CONFIRMATION OF TRICYCLIC ANTIDEPRESSANTS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

37.1 METHOD

This test method may be used to confirm the presence of tricyclic antidepressants (TCAs) and select metabolites amitriptyline, nortriptyline, imipramine, desipramine, clomipramine, desmethylclomipramine, doxepin, desmethyldoxepin and trimipramine in biological specimens.

The targeted compounds and corresponding internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of solid-phase extraction (SPE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

37.2 SPECIMENS

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: Matrix-matching of the full calibration curve and all positive control levels is not required for quantitation of TCA compounds in tissue homogenate samples, as determined through evaluation of alternative matrix (liver homogenate) during method validation (see 37.4.3). Note that low recovery of clomipramine and metabolite desmethylclomipramine in tissue homogenate may result in a higher LLOQ for these compounds. This will be evaluated on a batch-by-batch basis, with any adjustment to the LLOQ for these compounds clearly indicated in the batch record.

37.3 REAGENTS, MATERIALS AND EQUIPMENT

37.3.1 REAGENTS

NOTE: Laboratory general-use deionized water (DI H_2O) and reagent-grade organic solvents are used in reagent preparation, unless otherwise specified.

- Acetic acid, glacial
- 0.1M acetic acid

  Add 5.72 mL glacial acetic acid to 800 mL DI H_2O and mix. Dilute to 1 L with DI H_2O and mix. Store the acid in a glass bottle at room temperature for up to six months.
- Acetonitrile (ACN), reagent grade and LC-MS grade
- Ammonium hydroxide (NH_4OH), concentrated
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general use, and LC-MS grade H_2O (or equivalent from a high-purity filtration system)

- Elution solvent

  To 20 mL isopropanol, add 2 mL concentrated NH_4OH and mix. Add 78 mL CH_2Cl_2 and mix. Store the elution solvent in a glass flask/bottle at room temperature and use on date of preparation only.

- Formic acid (concentrated)
- 0.1% Formic acid

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade H_2O and mix. Store the solution in a glass bottle at room temperature for up to one year.

  NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H_2O must be used in place of LC-MS grade H_2O.

- Isopropanol (IPA)
- Methanol (MeOH), reagent grade and HPLC grade
- Methylene chloride (dichloromethane, CH_2Cl_2)
- 0.1M phosphate buffer (pH6)

  Dissolve 1.7 g Na_2HPO_4 and 12.14 g $NaH_2PO_4 \bullet H_2O$ in 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Check the pH and, if necessary, adjust to 6 ± 0.5 with concentrated NaOH. Store the solution in a glass bottle at room temperature for up to one year.

- Sodium hydroxide (NaOH), concentrated
- Sodium phosphate, dibasic anhydrous (Na_2HPO_4)
- Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$)

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

37.3.2 MATERIALS

- Polypropylene autosampler vials with integrated inserts and caps
- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent
- HPLC Column, Agilent Poroshell 120 EC-C18, 2.1x75 mm, 2.7 µM particle size, or equivalent
- Laboratory glassware (graduated cylinders, flasks)

37.3.3 EQUIPMENT

- Shimadzu HPLC, or equivalent
- Sciex API 3200 MS-MS, or equivalent
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipettes with disposable pipette tips

- General-use equipment (centrifuge, evaporator, pH meter/indicating paper, vacuum manifold, vortex mixer)

37.4 STANDARDS, CALIBRATORS AND CONTROLS

37.4.1 STANDARDS

- Working standard (WS): 10 ng/µL
- Working control standard (QC): 10 ng/µL
- Working internal standard (IS): 1 ng/µL

37.4.2 CALIBRATORS

37.4.2.1 Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 37.5 SAMPLE PREPARATION.

37.4.3 CONTROLS

37.4.3.1 At least one negative blood control and two positive blood controls are tested with every batch, prepared as described in 37.5.

37.4.3.2 One positive and one negative control must be included for each alternate matrix type tested in the batch, for qualitative or quantitative analysis.

37.4.3.3 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls. When the batch contains 20 or more specimens, a third positive control (low or high) must be extracted and analyzed mid-run.

