CONFIRMATION OF TRICYCLIC ANTIDEPRESSANTS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

37.1 POLICY

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 samples. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

37.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of TCA compounds and/or metabolites present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compounds.

37.3 PRINCIPLE

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). Following SPE, the specimens, now termed extracts, are injected into a high performance liquid chromatograph (HPLC) where they are separated between a liquid mobile and liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time.

The HPLC is coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion and multiple-reaction monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any target compound identified in a sample is determined from its calibration curve.

37.4 SPECIMENS

37.4.1 The specimen volume is 0.5 mL.

37.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

NOTE: Matrix-matching of calibrators and controls is not required for quantitation of TCA compounds in tissue homogenate samples, as determined through evaluation of alternative matrix (liver homogenate) during method validation. 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.

37.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

37.4.4 Analysis of larger specimen volumes must be approved and documented.

37.5 REAGENTS, MATERIALS AND EQUIPMENT

37.5.1 REAGENTS

37.5.1.1 Acetic acid, glacial

37.5.1.2 0.1M acetic acid

Add 5.72 mL glacial acetic acid to 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Store in a glass bottle at room temperature for up to six months. Adjustments to final volume are permitted as long as proportions are maintained.

37.5.1.3 Acetonitrile (ACN)

37.5.1.4 Ammonium hydroxide (NH_4OH), concentrated

37.5.1.5 Certified blank blood

37.5.1.6 Deionized water (DI H_2O)

37.5.1.7 Elution solvent

To 20 mL isopropanol, add 2 mL concentrated NH_4OH and mix. Add 78 mL CH_2Cl_2 and mix. Store in glass flask/bottle at room temperature and use on date of preparation only. Adjustments to final volume are permitted as long as the proportions of the elution solvent are maintained.

37.5.1.8 Formic acid (concentrated)

37.5.1.9 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the solution in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

37.5.1.10 Isopropanol (IPA)

37.5.1.11 Methanol (MeOH)

37.5.1.12 Methylene chloride (dichloromethane, CH_2Cl_2)

37.5.1.13 0.1M phosphate buffer (pH6)

Dissolve 1.7 g Na_2HPO_4 and 12.14 g NaH_2PO_4 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. Adjustments to final volume are permitted as long as the proportions are maintained.

37.5.1.14 Sodium hydroxide (NaOH), concentrated

37.5.1.15 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

37.5.1.16 Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$)

37.5.2 MATERIALS

37.5.2.1 Autosampler vials, inserts and caps

37.5.2.2 Disposable 16 x 100mm tubes with closures

37.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

37.5.2.4 Disposable pipette tips

37.5.2.5 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent

37.5.2.6 HPLC Column, Agilent Poroshell 120 EC-C18, 2.1x75 mm, 2.7µM particle size, or equivalent

37.5.2.7 Laboratory glassware (graduated cylinders, flasks)

37.5.2.8 Solvent filters (0.45 um pore size; reduced cellulose, other)

37.5.2.9 Volumetric glassware (flasks)

37.5.3 EQUIPMENT

37.5.3.1 Shimadzu HPLC, or equivalent

37.5.3.2 Sciex API 3200 MS-MS, or equivalent

37.5.3.3 Calibrated, adjustable piston pipettes

37.5.3.4 Centrifuge

37.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

37.5.3.6 pH Meter and/or indicating pH paper

37.5.3.7 Solvent filtration apparatus

37.5.3.8 Vortex mixer

37.5.3.9 Vacuum manifold

37.6 STANDARDS, CALIBRATORS AND CONTROLS

37.6.1 STANDARDS

37.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards which in turn are used to produce calibrators, positive controls and the working internal standard.

37.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a.	Amitriptyline:	1.0 mg/mL
b.	Amitriptyline-D_3:	0.1 mg/mL
c.	Clomipramine:	1.0 mg/mL
d.	Clomipramine-D_3:	0.1mg/mL
e.	Desipramine:	1.0 mg/mL
f.	Desmethylclomipramine:	1.0 mg/mL
g.	Desmethyldoxepin:	1.0 mg/mL
h.	Doxepin:	1.0 mg/mL
i.	Doxepin-D_3:	0.1mg/mL
j.	Imipramine:	1.0 mg/mL
k.	Imipramine-D_3:	0.1mg/mL
l.	Nortriptyline:	1.0 mg/mL
m.	Nortriptyline-D_3:	1.0 mg/mL
n.	Trimipramine:	1.0 mg/mL
o.	Trimipramine-D_3:	0.1mg/mL

37.6.1.3 Working standard (10 ng/µL)

a. Using a calibrated pipette, measure 250 µL of each stock standard into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working standard is 10 ng/µL. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

37.6.1.4 Working internal standard (1 ng/µL)

a. Using a calibrated pipette, measure 250 µL of each stock internal standard (25 µL nortriptyline-d_3) into a 25mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working internal standard is 1 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

37.6.2 CALIBRATORS

37.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 37.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure.

37.6.3 CONTROLS

37.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

37.6.3.2 Positive Controls

a. Two positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.
d. The control working standard (10 ng/µL) is prepared as described in 37.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 37.7 SAMPLE PREPARATION. Alternatively, quality assurance personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

37.7 SAMPLE PREPARATION

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

37.7.2 Add 1 mL DI H_2O to each tube.

37.7.3 Add 0.5 mL of certified blank whole blood into each of the five calibrator tubes, the positive control tubes and the negative control tube(s).

