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, $Na_2HPO_4 \cdot 7H_2O$)

s. Sodium phosphate, monobasic monohydrate (ACS grade, $NaH_2PO_4 \cdot H_2O$)

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<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 µ 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_4	flunitrazepam-d_7	nordiazepam-d_5
7-aminoflunitrazepam-d_7	ketamine-d_4	oxazepam-d_5
alprazolam-d_5	lorazepam-d_4	temazepam-d_5
clonazepam-d_4	midazolam-d_4	triazolam-d_4
diazepam-d_5		zolpidem-d_6

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 µL 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 µg/mL) and 2 mL of the Intermediate Internal Standard Mixture (0.5 µg/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 µL of Intermediate System Suitability Testmix (100 ng/mL) to an autosampler vial. Dilute with 810 µL of aqueous reconstitution solvent (5-qq) and 90 µL 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±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 +/- 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 µL Positive Control Solution to the Low and High Controls, respectively. Add 50 µL 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

Scan Mode	Turbo Spray	**Polarity**	Positive
Resolution	Unit	**Scan Type**	MRM
Curtain Gas	Nitrogen (30)	**Ionspray Voltage**	4000
Source Temperature	700°C	**Nebulizer Gas**	Nitrogen (75)
Entrance Potential	10	**Turbo Gas**	Nitrogen (75)
Dwell Time	15 ms	**Pause Time**	5 ms
Collision Gas	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 ±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 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 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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