Title: (TX-37 SOP) Drug Screening and Confirmation by Liquid Chromatography/High Resolution-Mass Spectrometry (LC/HR-MS)

1 Introduction

A rapid liquid chromatographic method using electrospray ionization (ESI) combined with high resolution accurate mass (HRAM) spectrometry (LC/MS) can be used to qualitatively screen biological specimens (e.g., blood, urine, vitreous fluid) for the presence of common drugs of abuse (DoA) or other compounds. The amount of a detected analyte can be estimated by comparing the responses (relative peak areas) 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 the same 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, barbiturates, opioids, and benzodiazepines, their respective metabolites, and other drugs (see list of target analytes and limits of detection). Other target compounds can be added, as needed, once validated.

3 Principle

Biological specimens are analyzed for the presence of drugs and/or their metabolites 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/plasma, vitreous fluid. Typically 0.5 mL of sample is consumed during the analysis. Serum and plasma samples will be considered synonymous to, and treated the same as, blood samples.
Varying volumes may be used, as necessary, but such changes shall be documented within technical records (e.g., worksheets, case notes). For example, dilution of samples due to limited specimen or due to suspicion of high drug or metabolite concentration or sample volumes over 0.5 mL are acceptable, given prior approval from the Technical Lead (or higher).

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 – Cerex, 48 sample (or equivalent)

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

h. Centrifuge

i. pH paper (Micro Essentials Lab ; or equivalent)

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

k. Liquid Chromatograph/High Resolution-Mass Spectrometer (LC/HR-MS) – Q-Exactive (or equivalent)

l. HPLC column – Accucore C18, 2.6 µm, 50 mmx3 mm (Phenomenex or equivalent)

m. Pre-Column – Accucore Defender guard 2.6 µm, 10 mm x 3 mm (Phenomenex or equivalent)

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

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

p. Ammonium formate (NH_4CHOO, LC/MS grade or equivalent)

q. Ammonium Hydroxide (NH_4OH, Reagent grade or equivalent)

r. Formic acid (HCOOH, LC/MS grade or equivalent)

s. Hydrochloric Acid ($HCl_{(conc)}$; Reagent Grade or equivalent)

t. Isopropanol (i-PrOH, IPA, 2-Propanol, Reagent grade or equivalent)

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

v. Methylene Chloride (CH_2Cl_2, Reagent grade or equivalent)

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

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

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

z. Water (H_2O ; Millipore, Deionized (DIW), or equivalent)

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

bb. 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.

cc. 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.

dd. 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.

ee. 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.

ff. 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.

gg. Solid Phase Extraction Elution Solution (e.g., 50 mL):
{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)

hh. 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.

ii. 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 $\leq$ 3 prior to use.

jj. 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) $NH_4CHOO_{(aq)}$.}

kk. 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 Control Solutions: 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. If not enough vitreous fluid is available for control solution preparation(s), water can be used in its place (document accordingly in case notes).

Internal Standard Components: Purchased from Cerilliant or other suitable vendors (e.g., 0.1 mg/mL solutions or neat solid). Storage and stability is determined by manufacturer(s). Additional or alternative deuterated compounds may be used.

a. Internal Standard (I.S.) Working Solution:
(Diazepam-D_5 + Butalbital-D_5 + Meprobamate-D_3 + Morphine-D_3 + Oxycodone-D_3):

(Diazepam-D_5 at 250 ng/mL ; Butalbital-D_5 at 1000 ng/mL ; Meprobamate-D_3 at 1000 ng/mL ; Morphine-D_3 at 250 ng/mL ; Oxycodone-D_6 at 250 ng/mL)

Can be prepared by placing the following into a 100 mL volumetric flask containing methanol:
Diazepam-D_5 reference standard solution: 250 µL of a 100 µg/mL
Butalbital-D_5 reference standard solution: 1 mL of a 100 µg/mL
Meprobamate-D_3 reference standard solution: 1 mL of a 100 µg/mL
Morphine-D_3 reference standard solution: 250 µL of a 100 µg/mL
Oxycodone-D_6 reference standard solution: 250 µL of a 100 µg/mL

Dilute all of the above to volume [100 mL] with MeOH. Store in glass in a freezer.

Note-01: If other deuterated drugs are used as internal standards, documentation will accompany affected batches and casefiles (e.g., minor deviation form).