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

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.

b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

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

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

37.7.6 Using the working standard and the prepared dilutions, spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Working Standard
Calibrator 1 - 25 ng/mL	125	0.1 ng/µL
Calibrator 2 - 50 ng/mL	25	1 ng/µL
Calibrator 3 - 100 ng/mL	50	1 ng/µL
Calibrator 4 - 500 ng/mL	25	10 ng/µL
Calibrator 5 - 1000 ng/mL	50	10 ng/µL

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

a. Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

37.7.8 Using the control working standard and prepared dilution, spike the positive controls according to the following table.

Control Description	Volume (µL) Added	Control Working Standard
Control 1 - 70 ng/mL	35	1 ng/µL
Control 2 - 800 ng/mL	40	10 ng/µL

37.7.9 If in-house positive controls are being used, transfer 0.5 mL of each into their labeled tubes.

37.7.10 Sample 0.5 mL of each case sample into its respective tube.

37.7.11 Add 125 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 250 ng/mL.

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

37.7.13 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500rpm.

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

37.7.15 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.

37.7.16 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.)

37.7.17 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

37.7.18 Dry the columns for 10 minutes under vacuum.

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

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

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

37.7.22 Reconstitute the extracts by the addition of 200 µL mobile phase (80:20 0.1% formic acid:ACN). Briefly vortex mix the tubes. If necessary, centrifuge the tubes for 2 minutes at 3000 rpm to collect the extracts at the bottom of the tubes.

37.7.23 Transfer the extracts to labeled polypropylene autosampler vials and cap.

37.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a batch worklist and set the data file/path in Analyst to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table ensure that the method listing in the table is TCA.dam (Shimadzu/Sciex) for each line. As needed, the sequence may conclude with an injection that rinses the column (e.g. using method RINSE.dam), or this may be done manually.

37.9 DATA ANALYSIS

37.9.1 Analysis of the batch data is conducted using MultiQuant quantitative instrumental data analysis software.

37.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration

curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

37.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves (reports do not need to be generated for batch entries added for column rinse or shutdown at the conclusion of acquisition).

37.9.4 Technical review of the batch is conducted according to the criteria listed below.

37.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

37.10.1 CALIBRATORS AND CALIBRATION CURVES

37.10.1.1 Chromatographic peaks for target compounds and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

37.10.1.2 Retention times shall be within ±3% and ion ratios shall be within ±20% of those in calibrator 3. These are inclusive ranges.

37.10.1.3 Quantitative results for target compounds in each calibrator shall be within ±20% of their target values with the exception of calibrator 1 which shall be within ±25% of their targets. These are inclusive ranges. Result comparisons will use whole integer, truncated results in units of ng/mL.

37.10.1.4 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

37.10.2 CONTROLS

37.10.2.1 The negative control(s) shall not identify target compounds above their limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

37.10.2.2 Positive controls

a. Chromatographic peaks for target compounds and internal standards shall appear symmetrical.
b. Retention times shall be within ±3% and ion ratios shall be within ±20% of those in calibrator 3. These are inclusive ranges.
c. Quantitative results for target compounds in each control shall be within ±20% of their target values. These are inclusive ranges. Result comparison will use whole integer, truncated results in units of ng/mL.
d. At least one positive control must meet these criteria for all compounds for the batch to be accepted.

37.10.2.3 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

37.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

37.11.1 Any chromatographic peak for target compounds shall appear symmetrical.

37.11.2 The retention times for target compounds are ±3% and the ion ratios are within ±20% of those in calibrator 3. These are inclusive ranges.

37.11.3 The quantitative results for target compounds must be within the dynamic range of the test method.

37.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

37.12 REPORTING

37.12.1 Results for target compounds are reported in units of milligrams per liter (mg/L).

37.12.1.1 The whole integer, truncated results are converted from ng/mL to mg/L.

37.12.1.2 Converted results are truncated to two significant figures for reporting.

a. Example 1: Clomipramine is measured as 632.89 ng/mL.

b. The unit conversion step truncates the result to 632 ng/mL and then represents the result as 0.632 mg/L.

c. The result is truncated to 0.63 mg/L (two significant figures) and reported.

37.12.2 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

37.13 METHOD PERFORMANCE

37.13.1 Limit of detection: 3.0 ng/mL

37.13.2 Lower limit of quantification: 25 ng/mL

37.13.3 Dynamic range: 25 – 1000 ng/mL

37.13.4 Upper limit of quantitation: 1000 ng/mL

37.13.5 Upper limit of linearity: 1200 ng/mL

37.14 TRACEABILITY

Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

37.15 REFERENCES

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37.15.4 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.

37.15.5 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.

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APPENDIX A
INSTRUMENTAL PARAMETERS

Shimadzu/Sciex LC-MSMS System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
Solvent B	ACN
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 MeOH:DI H_2O
Cooler Temperature	25°C

MASS SPECTROMETER

Scan type	(+) sMRM	Curtain/collision gas	Nitrogen
Ion mode	ESI	Curtain gas flow	30 L/min
Peak width	0.07	Collision gas flow	5 L/min
Resolution (MS1)	Unit	Gas 1 Temp	40°C
Resolution (MS2)	Unit	Gas 2 Temp	60 °C
Dwell time	50 msec	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