Title: Exclusionary Drug Screening by Liquid Chromatography/Mass Spectrometry (LC/MS)

1 Introduction

A rapid high performance liquid chromatography/mass spectrometry (LC/MS) method using electrospray ionization (ESI) can be used to qualitatively screen biological specimens (e.g., blood, urine) for common drugs of abuse, as well as some other drugs. The amount of a detected analyte can be estimated by comparing the response (peak area) for an analyte to that for a corresponding internal standard. Positive findings can be confirmed by using either a second technique or a second aliquot of sample. Confirmation can also be achieved during quantitative analyses using appropriate procedures.

2 Scope

This procedure is limited to the qualitative screening of biological specimens for the presence of cocaine and metabolites, opioids, and benzodiazepines and other drugs (see list of target analytes and limits of detection).

3 Principle

Biological specimens are analyzed for the presence of drugs of abuse (DoA) and/or their metabolites by extraction using solid phase extraction (SPE) columns. Final extracts are analyzed by LC/MS using ESI and can involve a combination of selected reaction monitoring (SRM) and full scan analysis modes.

4 Specimens

This procedure uses biological fluid(s) such as blood, urine, serum, and/or plasma. Typically 0.5 mL of sample is consumed during the analysis but varying volumes may be used, as necessary. Dilution of samples due to limited specimen or due to suspicion of high drug or metabolite concentration is acceptable.

5 Equipment/Materials/Reagents

a. General laboratory glassware

b. Disposable borosilicate test tubes (e.g., 16 x 100 mm, round bottom, borosilicate glass with Teflon caps)

c. Vortex mixer

d. Sonicator

e. Automatic pipettes (with disposable tips)

f. Positive pressure solid phase extraction device – SPEWare Cerex, 48 sample (or equivalent)

g. Sample concentrator with nitrogen – SPEWare Cerex 48 heated (or equivalent)

h. Centrifuge

i. pH meter (or equivalent)

j. Trace B Extraction Columns – SPEWare (or equivalent)

k. Liquid Chromatograph/Mass Spectrometer (Shimadzu LCMS-8030, or equivalent)

l. HPLC column – Kinetex, phenyl hexyl, 2.6 µm, 100Å, 50 mm x 4.6 mm (Phenomenex), or equivalent)

m. Pre-Column – SecurityGuard ULTRA Cartridge UHPLC Kinetex for 4.6mm ID Columns (Phenomenex or equivalent)

n. Autosample vials (LC/MS grade 1.8mL or equivalent)

o. Acetic acid, glacial ($CH_3COOH_{(l)}$, Reagent grade or equivalent)

p. Ammonium formate (NH_4CHOO, Reagent grade or equivalent)

q. β-Glucuronidase (p. vulgata ; Sigma or equivalent)

r. Formic acid (HCOOH, Reagent grade or equivalent)

s. Methanol (MeOH, Reagent grade, LC/MS Grade, or equivalent)

t. Morphine glucuronide (Morphine-3-β-glucuronide or Morphine-6-β-glucuronide) (Alltech, Sigma, or equivalent)

u. Sodium acetate trihydrate ($NaCH_3COO \cdot 3H_2O$, Reagent grade or equivalent)

v. Sodium bicarbonate ($NaHCO_3$; Reagent grade or equivalent)

w. Sodium carbonate (Na_2CO_3 ; Reagent grade or equivalent)

x. Water (Mobile Phase) (LC/MS Grade ; Optima or equivalent)

y. Ammonium $Formate_{(aq)}$: ($NH_4CHOO_{(aq)}$; 5M ; 31.5% (w/v)): Can be prepared by dissolving 3.15 g of ammonium formate in 10 mL of water. Stable for at least one (1) year in glass container when refrigerated.

z. Sodium Acetate $Buffer_{(aq)}$ ($NaCH_3COO$; pH~4.5): Can be prepared by combining 5.86 g of sodium acetate with 3.24 mL of glacial acetic acid in a 1 L volumetric cylinder and diluting to volume with water. Stable for at least one (1) year in glass container while at room temperature.

aa. Sodium $Bicarbonate_{(aq)}$ (0.1M ; $NaHCO_3$; 0.84% (w/v) ; pH~8): Can be prepared by dissolving 4.2 g of sodium bicarbonate in water within a 500 mL volumetric flask and diluting to volume with water. Stable for at least one (1) year in glass container while at room temperature.

