1. Introduction

A rapid liquid chromatographic method using heated electrospray ionization (HESI) combined with high resolution accurate mass (HRAM) spectrometry (LC/MS) can be used to qualitatively screen and confirm biological specimens (e.g., blood, serum/plasma, urine) for the presence of drugs and/or their metabolites. The amount of a detected analyte can be estimated by comparing the responses for an analyte to that for a corresponding internal standard. Any amount greater than the established cutoff level will be positive. Positive findings will be confirmed by using a second technique. Confirmation can also be achieved using quantitative analyses using appropriate procedures.

2. Scope

This procedure is limited to the qualitative screening and confirmation of biological specimens for the presence of prescription medications, over the counter medications and drugs of abuse. (See list of analytes in TX 37.3). 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 or dilute and shoot procedure. Final extracts are analyzed by LC/HRMS and can involve a MS1 technique (full scan analysis) or MS2 techniques (parallel reaction monitoring (PRM) and/or data independent acquisition (DIA)).

4. Specimens

This procedure uses biological fluid(s) such as blood, serum/plasma and urine. 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.

Dilutions due to low specimen volume or high concentration may be used with the approval from a FSE2 and above. Changes shall be documented within technical records (e.g., worksheets, case notes).

5. Equipment/Materials/Reagents

Equivalent equipment/material/reagent may be purchased. If a certified reference standard has a different concentration an appropriate volume (µL) shall be used.

5.1 General laboratory glassware

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

5.3 Vortex mixer

5.4 Sonicator

5.5 Automatic pipettes (with disposable tips)

5.6 Positive pressure solid phase extraction device (Cerex, 48 sample)

5.7 Sample concentrator with nitrogen (Cerex 48 heated)

5.8 Centrifuge

5.9 pH paper (Micro Essentials Lab)

5.10 Trace B Extraction Columns (Tecan)

5.11 Liquid Chromatograph/High Resolution-Mass Spectrometer (LC/HR-MS) (Q-Exactive)

5.12 HPLC column: Accucore C18, 2.6 µm, 50 mm x 3 mm (Phenomenex)

5.13 Pre-Column: Accucore Defender guard 2.6 µm, 10 mm x 3 mm (Phenomenex)

5.14 Autosampler vials (LC/MS grade 1.8 mL)

5.15 Acetic acid, glacial ($CH_3COOH_{(l)}$, Reagent grade)

5.16 Ammonium formate (NH_4CHOO, LC/MS grade)

5.17 Ammonium Hydroxide (NH_4OH, Reagent grade)

5.18 Formic acid (HCOOH, LC/MS grade)

5.19 Hydrochloric Acid ($HCl_{(conc)}$; Reagent grade)

5.20 Isopropanol (IPA, 2-Propanol, Reagent grade)

5.21 Methanol (MeOH, Reagent grade)

5.22 Methylene Chloride (CH_2Cl_2, Reagent grade)

5.23 Sodium acetate trihydrate ($NaCH_3COO \cdot 3H_2O$, Reagent grade)

5.24 Sodium bicarbonate ($NaHCO_3$; Reagent grade)

5.25 Sodium carbonate (Na_2CO_3 ; Reagent grade)

5.26 Water (H_2O ; Millipore, Deionized (DIW))

5.27 Ammonium $Formate_{(aq)}$: ($NH_4CHOO_{(aq)}$; 5M ; 31.5% (w/v)): 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.

5.28 Sodium $Acetate\ Buffer_{(aq)}$ ($NaCH_3COO$; pH~4.5): 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. Verify pH ~4.5 prior to use.

5.29 $Sodium\ Bicarbonate_{(aq)}$ (0.1M ; $NaHCO_3$; 0.84% (w/v) ; pH~8): 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.

5.30 Sodium Carbonate$_{(aq)}$ (0.1M ; Na_2CO_3 ; 1.1% (w/v) ; pH~11): 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.

5.31 Bicarbonate/Carbonate Buffer$_{(aq)}$ (pH~9.0): 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. Verify pH ~9.0 prior to use.

5.32 Solid Phase Extraction Elution Solution (CH_2Cl_2 : IPA : NH_4OH (80:18:2)): Prepared by adding 18 mL of isopropanol to 2 mL of ammonium hydroxide within a 100 mL graduated cylinder. To this mixture add 80 mL of methylene chloride and mix. This solution will be prepared fresh when needed for use.

5.32.1 The volume can be adjusted to account for number of samples that are to be extracted (2 mL are needed for each sample).