37.4.3.4 Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both blood and alternate matrix controls apply towards 10% of the batch.

37.5 SAMPLE PREPARATION

37.5.1 Label a clean extraction tube (16 x 100 mm recommended) for each member of the test batch. (i.e., calibrator, control, case sample).

37.5.2 Add 1 mL DI H_2O to each tube.

37.5.3 Using a calibrated pipette, add 0.5 mL of certified blank blood into each of the five calibrator tubes, the positive control tubes and the negative control tube(s).

37.5.4 Prepare a 1:10 dilution of the working standard. (1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

37.5.5 Prepare a 1:100 dilution of the working standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the 1:10 dilution with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

37.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 - 25 ng/mL	125	0.1 ng/µL	1:100
Calibrator 2 - 50 ng/mL	25	1 ng/µL	1:10
Calibrator 3 - 100 ng/mL	50	1 ng/µL	1:10
Calibrator 4 - 500 ng/mL	25	10 ng/µL	WS
Calibrator 5 - 1000 ng/mL	50	10 ng/µL	WS

37.5.7 Prepare a 1:10 dilution of the working control standard. (1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the working control standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

37.5.8 Using a calibrated pipette, spike the positive controls according to the following table, using the working control standard and prepared dilution.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 - 70 ng/mL	35	1 ng/µL	1:10
Control 2 - 800 ng/mL	40	10 ng/µL	QC

37.5.9 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.

37.5.10 Using a calibrated pipette or a verified repeater-pipette, add 125 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 250 ng/mL.

37.5.11 Add 2 mL of 0.1M phosphate buffer pH6 to each tube.

37.5.12 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

37.5.13 Place new, labeled SPE columns into the vacuum manifold.

37.5.14 Condition the SPE columns by passing each of the following solvents completely through under force of gravity.

a. 3 mL MeOH
b. 3 mL DI H_2O
c. 1 mL 0.1M phosphate buffer (pH6)

Do not let columns dry out between each conditioning step.

37.5.15 Transfer the contents of each tube to its respective SPE column and allow them to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

37.5.16 Wash the SPE columns by passing each of the following solvents completely through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

a. 2 mL DI H_2O
b. 1 mL 0.1M acetic acid
c. 3 mL MeOH

37.5.17 Dry the columns for 10 minutes under vacuum.

37.5.18 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.

37.5.19 Pass 3 mL of elution solvent through each SPE column and collect the extracts.

37.5.20 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

37.5.21 Reconstitute the extracts by the addition of 200 µL mobile phase (80:20 0.1% formic acid in LC-MS grade H_2O:LC-MS grade ACN). Briefly vortex mix the tubes. Centrifuge the tubes for 2 minutes at 2000 rpm (recommended) to collect the extracts at the bottom of the tubes.

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

37.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – TCA (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±3%, ion ratios ±20%) – Cal 3
- Result comparisons – all units in ng/mL

  Cal 1-5, Ctl 1-2: truncated to the whole integer value (acceptable range ±25% for Cal 1; ±20% for Cal 2-5 and Ctl 1-2)

Results are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L), truncated to two significant figures.

37.7 METHOD PERFORMANCE

- Limit of detection: 3.0 ng/mL
- Lower limit of quantification: 25 ng/mL
- Dynamic range: 25 – 1000 ng/mL
- Upper limit of quantitation: 1000 ng/mL
- Upper limit of linearity: 1200 ng/mL