bb. Sodium $Carbonate_{(aq)}$ (0.1M ; Na_2CO_3 ; 1.1% (w/v) ; pH~11): Can be prepared by dissolving 5.3 g of sodium carbonate in water within a 500 mL volumetric flask and diluting to volume with water. Stable for at least one (1) year in glass container while at room temperature.

cc. Bicarbonate/Carbonate Buffer$_{(aq)}$ (pH~9): Can be prepared by transferring 0.1M sodium bicarbonate$_{(aq)}$ solution into a beaker and checking pH (should be pH~8). Adjust the pH to ~9 using the 0.1M sodium carbonate$_{(aq)}$ solution. Stable for at least one (1) year in glass container while at room temperature.

dd. Solid Phase Extraction Elution Solution (e.g., 50 mL):
{Dichloromethane (CH_2Cl_2) : IPA : NH_4OH (80 : 18 : 2)}.
Can be prepared by adding 9 mL of isopropanol to 1 mL of ammonium hydroxide within a 50 mL volumetric cylinder. To this mixture add 40 mL of methylene chloride and mix. This solution will be prepared when needed for use.

Note: The volume should be adjusted to account for number of samples that are to be extracted (2 mL are needed for each extraction sample).

ee. $MeOH_{(aq)}$ (20% (v/v)): Can be prepared by adding 80 mL of water and 20 mL of methanol to a 100 mL volumetric cylinder and mixing. Stable for at least one (1) month in glass container when refrigerated.

ff. Methanol-HCl (1% (v/v)): Can be prepared by dissolving 10 µL of hydrochloric acid into 990 µL of methanol (or in different volume with an equivalent ratio). Stable for at least one (1) month in glass container when refrigerated. Verify pH is ≤ 3 prior to use.

gg. Hydrolysis Sodium Acetate Buffer$_{(aq)}$ (0.1 M ; $NaCH_3COO$; pH~5): Can be prepared by combining first making a 1M Sodium Acetate Buffer Solution: 42.9 g of $NaCH_3COO{\cdot}3H_2O_{(s)}$ with 400 mL of water. Add 10.4 mL of $CH_3COOH_{(l)}$ in a 500 mL volumetric cylinder and diluting to volume with water. Store in glass container at room temperature. Stable for at least one (1) year at room temperature. Check clarity and pH can be checked prior to use. Use 1M acetic acid to adjust pH to 5.0 (+/- 0.1). The 0.1M Sodium Acetate Buffer Solution can be made by diluting 20 mL of the 1M Sodium Acetate Buffer Solution in 200 mL water.

hh. β-Glucuronidase (in 0.1M $NaCH_3COO$ buffer solution ; pH~5): Prepare daily for use. The concentration of the enzyme buffer solution should be 5000 Units/mL. The volume of enzyme solution per [0.5 mL] urine specimen should be 0.5 mL. Each 0.5 mL urine sample requires 2500 enzyme F-Units to ensure adequate enzymatic hydrolysis. A slight excess of solution should be made to ensure enough is available for all of the samples.
Note: β-Glucuronidase has different activities based on the lot that is used. See enzyme activity information on individual bottles (i.e., Units/gram of material).

Example: Ten (10) urine specimens (0.5 mL each) that need hydrolyzing means there will need to be 5 mL of enzyme solution total. If the activity of the β-Glucuronidase is 1,439,000 Units/g, then the amount of β-$G_{(s)}$ needed is:
[(5 mL) x (5000 β-G Units/mL) x (1 g β-G/1,439,000 β-G Unit) x 1000 mg/g] = 17.4 mg
Dissolve 17.4 mg β-$G_{(s)}$ in 5 mL 0.1M $NaCH_3COO$ buffer solution (pH~5).

ii. Mobile Phase A – 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$:
Can be prepared by mixing 50 µL of formic acid with 0.5 mL of 5M ammonium formate in a 500 mL volumetric cylinder, diluting to volume with [Mobile Phase] water, and mixing well.

