CONFIRMATION OF TRICYCLIC ANTIDEPRESSANTS AND SELECTIVE SEROTONIN RE-UPTAKE INHIBITORS IN BIOLOGICAL SPECIMENS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

25.1 POLICY

This test method may be used to confirm the presence of select tricyclic antidepressants (TCA's) and selective serotonin re-uptake inhibitors (SSRI's) 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.

25.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the confirmation of select TCAs and SSRIs 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.

NOTE: This test method is only used for quantitation of SSRI compounds (fluoxetine, norfluoxetine, sertraline, norsertraline) or TCA compounds (amitriptyline, nortriptyline) in specimens unsuitable for use with test methods TCs12733 or TCt12737. It may also be used for qualitative confirmation of these compounds.

25.3 PRINCIPLE

The targeted compounds and internal standard are isolated from whole blood, serum, plasma, urine, or other submitted biological samples using liquid-liquid extraction. Following extraction, the specimens, now termed extracts, are injected onto 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 diode array detector (DAD), which measures compound absorbance at two assigned wavelengths.

Multi-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.

25.4 SPECIMENS

25.4.1 The specimen volume is 1 mL.

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

25.4.3 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.

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

25.5 REAGENTS, MATERIALS AND EQUIPMENT

25.5.1 REAGENTS

25.5.1.1 Acetonitrile

25.5.1.2 Borate buffer (pH9)

Add 630 mL solution A and 370 mL solution B in a 1 L beaker. Mix thoroughly. Check the pH and, if necessary, adjust to 9.0 ±0.5 with additional solution B or strong base (e.g., NH_4OH). Store the solution in a glass or plastic bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

Solution A: In a 1 L flask, dissolve 61.8 g H_3BO_3 and 74.6 g KCl in DI H_2O. Dilute to 1 L with DI H_2O and mix until dissolved (may require low heating).

Solution B: In a 500 mL flask, dissolve 53 g Na_2CO_3 in DI H_2O. Dilute to 500 mL with DI H_2O and mix until dissolved (may require low heating).

25.5.1.3 Boric acid (H_3BO_3)

25.5.1.4 n-butyl chloride

25.5.1.5 Certified blank blood

25.5.1.6 Deionized water (DI H_2O)

25.5.1.7 HPLC mobile phase

Combine 75% by volume mobile phase buffer and 25% by volume acetonitrile in a glass bottle and mix to ensure a homogenous product. Solution expires one week from the date of preparation.

25.5.1.8 Hydrochloric acid (HCl), concentrated 12N

25.5.1.9 0.025N Hydrochloric acid

Add 1 mL concentrated HCl to 300mL DI H_2O in glass flask. Dilute to 500 mL with DI H_2O and mix. 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.

25.5.1.10 Methanol (MeOH)

25.5.1.11 Nonylamine, 97% or better

25.5.1.12 Phosphate buffer (mobile phase)

Dissolve 1.2 g NaH_2PO_4 in 800 mL DI H_2O in a 1 L flask. Add 0.3 mL phosphoric acid and 0.6 mL nonylamine. It is normal for a precipitate to form. Add a Teflon stir bar and mix on a magnetic stir plate until fully dissolved, applying low to medium heat as needed. Dilute to 1 L with DI H_2O and mix. Store the solution in a glass bottle at room temperature. Solution expires one week from date of

preparation. Adjustments to final volume are permitted as long as the proportions are maintained.

25.5.1.13 Phosphoric acid

25.5.1.14 Sodium dihydrogen phosphate (NaH_2PO_4)

25.5.1.15 Sodium carbonate (Na_2CO_3)

25.5.2 MATERIALS

25.5.2.1 Autosampler vials, inserts and caps

25.5.2.2 Disposable 16 x 125mm or 16 x 150mm tubes with closures

25.5.2.3 Disposable screw-cap tubes or conical centrifuge tubes with closures

25.5.2.4 Disposable pipette tips

25.5.2.5 Glass serological and transfer pipettes

25.5.2.6 HPLC Column (Zorbax Eclipse XDB-C8, 150 mm x 4.6 mm ID d_p= 5µm, or equivalent)

25.5.2.7 Laboratory glassware (graduated cylinders, flasks)

25.5.2.8 Teflon stir bars

25.5.2.9 Volumetric glassware (flasks)

25.5.3 EQUIPMENT

25.5.3.1 Agilent/HP 1100 HPLC with diode array detector, or equivalent

25.5.3.2 Calibrated, adjustable piston pipettes

25.5.3.3 Centrifuge

25.5.3.4 Magnetic stir plate (with heater)

25.5.3.5 Rotary mixer

25.5.3.6 Vacuum aspirator

25.5.3.7 Vortex mixer

25.6 STANDARDS, CALIBRATORS AND CONTROLS

25.6.1 STANDARDS

25.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of the working standards and working internal standard.

