1 PRINCIPLE/SCOPE

This procedure is mainly used for detection of various stimulants and their respective metabolites within biological specimens (e.g., blood/serum) within a variety of forensic-related requests (e.g., driving under the influence, drug-facilitated assault). Deuterated internal standards are added to specimens for both qualitative measures (extraction monitoring) and quantitation purposes. In this procedure, blood specimens undergo a protein precipitation process to clean up any matrix before instrumental analysis. The supernatant is transferred to a clean test tube, evaporated to dryness and reconstituted using a 5% methanol solution. After sample preparation, samples are analyzed by LC/MS/MS.

2 SPECIMENS

This procedure uses biological fluids (e.g., blood, serum or plasma). Typically, 0.25 mL of sample is used during the analysis, but differing volumes may be necessary depending on the matrix. Dilution of samples due to limited specimen, due to suspicion of high drug or metabolite concentrations (as possibly indicated by presumptive results), due to possible matrix-effects, or similar reasons is acceptable. All dilutions and other significant changes should be recorded and communicated appropriately.

3 REAGENTS

3.1 In the event that a part number or item number listed is not available, an equivalent may be purchased.

3.2 If the equivalent reference material purchased has a different concentration, an appropriate volume (µL) shall be used.

3.3 All reagents must be ACS grade or better.

3.3.1	Formic Acid	Fisher 27048
3.3.2	Ammonium Formate	Fisher A11550
3.3.3	Water	Millipore, Deionized (DIW)
3.3.4	Acetonitrile (ACN)	Fisher A955
3.3.5	Hydrochloric Acid (HCL)	Fisher A144
3.3.6	Methanol	Fisher A456

3.3.7	Drug Free Blood	UTAK 44600-WB(F)
3.3.8	Amphetamine (1.0 mg/mL)	Cerilliant A-007
3.3.9	Amphetamine (1.0 mg/mL)	Lipomed AMP-95-HC-1LM
3.3.10	Amphetamine-d6 (1.0 mg/mL)	Cerilliant A-045
3.3.11	Ephedrine (1.0 mg/mL)	Cerilliant E-011
3.3.12	Ephedrine (1.0 mg/mL)	Lipomed EPH-888-HC-1LM
3.3.13	Ephedrine-d3 (1.0 mg/mL)	Cerilliant E-026
3.3.14	Pseudoephedrine (1.0 mg/mL)	Cerilliant P-035
3.3.15	Pseudoephedrine (1.0 mg/mL)	Lipomed EPH-776-FB-1LM
3.3.16	Methamphetamine (1.0 mg/mL)	Cerilliant M-009
3.3.17	Methamphetamine (1.0 mg/mL)	Lipomed AMP-301-HC-1LM
3.3.18	Methamphetamine-d5 (1.0 mg/mL)	Cerilliant M-023
3.3.19	MDA (1.0 mg/ml)	Cerilliant M-012
3.3.20	MDA (1.0 mg/ml)	Lipomed MDA-79-HC-1LM
3.3.21	MDMA (1.0 mg/mL)	Cerilliant M-013
3.3.22	MDMA (1.0 mg/mL)	Lipomed MDM-94-HC-ILM
3.3.23	Cocaine (1.0 mg/mL)	Cerillint C-008
3.3.24	Cocaine (1.0 mg/mL)	Lipomed COC-156-FB-1LA
3.3.25	Cocaine-d3 (1.0 mg/mL)	Cerilliant C-014
3.3.26	Cocaethylene (1.0 mg/mL)	Cerillint C-010
3.3.27	Cocaethylene (1.0 mg/mL)	Lipomed COC-207-FB-1LA
3.3.28	Benzoylecgonine (1.0 mg/mL)	Cerilliant B-004
3.3.29	Benzoylecgonine (1.0 mg/mL)	Lipomed COC-204-FB-1LM
3.3.30	Benzoylecgonine-d3 (1.0 mg/mL)	Cerilliant B-008

4 EQUIPMENT

4.1 LC-MS/MS

4.1.1 Agilent 6475AA QQQ with a 1260 Infinity II LC stack or equivalent

4.2 LC Column

4.2.1 50 x 3.0mm, 2.7 µm particle size, Poroshell 120 EC-C18, Agilent, or equivalent

4.3 Guard Cartridge

4.3.1 Poroshell 120 UHPLC Guard Column, EC-C18 3.0mm, Agilent, or equivalent

4.4 Centrifuge capable of at least 3000 rpm

4.5 Vortexer

4.6 Disposable borosilicate test tubes (e.g. 16x100 mm, round bottom, borosilicate glass with applicable caps)

4.7 Autosampler vials with inserts (1.8 mL or equivalent)

4.8 Sample concentrator with nitrogen or air (e.g. Cerex 48 heated)

5 SOLUTION PREPARATION

Different volumes may be prepared as long as the concentration/ratio is consistent.