Store in glass at room temperature. Stable for at least one (1) week while in a closed state. {Note: 5mM $NH_4CHOO_{(aq)}$ is equivalent to 0.0032 % (w/v) $NH_4CHOO_{(aq)}$.}

jj. Mobile Phase B – (MeOH or CH_3OH). Store in glass at room temperature – stable indefinitely in a closed state at room temperature.

Note: Unless otherwise noted, the above Reagent solutions are stable for at least one (1) year from either their creation or from their last verification. Check clarity and check pH (when indicated as having a pH value) for all solutions prior to use.

6 Standards and Controls

Positive and Negative Controls:
Can be purchased (e.g., UTAK Labs) or generated in-house (e.g., spiked blank blood, spiked blank urine, spiked vitreous). Store frozen, refrigerated, or obtain fresh. If purchased, the stability should be determined by the manufacturer. Appropriate positive and negative controls will be extracted and analyzed with each assay or batch.

Internal Standard Components:
Purchased from suitable vendor(s) in solution or neat solids (e.g., 0.1 mg/mL solution). Storage and stability is determined by manufacturer(s). Additional or alternative deuterated compounds may be used. If purchased solutions vary in concentration from above, analysts may adjust the preparation volumns to account for differences. Any variations will be noted on solution preparatation worksheets.

a. Internal Standard (I.S.) Working Solution (Diazepam–D_5 ; 250 ng/mL):
Can be prepared by adding 250 µL of the 100 µg/mL Diazepam-D_5 into a 100 mL volumetric flask and diluting with MeOH. Store refrigerated in glass. Stable for at least 1 year.

b. Morphine-β-glucuronide Stock Solution (0.1 mg/mL)
(Either morphine-3-β-glucuronide or morphine-6-β-glucuronide)
Purchased as 0.1 mg/mL solutions. Storage and stability is determined by manufacturer.

c. Morphine Glucuronide Control Working Solution (2.5 µg/mL):
Can be prepared by diluting 250 µL of the Morphine-β-glucuronide Stock Solution with $MeOH_{(aq)}$ (1:1 MeOH:H_2O) to a final volume of 10 mL. Store in glass in a freezer.
Stable for at least 6 months.

d. Hydrolysis Control Urine (~75 ng/mL morphine; ~125 ng/mL morphine glucuronide):
Can be prepared by adding 25 µL of the Morphine Glucuronide Control Working Solution (2.5 µg/mL) to 500 µL of Negative Control Urine on the day of analysis.

e. Optional Instrument Performance Standard Mix: ((Diazepam-D_5) ; 10 ng/mL)

This is optional and can be used to evaluate the instrument in addition to the performance check solution for the LC/MS instrument.

Dilute the I.S. Working Solution 1:25 with water to make a 10 ng/mL final concentration. This can be done by mixing 20 µL of the I.S. Working Solution, combining it with 500 µL of

deionized water, placing it into an autosample vial, and capping. This solution will be prepared when needed for use.

Note: Other analytes within the solution are not evaluated – just the Diazepam-D_5 analyte.

f. Negative Control Blood:
Purchased (e.g., Diagnostics Products Corporation, UTAK Labs), donated (e.g., American Red Cross, hospital blood bank), or in-house demonstrated negative control blood (e.g., from a proven blank specimen). Store frozen or refrigerated. If purchased or donated, stability should, when possible, be determined by manufacturer.

g. Negative Control Urine (or other matrix):
Purchased, acquired (e.g., Office of the Chief Medical Examiner (OCME)), or in-house demonstrated to be negative. Store refrigerated or frozen.

h. Negative Control [Other Matrix]:
Synthetic or in-house negative control [other matrix]. Store refrigerated or obtain fresh. If purchased, stability should be determined by manufacturer. A negative control [other matrix] should be extracted and analyzed with each appropriate assay or batch.

i. Positive Control:
If prepared in-house, can be prepared by adding 250 µL of UTAK DAU Level 2 solution to 250 µL blank matrix (e.g., blood, urine) and mixing. If a non-routine matrix is analyzed, then dilute with 250 µL of appropriate material. The resulting 0.5 mL positive control sample will contain (at least) the following analytes:
Methylenedioxymethamphetamine (MDMA), Temazepam, Benzoylecgonine (BE), Methadone, Methaqualone, Codeine, Phencyclidine (PCP), Nortriptyline. Prepare fresh and mix adequately.