25.6.1.2 Stock standards are purchased from an approved reference material supplier (generally at 1.0 mg/mL or 0.1 mg/mL concentrations) and may include amitriptyline, nortriptyline, fluoxetine, norfluoxetine, sertraline and desmethylsertraline.

NOTE: Methoxyverapamil internal standard is purchased as a solid reference material and weighed at time of stock standard preparation.

25.6.1.3 Working standard(s) (10 ng/µL)

a. Add 100 µL of the 1.0 mg/mL stock standard(s) to a 10-mL class-A volumetric flask. If using a 0.1 mg/mL stock standard, add 1 mL to the 10-mL 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).
d. Adjustments to final volume are permitted as long as the proportions are maintained.

NOTE: Working standards may contain a single target compound, target compound and metabolite(s), or mix of target compounds, as appropriate for the testing being performed.

25.6.1.4 Stock Internal Standard (1.0 mg/mL)

a. Using a calibrated balance, weigh 10 mg methoxyverapamil and add to a 10 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the stock internal standard is 1.0 mg/mL. The stock internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation.
d. Adjustments to final volume are permitted as long as the proportions are maintained.

25.6.1.5 Working internal standard (25 ng/µL)

a. Add 2.5 mL stock internal standard solution to a 100 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working internal standard is 25 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 the stock standard expires prior to 6 weeks from the date of preparation of the working standard, the expiration date of the working standard is the expiration date of the stock standard) .
d. Adjustments to final volume are permitted as long as the proportions are maintained.

25.6.2 CALIBRATORS

25.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standards. The preparation of the calibrators is detailed in 25.7 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.

25.6.3 CONTROLS

25.6.3.1 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.)

25.6.3.2 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 or supplier 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 25.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 25.7 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.

25.7 SAMPLE PREPARATION

25.7.1 Label a clean 16 x 125mm or 16 x 150mm tube for each member of the test batch. (i.e. calibrators, negative control, positive controls, case samples).

25.7.2 Add 1.0 mL borate buffer pH 9.0 to each tube.

25.7.3 Add 1 mL of certified blank whole blood into each of the calibrator tubes, and the negative control tube(s).

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

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

25.7.5 Using the working standard(s) and prepared dilution(s), spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Working Std Concentration
Cal 1 (100 ng/mL)	100	1 ng/µL
Cal 2 (250 ng/mL)	25	10 ng/µL
Cal 3 (500 ng/mL)	50	10 ng/µL
Cal 4 (1000 ng/mL)	100	10 ng/µL

NOTE: For qualitative confirmation only, a single calibrator (Cal 1) is prepared for retention time and wavelength absorbance ratio verification, and to serve as the positive cutoff level for the batch.

25.7.6 Using the working control standard(s), spike the positive controls according to the following table.

Control Description	Volume (µL) Added	Control Working Standard
Control 1 - 200 ng/mL	20	10 ng/µL
Control 2 - 800 ng/mL	80	10 ng/µL

25.7.7 Sample 1 mL of each case sample into its respective tube.

25.7.8 Add 100 µL of the working internal standard to each tube and briefly vortex-mix. Final concentration of the internal standard is 2.5 mg/L.

25.7.9 Add 6 mL or butyl chloride to each tube.

25.7.10 Cap the tubes and place on a rotary mixer for 10 minutes.

25.7.11 Centrifuge the tubes for 5 minutes at 2000 rpm.

25.7.12 Transfer the organic layer to clean, labeled 10 mL centrifuge or screw cap tubes.

25.7.13 Add 200 µL 0.025N HCl to each tube.

25.7.14 Cap the tubes and place on a rotary mixer for 10 minutes.

25.7.15 Centrifuge the tubes for 5 minutes at 2000 rpm.

25.7.16 Aspirate the organic layer to chemical waste.

25.7.17 Transfer the remaining aqueous layer to labeled autosampler vials with inserts and cap.

25.8 INSTRUMENTAL PARAMETERS

The instrumental parameters are found in Appendix A. Prepare a sequence table and set the sequence parameters to the date of the test and current operator. 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. As needed, the sequence may

conclude with an injection that rinses the column (e.g., using method RINSE), or this may be done manually.