5.33 Methanol-HCl (1% (v/v)): Prepared by dissolving 10 µL of hydrochloric acid into 990 µL of methanol (or in different volume with an equivalent ratio). This solution will be prepared fresh when needed for use. Verify pH is ≤ 3 prior to use.

5.34 Mobile Phase A – 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$: Prepared by mixing 100 µL of formic acid with 1 mL of 5M ammonium formate in a 1000 mL volumetric cylinder, diluting to volume with water, and mixing well. Store in glass at room temperature. Stable for at least one (1) week while in a closed state.

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

6. Standards and Controls

6.1 Internal Standard (IS) Working Solution (Diazepam-D_5 (250 ng/mL) + Butalbital-D_5 (1000 ng/mL)): Prepared by individually combining 250 µL of 0.1 mg/mL solution of Diazepam-D_5 and 1 mL of 0.1 mg/mL solution of Butalbital-D_5 into a 100 mL volumetric flask and diluting to volume with MeOH. Store in glass in freezer. Stable for one (1) year from date of preparation.

6.2 Cutoff Solution: Prepared using purchased reference standards and diluted with MeOH according to TX 37.1. Store in glass in freezer. Stable for six (6) months from date of preparation.

Note: Due to the large number of analytes in this method, there may be multiple Cutoff Working Standard Solutions.

6.3 Positive Control Solution: Prepared using purchased reference standards and diluted with MeOH according to TX 37.1. Store in glass in freezer. Stable for six (6) months from date of preparation.

Note: Due to the large number of analytes in this method, there may be multiple Positive Control Working Standard Solutions.

6.4 External Positive Control: When possible an external positive control will be run in addition to the in-house prepared controls. 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)).

7. Qualitative Analysis

This procedure may be used qualitatively through the construction of a single-point calibration graph for the analyte(s) of interest. Use the table below and analyze the extract solutions from the negative, cutoff and positive control solutions along with samples.

Table 1: Cutoff and Control Solutions

Level	Volume of each Cutoff Solution (µL)	Volume of each Positive Control Solution (µL)	Volume of Blank Matrix (mL)
Negative Control	-	-	0.5
Cutoff	10	-	0.5
3x Positive Control	-	30	0.5
3x Positive Control (end)	-	30	0.5

8. Sampling

Not applicable.

9. Sample Preparation Procedures

9.1 Qualitative screening analysis will be performed using the dilute and shoot procedure.

9.2 Qualitative confirmatory analysis will be performed using the solid phase extraction procedure.

9.3 Upon approval from a FSE2 and above, the solid phase extraction procedure may be used for qualitative screening.

10. Dilute and Shoot Procedure

10.1 Add 100 µL of the IS Working Solution to each test tube, cap and vortex-mix.

10.2 Prepare cutoff and control solutions according to Table 1 and label appropriately. These should contain 0.5 mL of sample each. Cap when not in use.

10.3 Add 0.5 mL of unknown specimen(s) and External Positive Control (if applicable) to properly labeled test tubes and cap when not in use.

10.4 Add 1.5 mL of cold ACN (stored in freezer) to sample while vortexing

10.5 Centrifuge tubes for 10 minutes at a minimum of 3000 rpm.

10.6 Transfer supernatant to test tube

10.7 Proceed to Section 12

11. Solid Phase Extraction Procedure

11.1 Add 100 µL of the IS Working Solution to each test tube, cap and vortex-mix.

11.2 Prepare cutoff and control solutions according to Table 1 and label appropriately. These should contain 0.5 mL of sample each. Cap when not in use.

11.3 Add 0.5 mL of unknown specimen(s) and External Positive control (if applicable) to properly labeled test tubes and cap when not in use.

11.4 Add 1 mL of 0.10 M sodium acetate buffer (pH ~4.5) into each test tube and cap.

11.5 Add 500 uL of water into each test tube and cap. Vortex-mix each test tube (~10 seconds).

11.6 For blood, sonicate for at least 15 minutes.

11.7 Centrifuge all tubes for ~8 minutes at ~5200 rpm.

11.8 For below steps, ensure to use appropriate waste collection container (organic or biohazard).

11.9 Precondition the SPE columns (do not allow the sorbent to dry):

11.9.1 Methanol (1 mL)

11.9.2 Water (1 mL)

11.10 Add the samples to the properly labeled SPE columns:

11.10.1 Slowly decant the supernatant from the samples (use caution to avoid debris) to the column.

11.10.2 Use pressure to push through the samples at 1-2 mL/minute.

11.11 Sequentially perform the following wash/rinse on each SPE column:

11.11.1 Bicarbonate/Carbonate $Buffer_{(aq)}$ (1 mL; pH ~9.0)

11.11.2 Water (1 mL)

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

11.13 Once the SPE columns are dry, replace the plastic waste tray with the SPE collection rack containing the collection tubes. Ensure that each tube is placed under the corresponding SPE column for elution.

