DRUG SCREENING OF BIOLOGICAL SPECIMENS BY LIQUID CHROMATOGRAPHY - TIME OF FLIGHT MASS SPECTROMETRY

41.1 METHOD

This screening test method may be used to identify drugs and drug metabolites in biological specimens. The target compounds and internal standards are isolated from submitted biological specimens through precipitation of blood with acetonitrile. The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer – time of flight (TOF) detector equipped with a dual spray atmospheric pressure electrospray ionization source, acquired in positive (POS) or negative (NEG) acquisition mode.

41.2 SPECIMENS

Specimen volume is 0.2 mL of blood. Other screening methods should be utilized for screening of alternate matrices. Dilutions of specimens may be analyzed at the discretion of the Forensic Scientist.

NOTE: Validation studies determined specimens run at a dilution do not need to be brought to standard volume.

41.3 REAGENTS, MATERIALS AND EQUIPMENT

NOTE: Only LC-MS grade (or equivalent from a high-purity filtration system) water (H_2O) and HPLC grade methanol (MeOH) are used in this procedure. Acetonitrile (ACN) used as the extraction solvent (with or without internal standard) is reagent grade. ACN used in preparation of TOF reference solution is LC-MS grade.

41.3.1 REAGENTS

- Acetonitrile (ACN), LC-MS and reagent grade
- Ammonium formate
- 5 mM Ammonium formate

  Add 0.31 g of ammonium formate to a 1 L flask containing approximately 20mL of LC-MS grade H_2O. Mix and dilute to 1 L with LC-MS grade H_2O. **Do not filter** this solution prior to use on the HPLC. Store the solution in a glass amber bottle at room temperature for no more than 5 days.

- API TOF reference mix (includes purine in ACN/H_2O and HP-0921 in ACN, Agilent part #G1969-85001)

  Prepare by adding 100 mL LC-MS H_2O to 900 mL of LC-MS grade ACN. Add 300uL of 5 mM purine in ACN/H_2O and 300uL of 2.5 mM HP-0921 to the ACN/H_2O mixture. Mix by inversion. Store the solution in a polypropylene or amber glass bottle at room temperature for up to one year.

- Certified blank blood
- H_2O, LC-MS grade (or equivalent from a high-purity filtration system)

- Methanol (MeOH), HPLC grade

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

41.3.2 MATERIALS

- Disposable 12 x 75 mm extraction tubes and screw cap or centrifuge tubes, with closures
- HPLC Column, Agilent Zorbax Eclipse Plus C18, 3.0 x100 mm, 1.8 µM particle size, or equivalent
- HPLC Guard Column, Agilent Zorbax Eclipse Plus C18, 4.6 x 5 mm, 1.8 µM particle size, or equivalent
- In-line HPLC filters (Agilent part #5023-0271)
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps

41.3.3 EQUIPMENT

- Agilent HPLC 1200 series or equivalent
- Agilent MS-TOF (6200) with API-ES source, or equivalent
- Calibrated, adjustable piston pipettes and verified, adjustable repeater pipettes with disposable tips
- General use laboratory equipment (centrifuge, evaporator, vortex mixer)

41.4 STANDARDS

- See APPENDIX A for list of compounds and concentrations for standards

41.5 BLANKS, CALIBRATORS AND CONTROLS

41.5.1 BLANKS

A blood matrix blank is included in the batch, with acetonitrile used as the extraction solvent.

41.5.2 CALIBRATORS

A semi-quant calibrator is prepared in certified blank blood at the time of analysis, as detailed in 41.6 SAMPLE PREPARATION. The semi-quant calibrator includes only target compounds identified in POS mode.

41.5.3 CONTROLS

41.5.3.1 At least one negative blood control and three positive blood controls (A, B, and C) are tested with every batch, as described in 41.6. If acquiring the batch in NEG mode, at least one negative control and two N positive controls are tested with the batch (to bracket specimens with known samples).

41.5.3.2 Samples with known target concentrations (semi-quant calibrator and positive/negative controls) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive known samples (semi-quant calibrator or positive control). When the batch (each acquisition mode, POS or NEG mode) contains more than 20 specimens, a known sample must be analyzed mid-run.

41.6 SAMPLE PREPARATION

NOTE: Specimens of poor quality (e.g., spleen squeeze, bile, oily specimen) should be analyzed at the end of the batch, followed by two solvent blank injections and reinjection of a positive control.

41.6.1 Label a clean 12 x 75 mm extraction tube for each member of the test batch. (i.e., blank, semi-quant calibrator, control, case specimen).