Note-02: As long as the final concentrations are met, variations in volumes, concentrations, and/or sets of reference standard solutions are allowed.

b. 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.

c. 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.

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.

d. In-House Cut-Off Positive Control Mixture (Blood, Urine):
Can be prepared by making analyte concentrations initially within methanol and then within the appropriate matrix according to the following table below. Concentrations are prepared based on purchased standard concentrations and diluted accordingly. The final mixture(s) of analytes will be in matrix-appropriate solutions.
Note: The urine In-House Cut-Off Positive Control Mixture will be used for vitreous humor samples.

In-House Cut-Off Positive Control Mixture (1 mg/mL stock soln's) (except where noted)	Volume to Add	Dilution #1 ('Stock A') (ng/mL) (in 100 mL of MeOH)	Dilution #2 ('Stock B') (ng/mL) (in 10 mL of MeOH)	Amount to add into 0.5 mL of matrix	In-House Cut-Off Positive Control Mixture (ng/mL) (0.5 mL of matrix)
Buprenorphine (100 µg/mL)	20 µL	20	–	25 µL	1
Fentanyl (100 µg/mL)	20 µL	20	–		1
6-MAM	10 µL	100	–		5
Citalopram	10 µL	100	–		5
Oxymorphone	10 µL	100	–		5
Pseudoephedrine	10 µL	100	–		5
Bupropion	20 µL	200	–		10
Cocaine	20 µL	200	–		10
Fluoxetine	20 µL	200	–		10
Lorazepam	20 µL	200	–		10
Methorphan	20 µL	200	–		10
Morphine	20 µL	200	–		10
Oxycodone	20 µL	200	–		10
PCP	20 µL	200	–		10
Sertraline	20 µL	200	–		10
Amphetamine	40 µL	400	–		20
Methadone	40 µL	400	–		20
Nordiazepam	40 µL	400	–		20
Venlafaxine	40 µL	400	–		20
Benzoylecgonine	100 µL	1000	–		50
Diphenhydramine	100 µL	1000	–		50
Tramadol	100 µL	1000	–		50

Lidocaine	100 µL	–	10,000		500
Meprobamate	100 µL	–	10,000		500
Secobarbital	100 µL	–	10,000		500

e. In-House Cut-off Verification Mixture (Blood, Urine):
Can be prepared by making analyte concentrations within the In-House Cut-Off Positive Control Mixture (Blood, Urine) at three times (3x) their listed levels. Concentrations are prepared based on purchased standard concentrations and diluted accordingly. The final mixture of analytes will be in a matrix solution.
Note: The urine In-House Cut-off Verification Mixture will be used for vitreous humor samples.

f. Positive Control Mixture (Blood, Urine, Vitreous):
When possible external positive control(s) will be used. These can be prepared by using an appropriate vendor's product (e.g., UTAK DOA Level 2 (blood), PM 100 (blood), DAU High Cutoff 2 (urine), PM Plus Low (urine), Miscellaneous Panel Level 1 (urine)). Only analytes which have concentrations listed at or above three-times (3x) the listed levels within the In-House Cut-Off Positive Control Mixture analyte concentration will be evaluated.
In-house positive control mixtures will be used when external controls are not feasable. At least five (5) analytes found within the In-House Cut-Off Positive Control Mixture (at concentrations at least three-times (3x) their levels) will be used for quality control (QC) evaluation. When possible, control solutions will be matrix-matched.

The Positive Control Mixture will be prepared as needed and its preparation recorded within appropriate case file documents (i.e., case notes, batch paperwork).

Notes (applicable to Section 6):

Note-01: For vitreous samples 6-monoacetylmorphine (6-MAM ; 6-AM) is the only analyte evaluated. Control solutions for vitreous samples can be a different, but similar, matrix (e.g., water).

Note-02: Control solutions containing 6-MAM levels lower than three-times (3x) the In-House Cut-Off Positive Control Mixture concentration are allowed.

Note-03: If other drugs are used when preparing the In-House Cut-Off Positive Control Mixtures then a minor deviation (or other paperwork) will accompany all batch work and case files.