Note-01: If other drugs are to be used when preparing the In-House Cut-Off Positive Control Mixtures then a minor deviation (or other paperwork) will be prepared prior to analysis and will accompany all batch work and case files.

Note-02: Unless otherwise noted, the above standard and control solutions are stable for at least one (1) year from either their creation or from their last verification. If solutions are used as purchased (i.e., without modification), storage and stability will be determined by manufacturer.

7 Calibration

This procedure is qualitative only.

8 Sampling

Not applicable.

9 Procedure

The Appendix contains an abbreviated version of this procedure. This form may be used at the bench by analysts who perform the procedure. If SPE cartridges are in a rack and are not removed during the extraction process and are always in a fixed position then labeling of SPE cartridges is optional, regardless of what is written in the following steps or in the Appendix. However, the vials which collect the final eluent must be properly labeled.

9.1. Specimen Preparation:

9.1.1. Add 0.5 mL of each sample to properly labeled test tubes.
Appropriate positive and negative controls will be analyzed with each batch of samples.

9.1.2. Add 100 µL of the I.S. Working Solution into each sample (25 ng each)

9.2. Urine Specimens Only (De-conjugate):
Note: Approval from Lead Examiner or Deputy Director needs to be obtained if urine specimens are to be hydrolyzed.

9.2.1. Add 250 µL of β–Glucuronidase in 0.1 M acetate buffer (pH ~ 5) to hydrolyze any conjugated drugs within urine samples.

9.2.2. Heat for 3 hours at 60 ˚C and then cool specimens to room temperature.

9.3. Add 1 mL of the Sodium Acetate Buffer (pH ~4.5) to each specimen.

9.4. Add 500 µL of water – cap and vortex-mix samples.
Sample test tubes may all be open during this step.

9.5. Sonicate for at least 15 minutes (not required for urine samples).

9.6. Centrifuge all samples (e.g., 8 minutes at ~5200 RPM)

9.7. Label Trace-B extraction [SPE] columns appropriately

9.8. Place labeled Trace-B extraction columns in the SPE column rack in the appropriate order. Position plastic waste tray underneath SPE column rack. {for Solvent Waste}

9.9. Condition each column with 1 mL MeOH, then drain (≈ 3 psi). {for Solvent Waste}

9.10. Remove solvent tray and replace with biohazardous tray.

9.11. Add 1 mL of water to each SPE column, then drain (≈ 3 psi) to biohazardous tray.

9.12. Individually transfer samples to the center of their respective [properly labeled] SPE column. Avoid splashing and/or transferring any sediment found at the bottom of the tube.

9.13. Allow samples to migrate through SPE column via gravity. Apply small pressure, if needed (< 3psi).

9.14. To each SPE column:

9.14.1. Wash with 1 mL of Bicarbonate/Carbonate buffer (pH~9), then drain (≈ 3 psi) to biohazardous waste.

9.14.2. Wash with 1 mL water, then drain (≈ 3 psi) to Biohazardous waste.

9.15. Dry the columns for ~10 minutes using maximum pressure (e.g., between 60-80 psi).

9.16. During this 10 minute window (or earlier) label autosampler vials and place in the appropriate position in the SPE collection rack underneath the corresponding SPE column. Prepare the elution solvent.

9.17. The elution solvent ratio is 80:18:2 CH_2Cl_2:IPA:NH_4OH.
(Approximately 2 mL will be needed for each tube).

9.18. After ~10 minutes, replace plastic waste tray with the SPE collection rack containing appropriately labeled autosample vials. Ensure that each vial is placed under their corresponding SPE column for elution.

9.19. Elute columns with two (2) – 1 mL aliquots of the Solid Phase Extraction Elution Solution. Collect eluent into appropriate autosampler vials using gravity or using low pressure (<3 psi).

9.20. Remove top SPE column rack and transfer collection rack from SPE manifold to sample concentrator.

9.21. Add ~1 drop of 1% MeOH-HCl to each tube.

9.22. Evaporate all eluents to dryness at <40 ˚C.

9.23. If samples are not to be immediately analyzed they should be placed in a refrigerator or freezer under proper evidentiary seal.