41.6.2 Using a calibrated pipette, add 0.2 mL of certified blank blood into each of the blank matrix, semi-quant calibrator, positive control tubes and the negative control tube(s).

41.6.3 Using a calibrated pipette, add 10 µL of the semi-quant working standard (1/2/4/5/10 ng/µL) to the semi-quant calibrator (SQ) tube.

41.6.4 Using a calibrated pipette, add the listed amounts of control working standards (QC) to each of the individual positive control tubes.

NOTE: Control N included only where NEG mode acquisition is run (two N positive controls to bracket specimens).

Control Description	Volume (µL) Added	Standard Concentration	Control Working Standard
Control A	60 µL	0.1/0.02 ng/µL	QC A1
	10 µL	4 ng/µL	QC A2
Control B	60 µL	0.1ng/µL	QC B1
	10 µL	1/4/10 ng/µL	QC B2
Control C	10 µL	2/4 ng/µL	QC C
Control N	30 µL	0.004/0.2 mg/mL	QC N

41.6.5 Using a calibrated pipette, transfer 0.2 mL of each case specimen into its respective tube.

41.6.6 Using a calibrated pipette or verified repeater-pipette, add 800 µL of the working internal standard solution to each tube (except blank matrices). Final concentration of the internal standard is 40 ng/mL MET-d_{14}, 400 ng/mL COC-d_3/MOR-d_6/DZP-d_5 and 800 ng/mL HXB. <u>For blank matrices, add 800 µL acetonitrile (no internal standard)</u>.

41.6.7 Cap the tubes and briefly vortex for at least 10 seconds. Centrifuge the tubes for 5 minutes at 3500 rpm.

41.6.8 Decant the supernatant into a conical centrifuge tube, and evaporate the extracts to dryness at 40°C.

NOTE: Should a specimen not evaporate fully within 45 minutes, DO NOT analyze the extract on the instrument (this will adversely affect subsequent samples and cause interference).

41.6.9 Reconstitute the extracts with the addition of 50 µL of 50:50 MeOH:H_2O (must be HPLC grade:LC-MS grade, as is used on the instrument). Briefly vortex, then centrifuge the tubes for 10 minutes at 3500 rpm to collect the extracts at the bottom of the tubes.

NOTE: Centrifugation is critical to removal of particulates in the extracts prior to transfer.

41.6.10 Transfer the extracts to labeled polypropylene autosampler vials with integrated conical inserts and cap.

41.7 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

Appendix B lists instrument acquisition parameters. The test batch is acquired in POS mode, with NEG mode analysis performed when target compounds identified in negative mode are suspected (e.g., case history, no compounds identified in positive mode). NEG mode analysis may be performed by re-injection of extracts following POS mode acquisition, provided that at least two N positive controls were extracted in the batch (to bracket specimens).

- Acquisition method – TOF-POS (POS mode), TOF-NEG (NEG mode)
- Semi-quant calibration curve – linear, equal weighting, origin included
- Update retention times with semi-quant calibrator (to set peak window only; retention time evaluated against criteria in qualitative data evaluation)
- Qualitative analysis – process all members of the test batch (POS and/or NEG mode) against the internal TLD database; the expanded database (Agilent forensic toxicology) may be used, as warranted, for extensive analysis of unknowns.

  NOTE: If no target compounds or internal standards are detected for an injection (e.g., solvent blank), the software may not generate a report for that sample.
- Semi-quantitative analysis – semi-quant calibrator, negative control(s) and any case specimens in which at least one semi-quant target compound is identified are processed (POS mode only).
- If, due to catastrophic sample preparation or instrument acquisition issues (e.g., incorrect standards/reagents used, reference solution line clogged), internal standards and/or semi-quant/positive control compounds are not found when analyzing data, and all members of the batch are re-extracted, the data is considered nonsensical. When this occurs, reports are not printed for the batch and a notation is made on the worklist.

41.8 CRITERIA FOR BATCH AND CASE SPECIMEN ACCEPTANCE

Review of the batch is conducted according to the criteria listed below.

41.8.1 No target compounds or internal standards shall be identified in the blank matrix (based on identification criteria in 41.8.2 below).

41.8.2 The negative control(s) shall not identify any target compound above its limit of detection. Identification is based on a) acceptable retention time difference (from database) b) distinct chromatographic peak present, c) acceptable mass accuracy, d) acceptable isotope abundance/spacing, and e) acceptable comparison score. All internal standards shall be identified in the negative control and meet criteria in 41.8.3 below.