Note-04: 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.

a. Appropriate cleaning (e.g., methanol or bleach solution) will be done on table/bench surfaces before each batch of samples is analyzed. Prior to casework samples being opened (or analyzed while in an exposed state), brown paper (or other similar barrier) will be placed on top of the table/bench surface to minimize/prevent contamination.

b. Specimen Preparation (Blood, Urine, Vitreous):

   i. Add 0.5 mL of each unknown sample to a properly labeled test tube.
   Only one unknown sample will be open during transfer. Test tubes containing unknown samples will be capped or their tube positions changed after each individual transfer.

   ii. Prepare 0.5 mL of each control (Negative, External Positive, In-House Cut-Off Positive) to their own properly labeled test tubes.
   The appropriate matrix will be used based on the samples being analyzed (i.e., blood, urine, vitreous).

   iii. Add 100 µL of the I.S. Working Solution (Diazepam-D_5 + Butalbital-D_5 + Meprobamate-D_3 + Morphine-D_3 + Oxycodone-D_6) into each unknown and control sample.
   This is equivalent to 25 ng of Diazepam-D_5, 100 ng of Butalbital-D_5, 100 ng of Meprobamate-D_3, 25 ng of Morphine-D_3, and 25 ng of Oxycodone-D_6 all being added to each [0.5 mL] sample.

   Note: Sample test tubes may all be uncapped during this step.

c. Add 1 mL of the Sodium Acetate buffer (pH ~4.5) to each specimen.
Sample test tubes may all be open during this step.

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

e. Sonicate, if needed, for at least 15 minutes (required for blood samples).

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

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

h. 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}

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

j. Remove solvent tray and replace with biohazardous tray

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

l. 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.

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

n. To each SPE column:

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

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

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

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

q. 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).

   Note: The Solid Phase Extraction Elution Solution ratio is 80:18:2 CH_2Cl_2:IPA:NH_4OH. (Approximately 2 mL will be needed for each tube).

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

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

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

u. 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.

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

w. 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.

x. Analyze extract solutions using LC/HR–MS.
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. The instrument conditions may be modified slightly to adjust or improve the procedure as needed. The appendix contains an abbreviated version of the procedure and this checklist can be used by analysts. Any changes in the instrumental parameters for a batch will be reflected in the instrumental parameter documents that accompany data.

HPLC Parameters:

Mobile Phase A: 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$
Mobile Phase B: $MeOH_{(l)}$ (neat)
HPLC Column: Accucore C18, 2.6 µm, 50 mm x 3 mm (Thermo Scientific)
Column Temperature: 40 °C
Flow Rate: 0.7 mL/min
Autosampler Temperature: 15 °C
Injection Volume: 10 µL
Needle Wash: 80 µL before and after aspiration

LC Gradient Program: Initial 5% B ; Flow 0.7 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 %
0.2	95 %	5 %
2.5	85 %	15 %
5.2	5 %	95 %
5.7	5 %	95 %
5.8	95 %	5 %
7.8	STOP	STOP

MS

Parameter	Value		Parameter	Value
Method duration	~8 min		Method duration	~8 min
Mode	ESI, Full scan MS, MS/MS		Mode	ESI, Full scan MS, MS/MS
Polarity	Positive		Polarity	Negative
Resolution	70,000		Resolution	70,000
AGC Target	1e6		AGC Target	1e6
Maximum IT	50 ms		Maximum IT	50 ms
Scan Ranges	1		Scan Ranges	1
Scan Range	80-500 m/z		Scan Range	80-500 m/z
Spectrum Data	profile		Spectrum data type	profile

Note: See instrument method for additional parameters

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) are used as the basis for detection. In most cases all of the criteria below should be met in order to detect the appropriate drugs within biological specimens.

a. Chromatography

The data from the performance check solutions for the LC/MS instrument, when analyzed, will contain peaks that possess good chromatographic quality (i.e., peak shape, peak width, signal-to-noise).

All chromatographic peaks for any reportable analytes of interest should show good chromatographic characteristics with reasonable peak shape, width, and resolution. In order to

be deemed acceptable as a chromatographic peak analyte peaks will compare favorably to corresponding chromatographic peaks within known samples.
Additionally, the following two criteria will be met:

i. Retention Time (RT)

The retention time of the chromatographic peak will be within ±0.2 minutes of the retention time obtained from injection of a reference standard or positive control.

ii. Signal-to-Noise Ratio (SNR)

To justify the existence of a peak, its baseline signal-to-noise ratio (SNR) 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 (corresponding to the analyte of interest) at a similar retention time in a negative control or blank injected prior to that sample.

iii. Reporting Limit (RL)

Only analytes at or above a certain cut-off level will be reported.

