CONFIRMATION OF SELECTIVE SEROTONIN REUPTAKE INHIBITOR COMPOUNDS BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

33.1 METHOD

This test method may be used to confirm the presence of fluoxetine (FLX) and norfluoxetine (NFX), using fluoxetine-d_6 (FLX-d_6) internal standard, and sertraline (SER) and desmethylsertraline (norsertraline, NSR), using protriptyline (PRO) internal standard, in biological specimens. The target compounds and internal standards are isolated from biological matrices by solid phase extraction (SPE). The extracts are subjected to chemical derivatization with heptafluorobutyric acid anhydride (HFBA or HFAA) and injected into a gas chromatograph (GC) coupled to a mass spectrometer (MS) detector equipped with an electron ionization source.

33.2 SPECIMENS

The specimen volume is 1 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate (see NOTE 1 and NOTE 2 in 33.5). 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 required for quantitation in serum/plasma or liver (tissue) homogenate specimens (see 33.4.3. and 33.4.4).

33.3 REAGENTS, MATERIALS AND EQUIPMENT

33.3.1 REAGENTS

NOTE: Organic solvents used are reagent grade.

- Acetic acid (glacial)
- 0.1M acetic acid

  To 800mL of DI H_2O, add 5.72 mL glacial acetic acid. Dilute to 1 L with DI H_2O. Store the acid in a glass bottle at room temperature for up to 6 months.
- Acetonitrile (ACN),
- Ammonium hydroxide (NH_4OH, concentrated)
- Certified blank blood and/or other biological matrices
- Deionized water (DI H_2O), laboratory general-use
- Elution solvent

  To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store the solvent in a glass flask/bottle at room temperature and use on date of preparation only.
- Ethyl acetate
- Heptafluorobutryric acid anhydride (HFBA, HFAA)
- Hydrochloric acid (HCl), concentrated

- Isopropanol (IPA)
- Methanol (MeOH)
- Methylene chloride (CH_2Cl_2, dichloromethane)
- 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 pH6 ± 0.5 with concentrated NaOH or HCl. Store the buffer 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.

33.3.2 MATERIALS

- 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
- GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 µm film thickness, or equivalent)
- Glass autosampler vials with inserts and caps
- Laboratory glassware (graduated cylinders, flasks)

33.3.3 EQUIPMENT

- Agilent GC (6890 or equivalent)
- Agilent MS (5973 or equivalent) with electron ionization source
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipettes with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heating block/oven, pH meter or paper, rotary mixer, vacuum manifold, vortex mixer)

33.4 STANDARDS, CALIBRATORS AND CONTROLS

33.4.1 STANDARDS

- Working standard: 10 ng/µL
- Working control standard: 10 ng/µL
- Working internal standard: 10 ng/µL

33.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 33.5 SAMPLE PREPARATION. Quantitation in serum/plasma or liver (tissue) homogenate specimens requires that a calibration curve be prepared in blank matrix. If testing only serum/plasma or tissue homogenate specimens, a whole blood calibration curve is not required.

33.4.3 CONTROLS

33.4.3.1 At least one negative whole blood control and two positive whole blood controls are included in the batch, prepared as described in 33.5.

33.4.3.2 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.

33.4.3.3 For qualitative analysis of any alternate matrices, one negative control and one positive control must be included for each alternate matrix type tested in the batch.

33.4.3.4 For quantitative analysis of serum/plasma or liver (tissue) homogenate specimens, matrix-matching of the full calibration curve and all positive controls (to meet 10% and bracket specimens in that matrix) is required.

33.5 SAMPLE PREPARATION

NOTE 1: Stability evaluation in method validation indicates that prepared extracts should be run on the instrument no more than 24 hours after extraction.

NOTE 2: If nortriptyline, citalopram or codeine has been identified in a case sample, an alternative test method must be used for confirmation of norsertraline. If clonidine or trazodone has been identified, an alternative test method must be used for confirmation/quantitation of norfluoxetine.

33.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample).

33.5.2 Add 4mL of 0.1M phosphate buffer (pH6) into each tube.

33.5.3 Using a calibrated pipette, add 1 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

33.5.4 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.

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

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

33.5.6 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.

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

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 – 75 ng/mL	75	1 ng/µL	1:10
Control 2 - 800 ng/mL	80	10 ng/µL	QC

33.5.8 Using a calibrated pipette, sample 1 mL of each case sample into its respective tube.

33.5.9 Using a calibrated pipette or verified repeater-pipette, add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 500 ng/mL.

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

33.5.11 Place new SPE columns in the vacuum manifold.

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

a. 3 mL methanol
b. 3 mL DI H_2O
c. 2 mL 0.1M phosphate buffer (pH6)

Do not let columns dry out between each conditioning step.