NOTE: Due to the sensitivity of TOF-MS, endogenous matrix peaks with low abundance present in blank matrices and/or negative control(s) may be integrated/identified as target compounds. Consult the LC-TOF-MS analysis reference sheets for estimate LOD peak abundance for target compounds, and/or abundances in the semi-quant calibrator or positive controls when evaluating acceptance of the blank matrices and negative control(s).

41.8.3 Identification

41.8.3.1 Comparison Score

The comparison score is derived from four individual components; retention time difference (relative to the database), mass difference from target mass (ppm), isotope abundance (IA) and isotope spacing (IS). However, each component should be evaluated individually, in addition to chromatography and comparison score, before determining an identification match.

- The final comparison score should be ≥ 60 for internal standards and those target compounds included in the TLD database (WSP_TLD_DB); however, a comparison score ≥ 60 alone does not automatically denote an identification, just as a score < 60 alone does not exclude a compound from identification. Irrespective of comparison score, consideration shall be given to the quality of individual components (Rt, ppm, IA, IS) when determining whether a compound has been identified in known samples or case specimens.

  Example: Amphetamine in control B has comparison score of 56.84; Rt, ppm, IA and IS are examined. The IA abundances for M+2 and M+3 are low (for amphetamine, it is common for M+2 and M+3 to have low abundance compared to M+1). This outcome is consistent for amphetamine (expected performance for the method), and amphetamine found in case specimens may be directly compared to amphetamine in the contemporaneous control B.

- For target compounds identified using the expanded Agilent (ForTox_AM_PCDL) database (i.e., without retention time reference), the final comparison score should be ≥ 90, including the considerations listed above. In order to report an identification, a standard must be run to obtain a retention time for the target compound.

41.8.3.2 An acceptable match for identification is based on the following criteria:

- Comparison score/individual component criteria described in 41.8.3.1 is satisfied
- The retention time (Rt) difference is within ±0.5 min (compared to database)

NOTE: Qualitative analysis applies ±0.5 min when searching against the database; however, the contemporary Rts for target compounds in the semi-quant/positive controls provide an additional manner of comparison for peaks found in case specimens.

Example: Tramadol and o-desmethylvenlafaxine (O-DMV) share an M+1 (tramadol peak Rt is between that of the two peaks for O-DMV). The TLD database may integrate any of these peaks as tramadol and/or O-DMV (multiple ID, based on M+1 and 0.5 min window). The Rt for tramadol in the contemporaneous semi-quant is used to determine which compound is actually present in the case specimen.

- The mass accuracy (ppm) difference is within 15 ppm
- The peak profile (M+1 or M-1) is symmetrical

NOTE: This method is designed to identify a broad spectrum of target compounds, while maximizing efficiency of instrument acquisition. Variations in chromatographic peak appearance (e.g., tailing, baseline resolution) are inherent in the test method. The method utilizes exact mass identification (no quantitation), with evaluation of multiple individual parameters, in addition to overall score. Chromatography is considered acceptable where chromatographic performance throughout the testing batch is consistent (i.e., semi-quant calibrator, controls, and specimens).

- Isotope abundance and isotope spacing

A visual evaluation of the predicted IA and IS for the target compound, overlaid with actual IA and IS, is used to confirm (or rule out) identification. See LC-TOF-MS analysis reference sheets for examples.

41.8.4 The specified target compounds and all internal standards shall be identified in the semi-quant calibrator and positive quality controls (A, B, C and/or N), and meet criteria in 41.8.3 above.

NOTE 1: Where a target compound is present, but one parameter for identification is not met, batch reporting is restricted for that compound, as appropriate (e.g., gabapentin exhibits ppm > 15, compound not reportable from batch).

NOTE 2: Meth-d_{14} response and ppm may vary throughout the batch. This is consistent with performance in validation and casework testing batches, and noted on the batch review sheet and/or data report for the affected case specimen(s), in addition to any reporting limitations (if necessary).

41.8.5 All internal standards shall be identified in case specimens. Internal standards and any identified target compounds shall meet criteria in 41.8.3 above (NOTE 2 in 41.8.4 also applies).

41.9 REPORTING

41.9.1 Any positive results reported from this screening are indicated as “positive.”

41.9.2 All positive results must be confirmed by a separate method, in the same matrix, or from a different matrix from the same individual. If the confirmation results are not reportable (e.g., < LOQ), the positive TOF results are removed from LIMS.

