

CONFIRMATION OF TRICYCLIC ANTIDEPRESSANTS AND SELECTIVE SEROTONIN RE-UP TAKE 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 the State Toxicologist, a Manager, or a Supervisor, 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.

25.3 PRINCIPLE

The targeted compounds and internal standards 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 0.025M Hydrochloric acid

Add 1 mL concentrated HCl to 300 mL 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.

25.5.1.13 Phosphoric acid

25.5.1.14 Sodium dihydrogen phosphate (NaH_2PO_4)

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- 25.5.1.15 Sodium carbonate (Na_2CO_3)
- 25.5.1.16 UTAK serum controls (UTAK High Level Tricyclic Antidepressants VI and UTAK SSRI 7 Plus High Range), kept refrigerated

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 $d_p=5\mu\text{m}$, 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 air-displacement 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 and include the following:
 - a. Amitriptyline: 1.0 mg/mL
 - b. Nortriptyline: 1.0 mg/mL
 - c. Doxepin: 1.0 mg/mL
 - d. Desmethyldoxepin: 1.0 mg/mL

- e. Fluoxetine: 1.0 mg/mL
- f. Norfluoxetine: 1.0 mg/mL
- g. Paroxetine: 1.0 mg/mL
- h. Sertraline: 1.0 mg/mL
- i. Desmethylsertraline: 100 µg/mL

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

25.6.1.3 TCA Working standard (10 ng/µL)

- a. Add 250 µL each of the amitriptyline, nortriptyline, doxepin, desmethyldoxepin, fluoxetine and norfluoxetine stock standards to 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.
- d. Adjustments to final volume are permitted as long as the proportions are maintained.

NOTE: Although fluoxetine and norfluoxetine are SSRI compounds, they are included in the TCA working standard due to co-elution with other SSRI compounds.

25.6.1.4 SSRI Working standard (10 ng/µL)

- a. Add 250 µL each of the paroxetine and sertraline stock standards, and 2.5 mL of the desmethylsertraline stock standard to 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.
- d. Adjustments to final volume are permitted as long as the proportions are maintained.

25.6.1.5 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

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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.

25.6.2 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. Commercially prepared serum controls are used:
 - UTAK High Level Tricyclic Antidepressants VI
 - UTAK SSRI 7 Plus High Range
- b. The TCA and SSRI serum positive controls are reconstituted with 5 mL DI H₂O using a volumetric pipette. Prepared controls are stored under refrigeration. TCA control must be used within 30 days of reconstitution and SSRI control must be used within 25 days of reconstitution.

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 individual 1:10 dilutions of the TCA and SSRI working standards. (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 standards and prepared dilutions, spike the calibrators according to the following table.

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

25.7.6 Sample 1 mL of the positive serum controls into the appropriately labeled tubes.

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 of 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 Two data analysis methods are used; one specific to compounds in the TCA working standard, and one specific to compounds in the SSRI working standard.
- 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. A report from both the TCA and SSRI data analysis methods should be generated for the negative control. If compounds from both the TCA and SSRI standards are present in a specimen, two reports will be generated; one for each method used for analysis.

NOTE: Calibrations for fluoxetine and norfluoxetine are generated using the TCA data analysis method. However, these compounds are contained in the SSRI control. The SSRI control will be analyzed with both the TCA and SSRI data analysis methods, with printed reports from each.

- 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 $\pm 2\%$ 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 $\pm 20\%$ of the target value with the exception of calibrator 1 where results shall be within $\pm 25\%$ 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 $\pm 2\%$ 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 $\pm 20\%$ of their nominal target values, as listed in the manufacturer's package insert. This is an inclusive range. Result comparisons will use values truncated after the second decimal place in units of mg/L.
- d. 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 $\pm 2\%$ 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

25.12.1.1 Results are reported in units of milligrams per liter (mg/L), reported to no more than 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.

NOTE: Desmethyldoxepin is only reported qualitatively from this assay.

25.12.2 Qualitative reporting

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.

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APPENDIX A
INSTRUMENTAL PARAMETERS

HIGH-PRESSURE LIQUID CHROMATOGRAPH – DIODE ARRAY DETECTOR

Gradient Elution	
Flow Rate	1.0 mL/min
Mobile Phase	75% NaH ₂ PO ₄ buffer 25 % ACN
Run Time	17 min
Column Temp	30.0°C
Autosampler	
Injection volume	30.0 µL
Needle Wash	DI H ₂ O
DAD	
Signals (λ)	230 nm - Primary
	214 nm - Secondary

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