Note: The word 'immediately' refers to instrumental sequences being started on the same day.

9.24. Reconstitute each extract sample with 500 µL of $MeOH_{(aq)}$ (20% (v/v)) when ready for instrumental analysis.

9.25. Check turbidity of solution. Remove undissolved particulates and treat as necessary (e.g., filtration or centrifugation). Controls (negative and positive) will be treated in same manner as samples.

9.26. Analyze extract solutions using the LC/MS instrument.
Blank solutions (i.e., solvent, mobile phase) will be analyzed in-between samples.

10 Instrumental Parameters

The following are the typical operating parameters for the instrument used in this procedure. With approval from the Lead Examiner and Deputy Director (or higher), the instrument conditions may be modified to adjust or improve the procedure. Documention must be included with batch data so that any instrumental parameter changes can be associated with data and casework until this procedure has been updated.
The appendix contains an abbreviated version of the procedure. The checklist can be used by analysts. Any changes within the instrumental parameters, if listed on the checklist, will be reflected on the checklist by the analyst filling it out.

HPLC Parameters:

Mobile Phase A: 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$
Mobile Phase B: $MeOH_{(l)}$ (neat)
HPLC Column: Kinetex, 2.6 µm Phenyl-Hexyl, 100 Å, 50 mm x 4.6 mm (Phenomenex)
Column Temperature: 40 °C
Constant Flow: 0.6 mL/min.
Autosampler Temperature: 15 °C
Injection Volume: 10 µL
Needle Wash: 500 µL before and after aspiration

Gradient Program: Initial 5% B ; Flow 0.6 mL/min

Time (min.)	Mobile Phase A [0.01 %(v/v) $HCOOH_{(aq)}$ + 5mM $NH_4CHOO_{(aq)}$] (%)	Mobile Phase B $MeOH_{(neat)}$ (%)
Initial	95	5
0.5	95	5
3.00	55	45
4.5	55	45
5.5	40	60
7.5	40	60
10.10	25	75
10.50	5	95
12.50	5	95
12.51	95	5
15.00	STOP	STOP

Mass Spectrometer Parameters:

Ionization Source	Electrospray	**Polarity**	Positive Ion
Ion Spray Potential	+4.5 kV	**Heat Block**	400 °C
Nebulizer Gas	Nitrogen	**Nebulizer**	2 L/min
Drying Gas	Nitrogen	**Drying Gas**	15 L/min.
Scan Type	MRM (&/or Full Scan)	**Resolution**	Unit
Desolvation Line	250 °C		

11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. Retention time (chromatographic characteristic), fragmentation pattern and qualified ion ratios (mass spectrometric characteristics), and other characteristics are used as the basis for detection and identification. In most cases all of the criteria below should be met in order to identify the appropriate drugs within biological specimens.

11.1. Chromatography

All chromatographic peaks for the analytes of interest should show good chromatographic characteristics, with reasonable peak shape, width, and resolution. For low concentrations of an analyte (<5 ng/mL), there may be transitions that are not optimal. In order to be determined as acceptable, a chromatographic peak in an unknown sample should compare favorably to the same analyte's chromatographic peak in a known sample which has been analyzed on the same system and in the same, or subsequent, analytical timeframe. Additionally, the following two criteria should be met:

11.1.1. Retention Time (RT)

The retention time of a peak of interest should be within ±2 % of the retention time (relative or absolute) of a reference standard or positive control.

Note: Instrumental evaluation of retention time using +/-0.1 minute as a criteria is acceptable. Using ±2 minute window is also acceptable for sample/control evaluation.

If analyzed, the retention times of the analytes within the Procedural Performance Solution should be within ±2 % of their previously analyzed times and a set value for acceptance. Minor changes in mobile phase percentage may account for slight retention time shifts.

11.1.2. Signal-to-Noise Ratio (SNR)

To justify the existence of a peak, its baseline signal-to-noise ratio (SNR) will exceed 3. Furthermore, the baseline signal for the peak of interest will be at least 10-fold greater than that for any observed peak (corresponding to the analyte of interest) at a similar retention time in a negative control or blank sample that was injected just prior to that sample.