41.10 METHOD PERFORMANCE

Limits of detection (LOD): See the LC-TOF-MS analysis reference sheets for LOD and 3x LOD reference peak abundances and for carryover information. LOD/3x LOD area counts and semi-quant concentration estimates provide direction for confirmation testing (e.g., dilution required, > LLOQ of confirmation test method).

41.11 REFERENCES

- D. Sklerov, in-house method development.
- F. Guale, S. Shahreza, J. Walterscheid, H. Chen, C. Arndt, A. Kelly and A. Mozayani, Validation of LC-TOF-MS Screen for Drugs, Metabolites, and Collateral Compounds in Forensic Toxicology Specimens, J Anal Tox. 37:17-24 (2013).
- S.Marin, J. Hughes, B. Lawlor, C. Clark, G. McMillin, Rapid Screening for 67 Drugs and Metabolites in Serum or Plasma by Accurate-Mass LC-TOF-MS, J Anal Tox. 36: 477-486 (2012).

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

NOTE: Working Controls A1 and B1 are prepared from a 1:100 dilution of stock solutions.

Internal standard

Cocaine (COC)-d_3 (0.1 ng/µL)
Diazepam (DZP)-d_5 (0.1 ng/µL)
Hexobarbital (HXB, negative mode) (0.2 ng/µL)
Methamphetamine (MET)-d_{14} (0.01 ng/µL)
Morphine (MOR)-d_6 (0.1 ng/µL)

Semi-quant working standard – 1/2/4/5/10 ng/µL

Target – 500 ng/mL (10), 250 ng/mL (5), 200ng/mL (4), 100ng/mL (2), 50ng/mL (1)

Alprazolam (5)	Morphine (10)
Citalopram (4)	Norfluoxetine (4)
Clonazepam (5)	Oxycodone (10)
Cocaine (10)	Quetiapine (10)
Dextromethorphan (1)	Sertraline (4)
Diphenhydramine (1)	Tramadol (2)
Lorazepam (5)	Trazodone (4)
Methadone (10)	Zolpidem (10)
Methamphetamine (10)	

Working control standard A – 0.02/0.1 ng/µL

Target (in pos ctrl A) – 30 ng/mL (fentanyl, hydromorphone 6 ng/mL)

Cocaethylene (0.1)	Fentanyl (0.02)
Codeine (0.1)	Hydromorphone (0.02)
Doxylamine (0.1)	Midazolam (0.1)

Working control standard A2 – 4 ng/µL

Target (in pos ctrl A) – 200 ng/mL

Bupropion	Fluoxetine

Working control standard B1 – 0.1 ng/µL

Target (in pos ctrl B) – 30 ng/mL

7-aminoclonazepam	Flualprazolam
Amphetamine	Hydrocodone
Clonazolam	Nordiazepam
Diazepam	

Working control standard B2 – 1/4/10 ng/µL

(in pos ctrl B)

Benzoylecgonine (1 ng/µL): Target – 50 ng/mL
Venlafaxine (4 ng/µL): Target – 200 ng/mL
Gabapentin (10 ng/µL): Target – 0.5 mg/L

Working control standard C – 2/4 ng/µL

Target (in pos ctrl A) – 200 ng/mL (cyclobenzaprine 100 ng/mL)

Amitriptyline (4)	Doxepin (4)
Cyclobenzaprine (2)	Imipramine (4)
Desipramine (4)	o-desmethylvenlafaxine (4)

Working control standard N – 0.2/0.004 mg/mL

Butalbital (0.004 mg/mL): Target – 0.6 mg/L
Topiramate (0.004 mg/mL: Target – 0.6 mg/L
Valproic Acid (0.2 mg/mL): Target – 30 mg/L

APPENDIX B
INSTRUMENTAL PARAMETERS

Agilent LC/MS – TOF 1

LIQUID CHROMATOGRAPH

Gradient Elution		
	Positive mode	Negative mode
Flow rate	0.5 mL/min	0.5 mL/min
Solvent A	5mM Ammonium formate	5mM Ammonium formate
Solvent B	Methanol	Methanol
90% A 10% B	Initial composition	Initial composition
10% B	0 – 0.5 min	0 – 0.5 min
90% B	0.5-3 min	0.5-3 min
90% B	8 min	6 min
Re-equilibration	5 min	3 min
Column temp	50°C	50°C
Autosampler		
Injection volume	2 µL	2 µL
Injection flush-port	Active	Active
Flush-port time	3.0 sec	3.0 sec
Flush-port solvent	75:25 HPLC MeOH: LC-MS grade H_2O	75:25 HPLC MeOH:LC-MS grade H_2O