A comparison of chromatographic peak area ratios (i.e., analyte-to-internal standard) between unknown samples compared to matrix-matched analytes found within control solutions will be used to determine when analytes will be reported. These peak area ratios will subsequently be termed, 'ratios.'
Note: Vitreous samples don't have to be compared to matrix-matched control solutions.

Analytes whose ratios are at or above the ratios from the same analytes within the In-House Cut-Off Positive Control Mixture will be reported.

Analytes not found within the In-House Cut-Off Positive Control Mixture whose ratios are at or above the ratios from the same drug class of analytes (e.g., amphetamines, opiates) within the In-House Cut-Off Positive Control Mixture will be reported.

Analytes not found within the In-House Cut-Off Positive Control Mixture which are outside the drugs and/or drug classes of those analytes within the In-House Cut-Off Positive Control Mixture will be reported when compared to an appropriate control. The comparison can either be from an extraction or from an unextracted positive control solution that contains an appropriate internal standard. Analytes who have ratios at or above the selected cut-off concentration ratios will be reported. Selected cut-off concentrations shall be determined from the Technical Lead (or higher) and will be documented within the batch and/or technical records (e.g., worksheet, case notes).
Note: Analyses of positive controls need not be done at the same time as unknown samples.

b. Mass Spectrometry (MS)

i. High Resolution Accurate Mass

In order for the MS instrument to be considered in good operating condition, the correct mass assignment for the internal standard will be present. The molecular ion will be present at the apex peak for each analyte with a tolerance of 5 mmu.

ii. Product Ion/Fragmentation Mass Spectrometry (MS/MS)

In order for the MS to be considered in good operating condition, the correct mass assignments for each reported analyte will be present. Independent MS/MS experiments are conducted for each analyte and ion ratios should compare favorably to ion ratios from reference standards (or extracted positive controls at comparable concentrations). Generally, ion ratios are within the following limits:

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

c. Batch Acceptance:

A batch of samples will be analyzed with appropriate controls. The decision of whether to accept the data within a batch will be determined by the following:

No analytes of interest should be detected within Negative Control samples. For this procedure, detected analytes of interest are defined as any analytes being listed in a final report (i.e., ratios greater than or equal to corresponding positive control (or class) ratios).

All batches containing reportable analytes shall have positive control data corresponding to each analyte. A reportable analyte is considered one which is toxicologically significant. When in doubt of whether an analyte is reportable or not, the Technical Lead (or higher) will be consulted. If an analyte is detected but not reported, documentation of this (along with a reason) will be included within the batch paperwork and/or within technical records (e.g., worksheet, case notes).

Internal standards within samples will contain acceptable chromatographic and mass spectral qualities (as defined above). Individual samples that don't contain acceptable relevant internal standard data will not be accepted and will need to be repeated. For example, if an analyte is an alkaline drug and the alkaline internal standard data is acceptable, but the weak acid and/or neutral internal standard data are unacceptable, the analyte in question can be considered reportable – but the Technical Lead will be consulted and documentation as to why it is acceptable will be included within the batch paperwork and/or within technical records (e.g., worksheet, case notes).

d. Screening versus Confirmation:

This procedure can provide presumptive (screening) results when only a single aliquot of sample is analyzed and a single mass spectrometric experiment is utilized. Confirmatory results will include either:

i. Analyzing a second aliquot (which includes re-extraction) using the same instrument method, and/or

ii. Analyzing the same sample extract using a different instrumental method (i.e., different chromatographic method or mass spectral technique). A different chromatographic method would include changing chromatographic columns or mobile phases such that analyte retention times differ.
A different mass spectral technique would include variations in mass spectral experiments (e.g., MS/MS). Such experiments can be included within the same instrumental method, thus allowing one aliquot of specimen to be simultaneously analyzed by two (2) mass spectral experiments (e.g., LC/HRMS (MS/MS)).

Any questions or concerns that are not addressed in this procedure regarding whether data should be used as screening versus confirmation will be answered or addressed by the Unit Lead (or higher). Decisions regarding whether to report analytes as presumptive positive or as confirmatory due to unusual circumstances (e.g., analytes not producing significant product ions or isobaric compounds having the same retention time) will be based on the above criteria in conjunction with the Unit Lead (or higher). Such decisions will be documented within case files.

Negative samples can be reported based on the single [screening] analysis.

