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

28.1 POLICY

This test method may be used to confirm the presence of fluoxetine (FLX), norfluoxetine (NFX), sertraline (SER), and desmethylsertraline (norsertraline, NSR) in biological samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. 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.

28.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of selective serotonin reuptake inhibitor (SSRI) compounds 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.

28.3 PRINCIPLE

The targeted compounds and 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 subjected to chemical derivatization with trifluoroacetic anhydride (TFAA).

Measured volumes of the extracts are injected into a gas chromatograph (GC) where they are separated between a gaseous mobile and liquid stationary phase. Each compound exits the GC at a reproducible time which is termed its retention time.

The GC is coupled to a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, selected-ion-monitoring (SIM) 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.

28.4 SPECIMENS

28.4.1 The specimen volume is 1mL.

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

28.4.3 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.

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

28.5 REAGENTS, MATERIALS AND EQUIPMENT

28.5.1 REAGENTS

28.5.1.1 Acetic acid (Glacial)

28.5.1.2 0.1M acetic acid

To 400mL of DI H_2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O. Store this in glass bottle at room temperature for up to 6 months. Adjustments to final volume are permitted as long as proportions are maintained.

28.5.1.3 Acetonitrile (ACN)

28.5.1.4 Ammonium hydroxide (concentrated)

28.5.1.5 Certified blank blood

28.5.1.6 Deionized water (DI H_2O)

28.5.1.7 Elution solvent

To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store in glass 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.

28.5.1.8 Ethyl acetate

28.5.1.9 Hydrochloric acid (HCl), concentrated 12N

28.5.1.10 1% Hydrochloric acid in methanol

Add 45 mL methanol to a graduated cylinder. Carefully add 0.5 mL concentrated HCl and bring total volume to 50 mL with methanol and mix. Store the solution in a glass bottle at room temperature for up to 1 month. Adjustments to final volume are permitted as long as proportions are maintained.

28.5.1.11 Isopropanol (IPA)

28.5.1.12 Methanol (MeOH)

28.5.1.13 Methylene chloride (dichloromethane, CH_2Cl_2)

28.5.1.14 0.1M Phosphate buffer (pH 6):

Dissolve 1.7 g Na_2HPO_4 and 12.14 g NAH_2PO_4 in 800mL DI H_2O. Dilute to 1L with DI H_2O and mix. Check the pH and, if necessary, adjust to 6 $\pm$0.5. Store the buffer in glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.

28.5.1.15 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

28.5.1.16 Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)

28.5.1.17 Trifluoroacetic anhydride (TFAA)

28.5.2 MATERIALS

28.5.2.1 Autosampler vials, inserts and caps

28.5.2.2 Disposable 16 x 100mm tubes with closures

28.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

28.5.2.4 Disposable pipette tips

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

28.5.2.6 GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 μm film thickness, or equivalent)

28.5.2.7 Laboratory glassware (graduated cylinders, flasks)

28.5.2.8 Volumetric glassware (flasks)

28.5.3 EQUIPMENT

28.5.3.1 Agilent GC (6890 or equivalent)

28.5.3.2 Agilent MS (5973 or equivalent)

28.5.3.3 Calibrated, adjustable air-displacement pipettes

28.5.3.4 Centrifuge

28.5.3.5 Evaporator (Biotage, formerly Zymark, TurboVap)

28.5.3.6 Oven, dry-bath, or wet-bath

28.5.3.7 pH Meter and/or indicating pH paper

28.5.3.8 Rotary mixer

28.5.3.9 Vortex mixer

28.5.3.10 Vacuum manifold

28.6 STANDARDS, CALIBRATORS AND CONTROLS

28.6.1 STANDARDS

28.6.1.1 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.

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

a. Fluoxetine: 1.0 mg/mL
b. Fluoxetine-d_6 (FLX-d_6): 0.1 mg/mL
c. Norfluoxetine: 1.0 mg/mL

d. Sertraline: 1.0 mg/mL
e. Desmethylsertraline (norsertraline): 0.1 mg/mL

28.6.1.3 Working standard (10 ng/μL)

a. Using calibrated pipettes, measure 250 µL each of FLX, NFX and SER, and 2.5 mL of NSR stock standards 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 date of preparation. Volumes may be adjusted provided that proportions remain constant.

28.6.1.4 Working internal standard (10 ng/μL)

a. Using calibrated pipettes, measure 2.5 mL of FLX-d_6 into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to designated volume.
c. The final concentration of the working internal standard is 10 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

28.6.2 CALIBRATORS

28.6.2.1 Calibrators are prepared in certified blank whole blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 28.7 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.

28.6.3 CONTROLS

28.6.3.1 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.)

28.6.3.2 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 should 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 28.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 28.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house or externally sourced positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

28.7 SAMPLE PREPARATION

NOTE: If oxazepam has been identified in a case sample, an alternative test method must be used for confirmation/quantitation of norfluoxetine. If 7-aminoflunitrazepam has been identified, an alternative test method must be used for confirmation/quantitation of sertraline. If citalopram, doxepin, desmethyldoxepin, amitriptyline, and/or nortriptyline have been identified, an alternative test method must be used for confirmation and/or quantitation of norsertraline (see 28.11.6).