11.2. Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the analytes in the performance standard should be present. Independent MS/MS experiments are conducted for each analyte. Ion ratios should compare favorably to ion ratios from reference standards or an extracted positive control at a comparable concentration. Generally, ion ratios are within the following limits:

Expected [Set] Ion Ratio	Allowance
>50 %	20%
20% - 50%	25%
<20%	30%

11.3. Batch Acceptance:

In order for a batch to be acceptable:

11.3.1. No analytes of interest will be detected in the Negative Control.

Note-01: An analyte is considered applicable if it is important to the batch. For example: OCME cases are not interested in methaqualone and nortriptyline, so these would not be considered as applicable analytes of interest for such samples.

Note-02: Analytes of interest are considered those compounds that are being reported.

11.3.2. All applicable analytes of interest within Positive Controls, as well as internal standards, will be identified.

11.3.3. For urine batches, morphine will be detected in the hydrolysis positive control.

11.4. Unknown Sample Compound Detection:

This procedure is intended to be used for qualitative screening purposes. However, if enough data is present, confirmations can be reported if a different chromatographic or mass spectrometric experiment is employed (or with approval of Lead Examiner and Deputy Director (or higher)).

12. Calibration

Not applicable.

13. Uncertainty

Not applicable – this is a qualitative procedure only.

14. Limitation

14.1. Limits of Detection (LOD)

This information should be found in validation paperwork.

14.2. Specificity

No known interferences. This procedure will generally be used as a screening technique. All forensically significant positive findings should be confirmed by a second technique unless alternative situations apply (e.g., Memorandum of Understanding (MOU), directive from customer, approval of Deputy Director (or higher)).

Note: A second technique could include a different LC/MS instrument or using the same instrument with a different chromatographic or mass spectrometric experiment.

15. Safety

This procedure is carried out in a laboratory environment and standard safety procedures appropriate for such an environment should be utilized, including gloves, safety glasses, and protective clothing (e.g., lab coat). Biological specimens will be handled using universal precautions and will be treated as biohazardous. Potentially contaminated items and surfaces will be cleaned prior to use, especially. When casework samples are being processed/analyzed brown paper (or other similar barrier) should be placed in between the surface and the specimens.

16. References

Screening and Confirmation of Benzodiazepines in Blood by Electrospray LCMSMS. West Chester County Department of Laboratories and Research: Division of Forensic Toxicology.

Solid-Phase Extraction Method for Trace B Columns. SPEWARE: Baldwin Park, CA.

A Comparison of the Validity of Gas Chromatography-Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry Analysis of Urine Samples for Morphine, Codeine, 6-Acetylmorphine, and Benzoylecgonine; Peter R. Stout, Nichole D. Bynum, John M. Mitchell, Michael R. Baylor and Jeri D. Ropero-Miller; J.Anal Toxicol (2009) 33 (8): 398-408

Exclusionary Drug Screen by UPLC-ESI-FTMS, FBI Laboratory.

Benzodiazepine Quant in Blood LCMSMS procedure, Toxicology Unit, Division of Scientific Services.

Processing of Opioids by LCMSMS procedure, Toxicology Unit, Division of Scientific Services.

Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update, Logan, et. al, Journal of Analytical Toxicology, 42, 2, 63-68

Appendix:
Bench Method:
Exclusionary Drug Screening by Liquid Chromatography/Mass Spectrometry (LC/MS)

The following is an abbreviated version of this procedure and a pdf-version can be used.
This form may be used at the bench level by the analyst performing the procedure.

Negative Control [drug-free] Blood/[other matrix]	**Lot:**
Positive Control Blood/[other matrix]	**Lot:**

Procedure:

