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 whole blood, serum, plasma, urine or other submitted biological specimens through precipitation 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

The specimen volume is 0.2 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate (see note in 41.6 regarding specimen quality).

NOTE: Matrix-matching of the semi-quant calibrator, all positive/negative controls and blank matrix is required for analysis of serum/plasma, urine and liver (tissue) homogenate specimens (see 41.5).

41.3 REAGENTS, MATERIALS AND EQUIPMENT

NOTE: Only LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI 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 HPLC grade.

41.3.1 REAGENTS

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

  Add 0.31 g of ammonium formate in a 1 L flask containing approximately 20mL of LC-MS grade DI H_2O. Mix and dilute to 1 L with LC-MS grade DI 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 DI H_2O to 900 mL of HPLC grade ACN. Add 300uL of 5 mM purine in ACN/H_2O and 300uL of 2.5 mM HP-0921 to the ACN/DI 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 and/or other biological matrices
- DI 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

- Semi-quant working standard – 5/10 ng/µL
- Working internal standard – 0.01/0.1/0.2 ng/µL
- Working control standard A – 2/10 ng/µL
- Working control standard B1 – 10 ng/µL
- Working control standard B2 – 5 ng/µL
- Working control standard N – 0.004/0.2 mg/mL

41.5 BLANKS, CALIBRATORS AND CONTROLS

41.5.1 BLANKS

A whole blood matrix blank is included in the batch, with acetonitrile used as the extraction solvent. Where an alternate matrix specimen is analyzed, a blank in that matrix is included in the batch. If testing only an alternate matrix, the whole blood blank is not required.

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. When alternate matrix specimens are analyzed (see NOTE in 41.2) in addition to whole blood specimens, the semi-quant calibrator must be included for that matrix. If testing only alternate matrix, a whole blood semi-quant calibrator is not required.

41.5.3 CONTROLS

41.5.3.1 At least one negative whole blood control and two positive whole blood controls (A, B) are tested with every batch, as described in 41.6. If testing only an alternate matrix, whole blood controls are not required. If acquiring the batch in NEG mode, one negative control and two N positive controls are tested with the batch (to bracket specimens with known samples).

41.5.3.2 Where alternate matrix specimens are analyzed in addition to whole blood specimens, both positive controls (A, B) and one negative control must be included for each alternate matrix type. If acquiring the batch in NEG mode, at least one N positive control and one negative control must be included for each alternate matrix.

41.5.3.3 Samples with known target concentrations (semi-quant calibrator and positive/negative controls) must make up at least 10% of the extracted batch for each matrix (based on number of case specimen samples), with case specimens bracketed by positive known samples (semi-quant calibrator or positive control).

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 whole 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 (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 to each of the individual positive control tubes.

Control Description	Volume (µL) Added	Standard Concentration	Control Working Standard
Control A	60 µL	0.1 ng/µL	QC A
Control B	60 µL	0.1 ng/µL	QC B1
	10 µL	5ng/µL	QC B2
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 sample 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 MOR-d_6/DZP-d_5 and 800 ng/mL HXB. For blank matrices, add 800 µL acetonitrile (no internal standard).

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:DI 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 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 (±2%) with semi-quant calibrator
- 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), a report will not generate 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).

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 Sheet* 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), taking into consideration the quality of individual score components (Rt, ppm, IA, IS).
- 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 criteria described in 41.8.3.1 is satisfied
- The retention time (Rt) difference is within ±0.5 min

- 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 Sheet* 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 and/or N), and meet criteria in 41.8.3 above.

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.

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 *LC-TOF-MS Analysis Reference Sheet* for LOD and 3x LOD reference peak abundances and 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

Internal standard

Methamphetamine (MET)-d_{14}
Morphine (MOR)-d_6
Diazepam (DZP)-d_5
Hexobarbital (HXB)

Semi-quant working standard 1 – 5/10 ng/µL correction:

Target – 500 ng/mL (5), 250 ng/mL (10) Target - 500 ng/mL (10), 250 ng/mL (5)

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

Working control standard A – 2/10 ng/µL

Target – 30 ng/mL (fentanyl 6 ng/mL)

Cocaethylene (10)
Doxylamine (10)
Fentanyl (2)
Fluoxetine (10)
Midazolam (10)
Morphine (10)
Temazepam (10)

Working control standard B1 – 10 ng/µL

Target (in pos ctl B) – 30 ng/mL

7-aminoclonazepam
Amphetamine
Benzoylecgonine
Diazepam
Nordiazepam
Oxycodone
Venlafaxine

hydrocodone replaced oxycodone in B1 as of 12/11/19. AB 12/16/19

Working control standard B2 – 0.01 mg/mL

Target (in pos ctl B) – 2 mg/L

Gabapentin

Working control standard N – 0.2/0.004 mg/mL

Butalbital (0.004 mg/mL): Target – 6 mg/L
Topiramate (0.004 mg/mL: Target – 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 MeOH:DI H_2O	75:25 MeOH:DI H_2O

TOF 1 pos mode run time extended to 10.5 min. AB 10/25/19

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 MeOH:DI H_2O	75:25 MeOH:DI H_2O

MASS SPECTROMETER-TIME OF FLIGHT

Scan type	100/1700	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
	Increased run time to 10.5 min on TOF 1 after parts replacement to capture all compounds in database. AB 10/25/19	
	Added watermarks to page 7; hydrocodone replaces oxycodone in ctl B1, corrected SQ concentration reference. AB 12/16/19	