MASS SPECTROMETER-TIME OF FLIGHT

Scan type	100-1100	Gas temp	325 °C
Ion mode	Dual ESI	Gas flow	11 L/min
Scan rate (spectra/scan)	1.5 Pos / 1.0 Neg	Nebulizer	40 psig
Time segment 1 (Time 0)	To waste	VCap	3500
Time segment 2 (Time 2.0 min)	To MS	Fragmentor	125
Time segment 3 (Time 7.9 min) Pos	To Waste	Skimmer 1	65
Time segment 3 (Time 5.9 min) Neg	To Waste	Octopole RF peak	750
Reference mass	Enable		
Reference nebulizer	10		

Agilent LC/MS – TOF 2.1

LIQUID CHROMATOGRAPH

Gradient Elution		
	Positive mode	Negative mode
Flow rate	0.5 mL/min	0.5 mL/min
Solvent A	5mM Ammonium formate	5mM Ammonium formate
Solvent B	Methanol	Methanol
90% A 10% B	Initial composition	Initial composition
10% B	0 – 0.5 min	0 – 0.5 min
90% B	0.5-3 min	0.5-3 min
90% B	11 min	8 min
Re-equilibration	5 min	3 min
Column temp	50°C	50°C
Autosampler		
Injection volume	2 µL	2 µL
Injection flush-port	Active	Active
Flush-port time	3.0 sec	3.0 sec
Flush-port solvent	75:25 HPLC MeOH: LC-MS grade H_2O	75:25 HPLC MeOH:LC-MS grade H_2O

MASS SPECTROMETER-TIME OF FLIGHT

Scan type	100-1100	Gas temp	325 °C
Ion mode	Dual ESI	Gas flow	11 L/min
Scan rate (spectra/scan)	1.5 Pos / 1.0 Neg	Nebulizer	40 psig
Time segment 1 (Time 0)	To waste	VCap	3500
Time segment 2 (Time 1.0 min)	To MS	Fragmentor	125
Time segment 3 (Time 10.8 min) Pos	To Waste	Skimmer 1	65
Time segment 3 (Time 7.9 min) Neg	To Waste	Octopole RF peak	750
Reference mass	Enable		
Reference nebulizer	10		

LIST OF CHANGES

Revision Date	Description	Page Number
8/29/2019	Method approved by Washington State Toxicologist. See DRA dated 7/31/19. Method released for use in evidentiary testing on 8/29/19.	All
3/30/21	Throughout procedure, references to alternate matrices were removed - at this time, only blood specimens are being analyzed with this test method. References to LC-MS DI H2O were corrected to LC-MS H2O. Specified in NOTE in 41.2 when specimens are analyzed at a dilution, the samples are not brought to standard volume. In 41.7, added specification that retention time comparisons are included in the qualitative data evaluation and section was added to describe nonsensical data. In 41.8.3.1, changed wording in first bullet regarding comparison score and individual score components. Additional information added in 41.8.3.2 for use of retention time to differentiate target compounds. NOTE 1 and 2 added to 41.8.4 for evaluation of target compound and internal standard performance. In Appendix A, Control A updated to replace morphine and temazepam with codeine and hydromorphone; Control B1 updated to replace oxycodone with hydrocodone and add flualprazolam and clonazolam. Added COC-d3 to 41.6.5 and Appendix A. Updated instrument parameters in Appendix B to reflect use of HPLC grade methanol.	All
2/1/22	In Appendix A Addition of QC Control A2 with compounds bupropion and fluoxetine and Control C of compounds amitriptyline, cyclobenzaprine, desipramine, doxepin, imipramine, o-desmethyl venlafaxine. Removal of fluoxetine from Control A. Correction of concentrations in Control A and Control B from 10 ng/uL to 0.1 ng/uL Addition of compounds benzoylecgonine and venlafaxine to control B2. Correction of gabapentin concentration from 2 mg/L to 0.5 mg/L. Change of concentration of compounds in semi-quant control. In 41.6.4 of the spiking table, added Control A2 of 10 uL to tube with Control A. In 41.4 change and addition of concentration for Control B2 to 1/4/10 ng/uL. Control compounds and/or concentrations were updated to reflect concentrations listed on updated drug scope (v. 8/2021), used in determining whether to move cases forward for confirmation testing, for select compounds.	2,3,8,9
8/15/22	Section 41.3 NOTE and 41.3.1 Reagents – changed HPLC grade ACN to LC-MS grade ACN for preparation of reference solution.	1