33.5.13 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.)

33.5.14 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. 3 mL DI H_2O
b. 2 mL 0.1M acetic acid
c. 3 mL methanol

33.5.15 Dry the columns for 10 minutes under vacuum.

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

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

33.5.18 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C. Extracts must be completely dry for efficient chemical derivatization.

33.5.19 In a fume hood, add 100 µL ethyl acetate and 50 µL HFBA to each tube and immediately cap and vortex. Minimize the time that HFBA is exposed to the atmosphere.

33.5.20 Incubate the tubes for 30 minutes at 50°C.

33.5.21 Remove the tubes from heat and cool to room temperature. Alternatively, transfer the tubes to a centrifuge and spin for 2 minutes at 2000 rpm.

33.5.22 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C. Make sure the extracts are evaporated to dryness before reconstitution.

33.5.23 Reconstitute the extracts by the addition of 50 µL ethyl acetate to each tube. Briefly vortex mix the tubes. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

33.5.24 Transfer the extracts to labeled glass autosampler vials with inserts and cap.

33.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – SSRIHF (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4
- Result comparisons –

  Cals 1-5, Ctls 1-2: truncated, whole integer values in units of ng/mL

33.7 REPORTING

Results for FLX and NFX are truncated to two significant figures for reporting, in units of milligrams per liter (mg/L). Results for SER and NSR are reported qualitatively from this test method.

33.8 METHOD PERFORMANCE

- Limit of detection: 5 ng/mL (0.005 mg/L)
- Lower limit of quantification: 25 ng/mL (0.025 mg/L)
- Dynamic range: 25 – 1000 ng/mL (0.025 – 1.0 mg/L)
- Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)
- Upper limit of linearity: 3000 ng/mL (3.0 mg/L)

33.9 REFERENCES

- Dawn Sklerov, in-house method development.
- Papoutsis, A. Khraiwesh, P. Nikolaou, C. Pistos, C. Spiliopoulou, S. Athanaselis, *A fully validated method for the simultaneous determination of 11 antidepressant drugs in whole blood by gas chromatography – mass spectrometry*, J Pharm. Biomed. Anal. 70 (2012) 557-562.

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Fluoxetine
Fluoxetine-d_6
Norfluoxetine
Norsertraline
Protriptyline (IS)
Sertraline

APPENDIX B
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Splitless
Inlet Liner	4mm splitless w/ glass wool plug
Temperature	250° C
Purge Flow	10 mL/min
Purge Time	0.7 min
Gas Type	Helium
Autosampler	
Injection Volume	1.0 µL
Solvent Wash A	3 (Ethyl acetate)
Solvent Wash B	3 (Ethyl acetate)
Sample Pumps	2

Oven / Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.5 mL/min
Initial Temperature	150° C
Initial Time	1.00 min
Ramp Rate 1	20° C/min
Final Temp 1	300° C
Final Time	5.0 min
Transfer Line Temp	280 ° C

MASS SPECTROMETER

Solvent Delay	4.00 min	MS Quad Temperature	150° C
EM Offset	Set in tune	MS Source Temperature	230° C
Resolution	Low	Dwell Time	50 msec

Signals	Ions	Ion ratios
Norfluoxetine	330,117, 226	117/330, 226/330
Fluoxetine-d6	350,123	123/350
Fluoxetine	344,117	117/344
Desmethylsertraline	487,274,239	274/487, 239/487
Protriptyline	191, 459	459/191
Sertraline	501,274,238	274/501, 238/501

LIST OF CHANGES

Revision Date	Description	Page Number
7/13/15	Method approved by Washington State Toxicologist. See DRA dated 7/9/15. Method released for use in evidentiary testing as of 7/13/15.	All
2/4/16	Added note regarding CRM expiration dates to 33.6.1.3 and 33.6.1.4.Edited 33.12.3 to reflect that only two significant figures are used for reporting. Changed wording in 33.12.4 to describe that quantitative results for samples that contain one or more SSRI compounds >ULOQ will be reported from the lowest dilution factor that brings all compounds within the dynamic range, and included an example in 33.12.4.a	9-10
5/8/17	Positive control acceptability criteria changed in 33.10.2.2.d. and 33.10.2.2.d. Sections 33.12.5 edited for qualitative reporting of SER and NSR. Use of a calibrated pipette for addition of blank blood, specimen samples and standards specified in 33.7. Other minor edits throughout.	1-11
8/5/19	Removed policy, purpose and principle sections, summarizing under new section METHOD. Modified specific wording regarding matrix-matching in 33.2 SPECIMENS, 33.4.2 CALIBRATORS and 33.4.3 CONTROLS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. 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. Added list of target compounds and internal standards in APPENDIX A, moved instrument parameters to APPENDIX B. Formatting and minor edits throughout.	All