25.9 DATA ANALYSIS

25.9.1 Analysis of the batch data is conducted using the ChemStation instrumental data analysis software.

25.9.2 Unique data analysis methods may be used, specific to those compounds in the batch. For example, if testing is performed for sertraline/norsertraline only (see NOTE in 25.4.2), the data analysis method will not include amitriptyline/nortriptyline.

25.9.3 Quantitative calculations are generated by internal standard, multi-point, linear regression with equal weighting. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

25.9.4 Printed reports for each vial in the batch, including calibration curves, are generated for review. If using multiple data analysis methods to process the negative control and blank, a report from each method should be generated.

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

25.10 CRITERIA FOR BATCH ACCEPTANCE

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

25.10.1 Calibrators and calibration curves

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

25.10.1.2 Retention times shall be within ±2% and the ratio of the absorbance measured at two wavelengths shall be within 20%. These are inclusive ranges.

NOTE: Each target compound and internal standard must have an "X" under the "IS Q" heading on the report, indicating that the ratio of the absorbance measured at two wavelengths is acceptable.

25.10.1.3 Quantitative results for target compounds in each calibrator shall be within ±20% of the target value with the exception of calibrator 1 where results shall be within ±25% of their targets. These are inclusive ranges. Result comparisons will use values truncated after the second decimal place in units of mg/L.

25.10.1.4 The calibration curves for each target compound shall have a correlation coefficient ≥0.99.

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

25.10.2 Controls

25.10.2.1 The negative control shall not identify any target compound. Identification is based on a) acceptable retention time matching, b) acceptable wavelength absorbance ratio match, c) criteria listed in 25.10.1.1 above.

25.10.2.2 Positive controls

a. Chromatographic peaks for target compounds and internal standard shall appear symmetrical.

b. Retention times of target compounds and internal standard shall be within ±2% and the ratio of the absorbance measured at two wavelengths shall be within 20%. These are inclusive ranges.

NOTE: Each target compound and internal standard must have an "X" under the "IS Q" heading on the report, indicating that the ratio of the absorbance measured at two wavelengths is acceptable.

c. Quantitative results for target compounds shall be within ±20% of their nominal target values. This is an inclusive range. Result comparisons will use values truncated after the second decimal place in units of mg/L.

d. Each target compound must meet these criteria in at least one positive control.

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

25.11 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.

25.11.1 Any chromatographic peak of target compounds shall appear symmetrical.

25.11.2 The retention times for target compounds and internal standard are ±2% and the ratio of the absorbance measured at two wavelengths shall be within 20%. These are inclusive ranges.

NOTE: Each target compound and internal standard must have an "X" under the "IS Q" heading on the report, indicating that the ratio of the absorbance measured at two wavelengths is acceptable.

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

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

25.12 REPORTING

25.12.1 Quantitative reporting

NOTE: Consult Appendix B – Relative Retention Times of Common Analytes when interpreting results. The presence of other analytes in case samples

should be evaluated, as some compounds co-elute with TCA or SSRI compounds on HPLC, which can result in an inflated concentration of the target compound(see 25.12.1.4) .

25.12.1.1 Results are reported in units of milligrams per liter (mg/L), to two significant figures.

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

25.12.1.3 Any compounds quantified using this method must be identified using a more specific test method (e.g., GC-MS), on a separate sampling before reporting.

25.12.1.4 When it is determined that other analytes present in a sample are interfering with the target compound(s), quantification may be performed using an alternative method such as GC-MS or GC-MS SIM, provided that the extraction procedure, instrument acquisition method and traceability of materials are recorded in the batch file.

25.12.2 Qualitative reporting

This method may be used for qualitative confirmation of amitriptyline, nortriptyline, paroxetine, fluoxetine, norfluoxetine, sertraline and norsertraline, as described below.

25.12.2.1 To appropriately identify and report a compound as present in a case sample, the following must be demonstrated:

a. Chromatography must meet criteria for acceptance found in 25.11.1 and 25.11.2.

25.12.2.2 Any compounds reported qualitatively using this method must be identified using a more specific test method (e.g., GC-MS), on a separate sampling before reporting.