5.1 **Mobile phase A (5mM Ammonium Formate/0.01% Formic Acid in water)**

5.1.1 Combine 0.315 g Ammonium Formate, 0.100 mL of formic acid to a final volume of 1000 mL in a glass bottle.

5.1.2 Make Fresh

5.2 **Mobile phase B (Methanol)**

5.2.1 Store in glass at room temperature.

5.3 **1% Methanol-HCL**

5.3.1 To a 10 mL volumetric flask partially filled with methanol, at 100 µL of HCL.

5.3.2 QS to volume using methanol. Mix.

5.3.3 Make Fresh

5.4 **5% Methanol Solution**

5.4.1 Combine 50 mL of methanol and 950 mL of water to a 1000 mL glass bottle.

5.4.2 Stable for 6 months when stored at room temperature.

5.5 **Stock Calibration Solution (Amp, EPH, PS, Meth, MDA, MDMA/Cocaine, CE/BE – 10/5/25 µg/mL)**

5.5.1 Add the following to a 10 mL volumetric flask

Analyte Name	Standard Concentration (Cerilliant)	Amount to Pipette(uL)
Amphetamine	1.0 mg/mL	100
Ephedrine	1.0 mg/mL	100
Pseudoephedrine	1.0 mg/mL	100
Methamphetamine	1.0 mg/mL	100
MDA	1.0 mg/mL	100
MDMA	1.0 mg/mL	100
Cocaine	1.0 mg/mL	50
Cocaethylene	1.0 mg/mL	50
Benzoylecgonine	1.0 mg/mL	250

5.5.2 QS to the line using methanol. Mix.

5.5.3 Stable for 6 months when stored in the freezer.

5.6 **Dilute Calibration Solution (Amp, EPH, PS, Meth, MDA, MDMA/Cocaine, CE/BE – 1.0/0.5/2.5 µg/mL)**

5.6.1 To a 10 mL volumetric flask, add 1000 µL of Stock Calibration Solution (10/5/25 µg/mL).

5.6.2 QS to the line using methanol. Mix.

5.6.3 Stable for 6 months when stored in the freezer.

5.7 **Working IS Solution (Amp-D6, EPH-D3, Meth-D5/Cocaine-D3/BE-D3 – 1.0/0.5/2.5µg/mL)**

5.7.1 Add the following to a 10 mL volumetric flask

Analyte Name	Standard Concentration (any manufacturer)	Amount to Pipette(uL)
Amphetamine-D6	1.0 mg/mL	10
Ephedrine-D3	1.0 mg/mL	10
Methamphetamine-D5	1.0 mg/mL	10
Cocaine-D3	1.0 mg/mL	5
Benzoylecgonine- D3	1.0 mg/mL	25

5.7.2 Dilute to the line using methanol. Mix.

5.7.3 Stable for 6 months when stored in the freezer.

5.8 **Stock Control Solution (Amp, EPH, PS, Meth, MDA, MDMA/Cocaine, CE/BE – 10/5/25 µg/mL)**

5.9 Add the following to a 10 mL volumetric flask

Analyte Name	Standard Concentration (Lipomed or Cayman)	Amount to Pipette(uL)
Amphetamine	1.0 mg/mL	100
Ephedrine	1.0 mg/mL	100
Pseudoephedrine	1.0 mg/mL	100
Methamphetamine	1.0 mg/mL	100
MDA	1.0 mg/mL	100
MDMA	1.0 mg/mL	100
Cocaine	1.0 mg/mL	50
Cocaethylene	1.0 mg/mL	50
Benzoylecgonine	1.0 mg/mL	250

5.9.1 Dilute to the line using methanol. Mix.

5.9.2 Stable for 6 months when stored in the freezer.

5.10 **50**

5.10.1 To a 10 mL volumetric flask, add 1000 uL of Stock Control Solution (10/5/25 µg/mL).

5.10.2 Dilute to the line using methanol. Mix.

5.10.3 Stable for 6 months when stored in the freezer.

6 PROCEDURE

6.1 Sample Preparation

6.1.1 Label clean test tubes accordingly: Calibrators, controls and case specimen identifier.

6.1.2 Add 50 µL of Working IS Solution to all tubes.

6.1.3 Pipette 0.25 mL of case specimen to the appropriately labeled tube unless directed to run the sample diluted.

6.1.3.1 If a dilution is needed, make up remaining blood volume using blank blood to ensure total volume used is 0.25mL (i.e., x2 dilution would require 0.125mL blank blood in addition to 0.125mL case sample). NOTE: Serum/Plasma case specimen will not receive any additional blank matrix, resulting in total volume less than 0.25mL.