	For Blood Specimens	
Add 0.5 mL sample/negative control/positive control into properly labeled test tubes (e.g., 16 x 100 mm)		
Add 100 µL of I.S. Working Sol'n to each sample (25 ng each drug):		
	Diazepam-D_5 (250 ng/mL)	**Lot:**
Deconjugate urine specimens (approval needed):		
	Enzyme	**Lot:**
	Hydrolysis Sodium Acetate Buffer solution (pH~5) – heat for 3 hours at 60 ˚C	**Lot:**
Add 1 mL of Sodium Acetate $Buffer_{(aq)}$ (pH 4.5) to each test tube.		**Lot:**
Add 500 µL of water to each test tube, cap and vortex-mix samples, sonicate, if blood. Centrifuge.		
Label Trace-B extraction [SPE] columns appropriately.		**Lot:**
Place labeled Trace-B extraction columns in the SPE column rack in the appropriate order.		
Add 1 mL MeOH (to condition SPE), drain (≈3 psi)		**Lot:**
Add 1 mL of water (to wash each SPE column), then drain (≈ 3 psi)		**Lot:**
Individually transfer samples/controls to the center of their respective [properly labeled] SPE column. Avoid sediment / splashing. Allow to migrate through SPE column via gravity. Apply small pressure, if needed (< 3psi).		
To each SPE column:		
	Wash with 1 mL of Bicarbonate buffer (pH ~9), then drain (≈ 3 psi)	**Lot:**
	Wash with 1 mL water, then drain (≈ 3 psi)	**Lot:**
Dry the columns for 10 minutes using maximum pressure (e.g., between 60-80 psi)		
During this 10 minute window (or earlier) label autosample vials, place in SPE rack. Prepare the elution solvent.		
The Solid Phase Extraction Elution Solution ratio is 80:18:2 CH_2Cl_2:IPA:NH_4OH. (Approximately 2 mL per each tube).		
	Methylene Chloride (CH_2Cl_2)	**Lot:**
	Isopropyl Alcohol (2-Propanol ; IPA)	**Lot:**
	Ammonium Hydroxide (NH_4OH)	**Lot:**
After ~10 minutes, replace plastic waste tray with SPE rack containing autosample vials. Ensure vials are properly placed.		
Elute columns with two (2), 1 mL aliquots of the Elution Solution Flow using gravity (<3 psi if needed)		**Lot:**
Transfer collection rack from SPE manifold to sample concentrator		
Add ~1 drop of MeOH-$HCl_{(aq)}$ and evaporate to dryness (<40 ˚C) ; Store refrigerated and properly sealed if not immediately analyzed		
Reconstitute each extract sample with 500 µL of 20% $MeOH_{(aq)}$; only if analyzing immediately		**Lot:**
Analyze by the LC/MS instrument		

Appendix (Cont'd):

Specimen Procedure (Cont'd)

Analyze by LC/MS/MS:

Instrument: Shimadzu 8030	Instrument Identifier:
HPLC Column: Phenomenex ; Kinetex Phenyl-Hexyl ; 100 Å ; 2.6 µm ; 50 mm x 4.6 mm):	Lot:
Mobile Phase A: [0.01 %(v/v) $HCOOH_{(aq)}$ + 5mM $NH_4CHOO_{(aq)}$]	Lot:
Mobile Phase B: MeOH (CH_3OH) – 100%	Lot:

Gradient Program:
Initial 5% B ; Flow 0.6 mL/min

Time (min.)	%B
Initial	5
0.50	5
3.0	45
4.5	45
5.5	60
7.5	60
10.10	75
10.50	95
12.50	55
12.51	5
15.00	STOP

Mass Spectrometer Parameters

Ion Source	Electrospray
Spray Voltage	+4.5
Polarity	Positive
Nebulizer Gas	Nitrogen (2 L/min.)
Drying Gas	Nitrogen (15 L/min.)
Scan Type	MRM (&/or Full Scan)
Heat Block	400 °C
Resolution	Unit

Rev. #	History
1	New procedure for urine, blood or other appropriate matrices.
2	Updated the concentration of the Internal Standard to 250 ng/mL, also remove the use of Amobarbital-D_5 and Ethinimate as part of the IS solution. Uppdated the instrument parameters to match current parameters. Updated the IS information on the run sheet. Appropriate positive and negative controls will be extracted and analyzed with each assay or batch. Approval needed from Lead Examiner and Deputy Director if instrument conditions/parameters are changed – documention of charnge(s) must be included with batch data ; appendix must contain any changes, if listed. Section 11.1.1: Retention times of the analytes within the Procedural Performance Solution should be within ±2 % of their previously analyzed times and a set value for acceptance. 14.2: A confirmation could include using a different LC/MS instrument or using the same instrument with a different chromatographic or mass spectrometric experiment. Updated verbiage throughout to be similar to TX 37 SOP. Added direction describing when analytes can be confirmed using this procedure.