25.13 METHOD PERFORMANCE

25.13.1 Lower limit of quantification: 0.1 mg/L

25.13.2 Dynamic range: 0.1 mg/L – 1.0 mg/L

25.13.3 Upper limit of quantitation: 1.0 mg/L

25.14 TRACEABILITY

25.14.1 Traceability of the reference materials is provided through the certificate of analysis provided by the approved reference material supplier.

APPENDIX A
INSTRUMENTAL PARAMETERS

HIGH-PRESSURE LIQUID CHROMATOGRAPH – DIODE ARRAY DETECTOR

Gradient Elution	
Flow Rate	1.0 mL/min
Mobile Phase	75% NaH_2PO_4 buffer 25 % ACN
Run Time	17 min
Column Temp	30.0°C
Autosampler	
Injection volume	30.0 µL
Needle Wash	DI H_2O
DAD	
Signals (λ)	230 nm - Primary
	214 nm - Secondary

APPENDIX B
RELATIVE RETENTION TIMES OF COMMON ANALYTES

Compound	RRT	Compound	RRT
oxycodone	0.1093	**amitriptyline**	**0.8139**
mirtazapine	0.1979	duloxetine	0.8281
hydrocodone	0.1981	methadone	0.8286
tramadol	0.1981	maprotiline	0.8489
o-desmethylvenlafaxine	0.1983	aripiprazole	0.9455
cocaine -1	0.1992	**desmethylsertraline**	**1.1599**
cocaine -2	0.2247	**norfluoxetine**	**1.2162**
zolpidem	0.2023	**sertraline**	**1.2625**
meperidine	0.2028	**fluoxetine**	**1.3819**
venlafaxine	0.2137	clomipramine	1.4298
bupropion	0.2171	carbamazepine	1.5278
benzoylecgonine	0.2248		
quetiapine -1	0.2303		
quetiapine -2	0.3359		
trazodone	0.2446		
phencyclidine	0.2521		
dextromethorphan -1	0.2706		
dextromethorphan -2	0.2898		
citalopram -1	0.2720		
citalopram -2	0.4037		
norpropoxyphene -1	0.3324		
norpropoxyphene -2	0.7135		
norpropoxyphene -3	0.7400		
norpropoxyphene -4	0.7983		
diphenhydramine	0.3444		
desmethyldoxepin	**0.3777**		
olanzapine	0.4037		
doxepin	**0.4085**		
amoxapine	0.4772		
loxapine	0.4995		
atomoxetine	0.5640		
desipramine	0.6218		
cyclobenzaprine	0.6694		
imipramine	0.6728		
paroxetine	**0.6794**		
norverapamil	0.7290		
nortriptyline	**0.7514**		
hydroxyzine	0.7707		
propoxyphene	0.7965		
verapamil	0.7979		

LIST OF CHANGES

Revision Date	Description	Page Number
07/08/13	Method approved by Washington State Toxicologist. See DRA dated 7/07/13. Method released for use in evidentiary testing on 7/08/13.	All
02/01/14	Corrected calibrator concentration units to ng/mL in section 25.7.5. Added Appendix B – Relative Retention Times of Other Analytes and wording to section 25.12.1 referencing Appendix B for use when interpreting results (with follow- up described in section 25.12.1.4).	6, 8-9, 11
08/24/15	Procedure revised to reflect use of this test method for quantitation of amitriptyline/nortriptyline, qualitative confirmation of selected analytes (25.12.2), and as an alternative method for confirmation/quantitation of SSRI compounds (25.2). Sections 25.6.1 (standards) and 25.7 (sample preparation) were also modified, and the use of UTAK reconstituted serum controls has been replaced with positive controls spiked at time of extraction (25.6.3.2 and 25.7.6).	1, 3-10
9/30/16	Wording was added to 25.5.1.12 to allow for volume adjustments and removed 25.5.1.16, as UTAK serum controls have been replaced with spiked positive controls. Added clarification to 25.6.3.2.c for use of same CRM in preparation of working standard and working control standard and note regarding CRM expiration dates in 25.6.1.3. Wording added to 25.10.2.2 to specify that each compound must pass in at least one positive control. Edited 25.12.1.1 to reflect that only two significant figures are used for reporting and added “Printed Copies are Uncontrolled” to the footer. Other minor edits throughout.	All