Direct Solvent Extraction of Acid/Neutral Drugs from Biological Fluids

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1 INTRODUCTION

This procedure detects common acidic and neutral drugs in biological fluids.

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☐ Quantitation
Matrices	Blood, Urine
Analytes	Selected acid-neutral drugs
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 PRINCIPLE

Specimens are mixed with an internal standard, adjusted to an acidic pH, and extracted with an ether:toluene mixture. 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 SPECIMEN CRITERIA

This procedure uses 0.5 mL of blood or urine.

5 EQUIPMENT

5.1 Equipment

A. Vortex mixer
B. Centrifuge
C. Rotator
D. Evaporator with nitrogen

5.2 Consumables

A. 16 x 125/100 mm screw-top tubes
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. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.
E. 10 mL glass centrifuge tubes (with conical bottom)
F. ALS (automatic liquid sampler) vials 12x32 mm with glass inserts
G. Mid-Range pH paper

5.3 Instruments

A. Gas Chromatograph / Mass Spectrometer with electron impact (EI) source equipped with a 30 m x 0.25 mm x 0.25 µm Rtx-5MS (or equivalent) column

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	7 Pro SP 1
GC/MS	Enhanced Chemstation	F.01.03.2357
Autosampler	Gerstel Maestro 1	1.5.3.3/3.5
Data Analysis	Xcalibur	4.0

5.5 Chemicals/Reagents

5.5.1 Purchased

A. N- Hexane (≥ 95%)
B. Potassium phosphate, monobasic (ACS grade or equivalent, KH_2PO_4)
C. Diethyl ether (High purity grade or equivalent)
D. Toluene (HPLC grade or equivalent)
E. Chloroform (GC^2 grade or equivalent)
F. Methanol (Optima, GC^2 grade or equivalent)
G. Ethanol (Pharmaceutical grade or equivalent)
H. Water (Deionized)
I. Dichloromethane (Optima grade or equivalent)

5.5.2 Prepared

A. 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<pH<5.0. Store refrigerated in glass. Stable 1 month.

B. 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.

C. Chloroform:Methanol ($CHCl_3$:MeOH) (4:1 v:v):

Combine 40 mL chloroform with 10 mL methanol. Mix well. Store in brown glass at room temperature. Stable 1 month.

D. 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.

5.6 Standards/Controls

5.6.1 Purchased

Storage and stability determined by manufacturer, unless otherwise noted.

A. Methylphenylhydantoin (MPH)

B. Negative Control:

Purchased from Cliniqa or an equivalent supplier, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh.

C. 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.

D. Positive Control Solution Components:

In addition to the Barbiturate Mix-5, target analytes (carbamazepine, carisoprodol, ibuprofen, meprobamate, and phenytoin) are obtained from an approved vendor in liquid (1 mg/mL) or solid form.

E. Acetaminophen Standard (1 mg/mL):

Purchased as a 1 mg/mL solution in methanol from Cerilliant or another approved supplier.

5.6.2 Prepared

A. 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.

B. Methylphenylhydantoin Working Internal Standard (30 µg/mL):

Add 0.75 mL of the MPH Stock Standard to a 25 mL volumetric flask. Dilute to the mark with deionized water. Store refrigerated in glass. Stable for at least 2 years.

C. Positive Control Solution

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 the table below:

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.)

D. Positive Control:

100 µL of the Positive Control Solution is added to 0.50 mL of the Negative Control matrix on the day of analysis. Optional: 50 µL of a 1 mg/mL acetaminophen standard can be added directly to the Positive Control as well (yields a 100 µg/mL concentration). Other positive controls preparations may be used as is appropriate.

6 PROCEDURE

	Step	Note	Reference/Lot
	A. Samples		
	1. To labeled 16 x 125/100 mm screw-top tubes add:		
☐	i. 0.5 mL of blood or urine		
	B. Controls		
☐	1. Prepare Negative Control(s)		
☐	2. Prepare Positive Control(s)		
☐	**C. QS to 1 mL with deionized water**		
	D. Internal Standard(s)		
☐	1. Add 25 µL of MPH Internal Standard Solution		
	E. Buffer		
☐	1. Add 1 mL of 5% KH_2PO_4 buffer solution		
☐	2. Check urine pH to ensure pH is between 4 and 6		
	F. Extract		
☐	1. Add 5 mL of ether:toluene (1:1)		
☐	2. Extract for 20 minutes on a rotator		
☐	3. Centrifuge 5 minutes		
	i. If emulsions develop, break up with wooden stick and recentrifuge		
☐	4. Transfer organic (top) layer to a 16 x 100 mm tube		
	G. Concentrate/Clean Up		
☐	1. Evaporate solvent to dryness under nitrogen at 50°C.		
☐	2. Transfer dried residue to a 10 mL glass centrifuge tube with two successive 1 mL washes of $CHCl_3$:MeOH (4:1)		
☐	3. Evaporate solvent to a dry residue under nitrogen at 50°C.		
☐	4. Add 2 mL hexane		
☐	5. Add 200 µL 80% ethanol		
☐	6. Vortex for 30 seconds		
☐	7. Centrifuge for 5 minutes		

☐	8. Discard the hexane (top) layer (optional)		
☐	9. Transfer ethanol layer to a fresh 12 x 75 mm tube		
☐	10. Evaporate ethanol layer to a dry residue under nitrogen at 50°C		
	H. Reconstitute		
☐	1. Add 50 µL of $CHCl_3$:MeOH (4:1) 2. Vortex		
☐	3. Transfer to ALS vial		
☐	**I. Analyze 2 µL by GC/MS** 1. Analyze methylene chloride blanks at the sequence start and after positive control to reduce carryover. Analyze methanol blanks prior to each case sample.		