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

28.7.2 Place 2 mL of DI H_2O into each tube.

28.7.3 Place 2 mL of 0.1M phosphate buffer (pH6) into each tube.

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

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

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

28.7.6 Using the working standard and the prepared dilution, spike the calibrators according to the following table.

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

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

a. Using calibrated pipettes, combine 0.1 mL of the control working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.

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

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

Control Description	Volume (µL) Added	Control Working Standard
Control 1 (75 ng/mL)	75	1 ng/ µL
Control 2 (800 ng/mL)	80	10 ng/µL

28.7.9 If in-house positive controls are being used, transfer 1 mL of each into their labeled tubes.

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

28.7.11 Add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 500 ng/mL.

28.7.12 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 5 minutes between 3000-3500 rpm.

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

28.7.14 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 0.1 M phosphate buffer (pH6)

Do not let columns dry out between each conditioning step.

28.7.15 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).

28.7.16 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. 3 mL DI H_2O
b. 3 mL 0.1 M acetic acid
c. 3 mL methanol

28.7.17 Dry the columns for 10 minutes under vacuum.

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

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

28.7.20 Add 100 µL 1% HCl in MeOH to each tube and vortex-mix.

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

28.7.22 In a fume hood, add 50 µL of ethyl acetate and 50 µL of TFAA to each tube and immediately cap and vortex.

28.7.23 Incubate the tubes for 20 minutes at 55-60^{o}C.

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

28.7.25 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40^{o}C. Make sure the extracts are evaporated to dryness before reconstitution.

28.7.26 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.

28.7.27 Transfer the extracts to labeled glass autosampler vials and cap.

28.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation 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 SSRI for each line.

28.9 DATA ANALYSIS

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

28.9.2 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.

28.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.

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

28.10 CRITERIA FOR BATCH ACCEPTANCE

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

28.10.1 Calibrators and calibration curves

28.10.1.1 Chromatographic peaks for FLX, NFX, SER, NSR, and internal standard shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

28.10.1.2 Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

28.10.1.3 Quantitative results for FLX, NFX, SER and NSR in each calibrator shall be within ±20% of their target values with the exception of calibrator 1, which shall be within ±25% of its target. These are

inclusive ranges. Result comparisons will use whole integer, truncated results in units of ng/mL.

28.10.1.4 The calibration curves for FLX, NFX, SER and NSR shall have correlation coefficients ≥0.99.

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

28.10.2 Controls

28.10.2.1 The negative control(s) shall not identify FLX, NFX, SER, or NSR above their respective limits of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

28.10.2.2 Positive controls

a. Chromatographic peaks for FLX, NFX, SER, NSR, and internal standards shall appear symmetrical.
b. Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4 for each compound in a positive control. These are inclusive ranges.
c. Quantitative results for FLX, NFX, SER, and NSR 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. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
e. At least one positive control must meet these criteria for all compounds for the batch to be accepted.

28.11 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.

28.11.1 Any chromatographic peaks for FLX, NFX, SER, NSR and internal standard shall appear symmetrical.

28.11.2 The retention times for any reportable compounds are ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

28.11.3 The quantitative result for each identified compound must be within the dynamic range of the test method.

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

28.11.5 If any target compound in a given case sample is outside of the dynamic range it does not invalidate the result for other compounds.

28.11.6 The presence of oxazepam, 7-aminoflunitrazepam, citalopram, doxepin, desmethyldoxepin, amitriptyline and nortriptyline in a sample has been shown to interfere with select SSRI compounds (see NOTE in 28.7). In these cases, an alternative confirmation/quantitation method may be used (e.g., GC-MS without derivatization or high performance liquid chromatography) , with the

sample preparation, instrument acquisition method and data analysis method included with the batch.

28.12 REPORTING

28.12.1 Results are reported in units of milligrams per liter (mg/L).

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

28.12.3 Converted results are truncated to no more than two significant figures for reporting.

a. For example: sertraline is measured as 642.73 ng/mL.

b. The unit conversion step truncates the result to 642 ng/mL and then represents the result as 0.642 mg/L.

c. The result is truncated to 0.64 mg/L (two significant figures) and reported.

28.13 METHOD PERFORMANCE

28.13.1 Limit of detection: 5.0 ng/mL

28.13.2 Lower limit of quantification: 25 ng/mL

28.13.3 Dynamic range: 25 ng/mL to 1000 ng/mL

28.13.4 Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)

28.14 TRACEABILITY

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

28.15 REFERENCES

28.15.1 Dawn Sklerov, in-house method development.

28.15.2 United Chemical Technologies (UCT) Solid Phase Extraction Applications Manual, 2007

APPENDIX A
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
Gas Saver	On
Gas Saver Flow	20 mL/min
Gas Saver Time	2.00
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	2.5 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	230,104,117	104/230, 117/230
Fluoxetine-d6	250,123	123/250
Fluoxetine	244,117	117/244
Norsertraline	387,202,274	202/387, 274/387
Sertraline	401,302,238	302/401, 238/401

LIST OF CHANGES

Revision Date	Description	Page Number
8/20/14	Method approved by Washington State Toxicologist. See DRA dated 8/14/14. Method released for use in evidentiary testing as of 8/20/14.	All