Processing of additional analytes using high-resolution mass spectral data (i.e., M+H) can be performed using extracted ion chromatography. This is useful in attempts to detect other potential analytes of interest that are not within the instrument method. Alternatively, multiple injections of sample extracts may be performed using additional instrumental methods so as to identify other analytes of interest.

When an analyte that is not found in the positive control is detected within a sample, the sample will be compared to a known control, if possible, for retention time and mass spectral verification (both HR-MS and MS/MS). A general description of how to prepare such analytes can be found within the Reporting Limit (RL) section below (with respect to concentrations). If positive controls for detected analytes are not available, the Technical Lead (or higher) will be consulted for guidance. Decisions will be documented within batch paperwork and/or within technical records (e.g., worksheet, case notes).

Note: When analytes are known to give potential false positive results using a particular experiment (e.g., amphetamine using only HR-MS), then simply analyzing a second aliquot using the same experimental method will not be done for confirmation purposes ; and alternate experiment(s) will be employed for confirmatory results to be reported.

12. Uncertainty

Not applicable – this is a qualitative procedure only.

13. Limitation

a. Limits of Detection (LOD)
This information will be found in the validation paperwork for the instrument.

Extraction LOD for analytes are administratively set to be at least at cut-off concentrations within this procedure.

b. Reporting Limits (RL):
Administratively set analyte concentrations within positive control solutions (e.g., In-House Cut-Off Positive Control Mixture). The same concentrations will be used for all biological matrix types (e.g., blood, urine, vitreous) and are based on recommended blood level concentrations related to driving while impaired data and literature values (e.g., therapeutic concentrations).

c. Specificity
No known interferences have been found within biological specimens.

d. Experimental
There are a limited number of analytes (i.e., ~44) which can be detected during the MS/MS experiment within an instrumental method. Alternate instrumental experiments can be used for the detection of additional analytes. Refer to the specific instrumental methods for actual list of analytes. However, full scan data is still collected (i.e., M+H data) and extracted ion chromatography during data processing can be applied to help in the detection of analytes that are not found within instrumental experiments.

14. Safety

This procedure is carried out in a laboratory environment and standard safety procedures appropriate for such an environment will 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.

15. References

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

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:
Drug Screening and Confirmation by LC/HR-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, Urine, Vitreous)	**Lot:**
External Positive Control (Blood, Urine, Vitreous)	**Lot:**
In-House Cut-Off Positive Control (Blood, Urine, Vitreous)	**Lot:**

Procedure:

	For Blood or Urine (or Other Matrix) 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/control :		
	Diazepam-D_5 + Butalbital-D_5 + Meprobamate-D_3 + Morphine-D_3+ Oxycodone-D_3	**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) to Solvent Waste tray		**Lot:**
Add 1 mL of water (to wash each SPE), drain (≈ 3 psi) to Biohazardous tray		**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/Carbonate buffer (pH ~9.0), then drain (≈ 3 psi) to Biohazardous waste	**Lot:**
	Wash with 1 mL water, then drain (≈ 3 psi) to Biohazardous waste	**Lot:**
Dry the columns for ~10 minutes using maximum pressure (e.g., between 60-80 psi)		
During this 10 minute window (or earlier) label autosampler vials, place in SPE rack. Prepare the elution solvent.		
The Solid Phase Extraction Elocution 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 autosampler 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		**Lot:**
Reconstitute each extract sample with 500 µL of 20% $MeOH_{(aq)}$; only if analyzing immediately		**Lot:**
Check turbidity of solution. Remove undissolved particulates and treat as necessary.		
Analyze by HR-LC/MS		

Appendix (Cont'd):

Specimen Procedure (Cont'd)

Analyze by LC/HR-MS:

Instrument: Q-Exactive	Instrument Identifier:
HPLC Column: Accucore C18, 2.6 µm, 50 mm x 3 mm (Thermo Scientific)	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 0.7 mL/min

Time (min.)	%B
Initial	5
0.2	5
2.5	15
5.2	95
5.7	95
5.8	5
7.8	STOP

Mass Spectrometer Parameters

Ion Source	Positive and Negative ion
Mode	ESI ; Full Scan ; MS/MS
Resolution	70,000
Range	100-500 m/z
Max ionization time	50 ms
AGC	1E+06

AGC = Automatic Gain Control

Rev. #	History
1	New procedure for urine, blood or other appropriate matrices.