6.1.4 To prepare the calibration curve, pipette the following volumes of the Designated Calibration Solution into 0.25 mL of drug-free blood:

Calibrator	**Dilute Cal Solution (µL)**	**Stock Cal Solution (µL)**	**Final Concentration of Target Analytes (ng/mL)** Amp, EPH, PS, Meth, MDA, MDMA/Cocaine,CE/BE
Level 1	5	N/A	20/10/50
Level 2	10	N/A	40/20/100
Level 3	25	N/A	100/50/250
Level 4	N/A	5	200/100/500
Level 5	N/A	12.5	500/250/1250
Level 6	N/A	25	1000/500/2500
Level 7	N/A	50	2000/1000/5000

6.1.5 To prepare the in-house controls, pipette the following volumes of the Designated Control Solution into 0.25 mL of drug-free blood.

Control	**Dilute Control Solution (µL)**	**Stock Control Solution (µL)**	**Final Concentration of Target Analytes (ng/mL)** Amp, EPH, PS, Meth, MDA, MDMA/Cocaine,CE/BE
Negative	0	0	0
Low	12.5	N/A	50/25/125
Mid	N/A	10	400/200/1000
High	N/A	40	1600/800/4000

6.1.6 Add 0.50 mL cold ACN (stored in Freezer) dropwise to the sample while vortexing.

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

6.1.8 Transfer supernatant to appropriate test tube

6.1.9 Add 10µL of 1% MeOH-HCL solution to each tube

6.1.10 Evaporate tubes to dryness at approximately 40°C

6.1.11 Add 500 µL 5% MeOH solution to each test tube. Vortex

6.1.12 Transfer 200 µL supernatant to appropriate LC-MS/MS vial.

6.1.13 Cap the LC-MS/MS vials and mix.

6.1.14 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.

6.1.15 Prepare the sequence and enter the samples in appropriate order. Negative control will be analyzed prior to evidentiary samples. Blank samples (i.e., those containing methanol) may be analyzed in between evidentiary samples to avoid carry-over.

6.1.16 Verify the sequence:

6.1.16.1 Print the sequence list

6.1.16.2 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.

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

7 INSTRUMENT PARAMETERS

For complete method parameters see TX 46.2. 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.

8 DATA PROCESSING

8.1 Refer to TX 43 TOX Quality Control Manual for specific batch processing criteria.

8.2 Ion ratios should compare favorably to ion ratios of an extracted calibrator or positive control at a comparable concentration. Generally, ion ratios are within the limits specific within the section procedure related to mass spectral comparisons. NOTE: With the exception of the internal standard, it is recognized that some ion ratios are concentration dependent; thus, concentrations at the ends of the calibrations curve may not be within the updated ratios and may be acceptable.

8.3 STABILITY POST-EXTRACTION

8.3.1 Calibrators, controls or case specimens may be re-injected within 48 hours of being injected with a solvent blank prior. The chromatograms for both injections are saved with the data packet. A positive control must be re-injected with case specimens to verify that the curve is still acceptable if the sequence was completed.

8.3.2 If a case specimen(s) is re-injected after 48 hours, the calibrators and controls shall be re-injected along with the case specimen(s). NOTE: This would be considered a new analytical run and prepared separately from the original runs.

8.3.3 If a case specimen(s) was inadvertently not injected and it is more than 48 hours since the first calibrator was injected, you shall re-inject the calibrators and controls with the case specimen(s). NOTE: This would be considered a new analytical run and prepared separately from the original runs. ASSAY CHARACTERISTICS

8.4 The limit of detection for each analyte is listed below:

Analyte	LOD
Amphetamine	20 ng/mL
Ephedrine	20 ng/mL
Pseudoephedrine	20 ng/mL
Methamphetamine	20 ng/mL
MDA	20 ng/mL
MDMA	20 ng/mL
Cocaine	10 ng/mL
Cocaethylene	10 ng/mL
Benzoylecgonine	50 ng/mL

8.4.1 Results with values greater than or equal to 10 ng/mL will be reported as a whole number.

8.4.2 All analytes demonstrate a linear regression model using 1/x weighting except MDA.

8.4.3 MDA demonstrates a quadratic regression model using 1/x weighting.

8.4.4 LOQ is equal to the lowest cal

8.4.5 ULOQ is equal to the highest cal

8.4.6 Dilutions of up to x5 (1:4) may be performed.

9 SAFETY PRECAUTIONS

Refer to the DSS GL 2 Safety Manual for precautions.