11.14 Elute the SPE columns:

11.14.1 Add 2 mL of the Solid Phase Extraction Elution Solution (CH_2Cl_2 : IPA : NH_4OH (80:18:2)) to each SPE column.

11.14.2 Collect the eluent using gravity or low pressure (<3 psi).

11.15 Remove the top SPE column rack and transfer the collection rack from the SPE manifold to the sample concentrator. Ensure concentrator parts are clean and free of contaimination.

11.16 Proceed to Section 12

12. Post Extraction Procedure

12.1 Add 10 µL of 1% MeOH-HCl to each tube.

12.2 Evaporate all samples to dryness at ~<40°C.

12.3 Reconstitute each sample extract with 50 µL MeOH, vortex, then 450 µL H_2O, vortex again.

12.4 Transfer samples into properly labled autosampler vials.

12.5 Ensure the appropriate instrumental quality assurance/quality control (QA/QC) procedures were performed. The instrument must have passing QA/QC results prior to preparing and loading of samples.

12.6 Prepare the sequence and enter the samples in appropriate order. Negative controls will be analyzed prior to evidentiary samples. Blank samples (i.e., those containing just $MeOH_{(aq)}$ (10% (v/v)) may be analyzed in between evidentiary samples to avoid carry-over and shall be analyzed after the 3x Positive Control is injected.

12.7 Verify the sequence:

12.7.1 Print the sequence list.

12.7.2 Check that the physical placement of the autosampler vials and the vial positions within the instrument's sequence list match. Indicate completion of this check using sequence checked, sequence verified or similar on the sequence page along with analysts initials and date.

12.8 Print the instrument method and include both the method and sequence printouts with the batch documents.

13. Instrumental Parameters

For complete method parameters see TX 37.2. Documentation of changes must be included with batch data so that any instrumental parameter change can be associated with data and casework until this procedure has been updated.

14. 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.

14.1 Solvent Blanks

14.1.1 If a solvent blank was injected, it must be reviewed for possible carryover.

14.1.2 The solvent blank shall not contain any analyte measured by this assay, at a response greater than 10% of the cutoff, which meets reporting criteria (retention time and peak shape). If an analyte is present in the solvent blank and the following injection, this analyte shall not be reported in that injection. Upon re-injection of the solvent blank and the corresponding sample, if the solvent blank is acceptable, proceed with analysis.

14.2 Chromatography

14.2.1 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, there may be transitions that are not optimal. In order to be determined as acceptable, a chromatographic peak in a 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.

14.3 Retention Time (RT)

14.3.1 The retention time of a peak of interest should be within 0.1 minute of the retention time of a reference standard (i.e., cutoff or positive control).

14.4 Mass Spectrometry

14.4.1 MS1: The molecular ion will be present at the apex peak for each analyte with a tolerance of 5 ppm.

14.4.2 MS2: Ion ratios should compare favorably to ion ratios of an extracted cutoff or positive control at a comparable concentration. See section procedure for mass spectral comparisions for set-point tolerance ranges.

14.5 Batch Acceptance

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

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

14.5.2 Significant carryover will be brought to the attention of a FSE2 or higher to determine if evidentiary samples have been negatively impacted. If so, re-

injection will occur and sample re-extraction may be necessary. Appropriate case documentation will accompany these instances within affected case files to record events.

14.5.3 All applicable analytes of interest within positive controls, as well as internal standards, will be identified.

14.5.3.1 When in doubt of whether an analyte is reportable or not, a FSE2 or higher will be consulted.

14.5.3.2 Internal standards will also contain acceptable chromatographic and mass spectral qualities as defined above. Responses must be within +/- 20% of a cutoff or control. If internal standard responses are outside 20%, a FSE2 or higher will be consulted.

14.6 Reporting Limit

14.6.1 The reporting limit for each analyte is administratively set and will be used for all matrix types (blood, urine). Reporting limits are based on recommended blood level concentrations related to driving while impaired data and literature values. See TX 37.3 for all reporting limits.

14.6.2 Analytes will be reported as "positive" in the batch if greater than the reporting limit.

15. Uncertainty

Not applicable.

16. Limitations

16.1 Case samples that need to be re-injected, will be injected along with a negative control, cutoff and positive control.

16.2 Case samples that were inadvertently not injected and the sequence has completed, will be injected along with a negative control, cutoff and positive control.

17. 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. Refer to Safety Manual for further guidance.

18. 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