7 ANALYTICAL PARAMETERS

7.1 Agilent Gas Chromatograph

7.1.1 Oven – Standard Conditions

Step	Temperature (°C)	Hold (min)	Ramp (°C/min)
1	45	1	25
2	150	2	15
3	280	14	

Total Run Time (min): 29.87

7.1.2 Inlet/Carrier/Column

Inlet		**Carrier**		**Column**	
Temperature (°C)	220	**Gas**	ultrapure helium	**Type**	DB-5MS
Injection Mode	Split	**Mode**	constant flow	**Length (m)**	30
Split Flow	12	**Flow (mL/min)**	1.2	**Internal Diameter (mm)**	0.25
Split Ratio	10:1			**Film Thickness (µm)**	0.25

7.2 Agilent Mass Spectrometer

	Standard Conditions
Ionization Mode	Electron Impact (+)
Scan Mode	Full Scan
Scan Range (m/z)	35-500
Solvent Delay (min)	3.0
Temperatures (°C)	
Source	230
Quadrupole	150
Transfer Line	280

8 DATA ANALYSIS

Data for the batch is converted to Xcalibur RAW files. Layouts with extracted ion chromatograms (EIC) are applied in Xcalibur according to the Scope required for the case sample(s).

Analyte	Applicable Scope(s)	Extracted Ions (m/z)
Acetaminophen	Postmortem (Full)	109, 151, 80
Amobarbital	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	156, 141, 157
Butalbital	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	168, 167, 41
Carbamazepine	Postmortem (Full)	193, 192, 191
Carisoprodol	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	104, 55, 158
Levetiracetam	Postmortem (Full)	126, 69, 41
Meprobamate	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	83, 55, 71
Pentobarbital	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	156, 141, 157
Phenobarbital	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	204, 117, 115
Phenytoin	Drug-Facilitated Crime Postmortem (Full)	180, 223, 252
Secobarbital	DUI/Human Performance Drug-Facilitated Crime Postmortem (Full)	168, 167, 195

8.1 Decision Criteria

8.1.1 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 target analytes should be detected in the Positive Control.

8.1.2 Chromatography

The EICs for 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.

8.1.2.1 Retention Time

The retention time of the peak will be within ±2% of the retention time (relative or absolute, as appropriate) obtained from an extracted Positive Control.

8.1.2.2 Response

The 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.

8.1.3 Mass Spectrometry

When necessary, the mass spectrum of the analyte of interest is compared to an extracted Positive Control. See the Guidelines for Comparison of Mass Spectra (TOX-104) for further guidance.

9 REPORTING

See TOX-100 for reporting criteria.

10 CORRECTIVE MEASURES

Refer to Quality Control for Toxicology Examinations (TOX-101) for guidance on action steps in the event of a quality control failure.

11 PERFORMANCE CHARACTERISTICS

11.1 LOD

Detection limits for common acidic and neutral analytes are listed in the table below. Note: When LODs were evaluated in 2023, it became apparent that when mass spectral criteria are used as part of detection limit verification, previously validated detection limits could not be reached for all analytes. Therefore, detection limits are not determined for all analytes, as evident by the “>” signs in the table below.

Analyte	Blood (µg/mL)	Urine (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
Levetiracetam	>2.5	>2.5
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
***LODs for analytes marked with an asterisk were not evaluated in 2023.**		

11.2 Carryover

Carryover may occur after samples containing higher amounts of analyte; reinjection with appropriate solvent blanks may be performed. Appreciable carryover was identified for naproxen (~2.3%), phenytoin (~1.5%), and phenobarbital (~0.53%) in blood. Appreciable

carryover was identified for naproxen (~2.4%), phenytoin (~1.7%), and phenobarbital (~0.66%) in urine.

12 LIMITATIONS

12.1 Interferences

None known. Grossly decomposed or putrefied samples may affect detection limits.

12.2 Processed Sample Stability

Most analytes are stable in blood and urine extracts for up to 6 days when stored in unpunctured screw-top ALS vials at 2-8°C or when vials are punctured and left at room temperature on the autosampler to evaporate. See Validation Records for further details.

13 SAFETY

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

14 REVISION HISTORY

Revision	Issued	Changes
09	02/11/2022	Document reformat. 1-Simplified statement 2-Reformatted scope statement 5-Reorganized equipment listing, updated acetaminophen control amount 6-Reformat of procedure for readability, eliminate form, specify injection amount 7-Reformat GC/MS information Other minor edits and reorganization
10	02/15/2024	Updated Scope (2), Specimen Criteria (4), and Procedure (6) to cover only blood and urine. Updated 5.2.A to allow for 16x100 mm tubes and removed requirement for Teflon inserts. Added 5.2.G Updated 5.3.A Clarified 5.6.2.B Removed volatiles analysis in Sections 5.6, 6, and 7. Updated 6.H.1. to specify 50 µL reconstitution volume. Updated 6.I.1. to include methanol blanks prior to each sample. Included data analysis details in Section 8. Clarified 8.1.1. Updated 8.1.2.2. Updated 9 and 11.1. Added 12.2. Removed reference to embalmed tissues in 12.1.