37.8 REFERENCES

- Fast Extraction of 10 Tricyclic Antidepressant Drugs from Urine using ISOLUTE® SLE+ Columns Prior to LC-MS-MS Analysis, Biotage Application Note AN760, Aug 2012.
- K. Harris, J. Knoy, in-house development.
- S. Huq, S. Sadjadi, and E. Pike, Rapid, Automated Extraction and LC/MS/MS Analysis of Tricyclic Antidepressants from Plasma using Strata™-X Drug B SPE and a Kinetex® Core-Shell HPLC/UHPLC Column, Phenomenex Application TN-0056, 2013.
- D. Montenarh, M.P. Wernet, M. Hopf, H.H. Maurer, P.H. Schmidt and A.H. Ewald, Quantification of 33 antidepressants by LC-MS/MS – comparative validation in whole blood, plasma and serum, Anal Bioanal Chem (2014) 406:5939-5953.
- M. Youssef, V.P. Miller, Ultrafast Analysis of a Tricyclic Antidepressant Drug Panel in Human Serum by the Agilent RapidFire High-Throughput Triple Quadrupole Mass Spectrometry System, Agilent Application Note 5991-3494EN, Aug 2014.
- K. Titier, N. Castaing, M. Le-Deodic, D. Le-bars, N. Moore and M. Molimard, Quantification of Tricyclic Antidepressants and Monoamine Oxidase Inhibitors by High Performance Liquid Chromatography – Tandem Mass Spectrometry in Whole Blood, J Anal Tox, (2007) 31:200-207.

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Amitriptyline
Amitriptyline-d3
Clomipramine
Clomipramine-D_3
Desipramine
Desmethylclomipramine
Desmethyldoxepin
Doxepin
Doxepin-D_3
Imipramine
Imipramine-D_3
Nortriptyline
Nortriptyline-D_3
Trimipramine
Trimipramine-D_3

APPENDIX B
INSTRUMENTAL PARAMETERS

Shimadzu/Sciex LC-MSMS System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid in LCMS-grade H_2O
Solvent B	ACN (LCMS-grade)
Initial composition	80% A, 20% B
0 – 1.0 min	30% B
1.0 – 5.5 min	50% B
5.5 – 7.0 min	50% B
7.0 – 7.1 min	20% B
7.1 – 10.0 min	20% B
Column temp	35°C
Autosampler	
Injection volume	2 µL
Rinsing Volume	1000uL
Flush-port solvent	75:25 HPLC grade MeOH:LC-MS grade H_2O
Cooler Temperature	25°C

MASS SPECTROMETER

Scan type	(+) sMRM	Curtain/collision gas	Nitrogen
Ion mode	ESI	Curtain gas flow	30 L/min
MRM detection window	40 sec	Collision gas flow	5 L/min
Resolution (MS1)	Unit	Gas 1 Temp	40°C
Resolution (MS2)	Unit	Gas 2 Temp	60 °C
Target scan time	1 sec	Ion voltage	3.0 kV
Time segment 1 (Time 0)	To waste	Interface Temp	600°C
Time segment 2 (Time 1.0 min)	To MS		
Time segment 3 (Time 8.4 min)	To Waste		

Compound	MRM Transitions
Desmethyldoxepin	266.0→ 107.1, 235.2
Doxepin-D_3	283.0→ 115.1, 165.1
Doxepin	280.0→ 115.0, 165.1
Desipramine	267.0→ 44.2, 208.2
Imipramine-D_3	284.1→ 61.2, 208.2
Imipramine	281.0→ 208.2, 193.2
Nortriptyline-D_3	267.0→ 115.2, 202.2
Nortriptyline	264.1→ 91.2, 105.2
Amitriptyline-D_3	281.1→ 117.2, 105.1
Amitriptyline	278.0→ 117.1, 191.1
Trimipramine-D_3	298.1→ 193.0, 208.2
Trimipramine	295.1→ 208.1, 193.2
Desmethylclomipramine	301.0→ 241.8, 44.1
Clomipramine-D_3	318.0→ 89.2, 227.0
Clomipramine	315.0→ 227.1, 86.2

LIST OF CHANGES

Revision Date	Description	Page Number
3/16/16	Method approved by Washington State Toxicologist. See DRA dated 3/7/16. Method released for use in evidentiary testing as of 3/16/16.	All
11/15/21	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 37.2 SPECIMENS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 37.3.1. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Target compound/internal standard list added in APPENDIX A, with test method parameters moved to APPENDIX B. Formatting and minor edits throughout.	All
4/10/23	Updated 37.4.3.3 to change requirement for mid-run control from more than 20 specimens to 20 or more specimens. Correction in 37.6 in Results Comparisons of Cal Level, from 6 to 5.	5