 13 Limitations

a. Linear range using 1/x weighting and a linear calibration model is 5 – 100 µg/mL.

b. Limit of Detection is 1 µg/mL

c.

	6 µg/mL	40 µg/mL	80 µg/mL
Bias (n=15)	-13.66	1.38	-2.96
Repeatability (n=15)	4.82	5.75	6.00
Intermediate Precision (n=15)	7.53	8.98	8.27

d. Interferences: None observed.

e. Carryover: For extracted negative control samples analyzed immediately following extracted 100 µg/ml calibrator samples, no carryover was observed.

f. Processed sample stability: the calculated difference between day 0 and day 1, 3, and 7 never exceeded ±10%.

### 14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

### 15 References

*Guidelines for Toxicological Quantitations* (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Practices for Validating Chemical Procedures*; FBI Laboratory Operations Manual.

*Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

*Guidelines for Comparison of Mass Spectra* (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

*FBI Laboratory Safety Manual.*

*Baselt, Randall; Disposition of Toxic Drugs and Chemicals in Man, 9<sup>th</sup> ed.; 2011*

*Neurontin<sup>®</sup> (Gabapentin) Product Monograph. Parke-Davis, Division of Pfizer Inc. NY, NY 10017; Apr2009.*

Rev.#	Issue Date	History
0	08/20/15	New document.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Quantitation and Confirmation of Gabapentin  
Procedure for bench use**

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Redacted - Form on File

**Appendix 2: Abbreviated version of the Instrumental Parameters for bench use**

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