SOP 409 - SSRI Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

Table of Contents

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SOP Name:		SOP #:
SSRI Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)		**409**
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:**	**Revision Date/Initials:**
	10.1.1.3 – Added IS recovery acceptance range	MSF – 04/14/2015
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	10.1.3.3 – Added reference to QA Manual (Cal point exclusion)	MSF – 04/04/2016
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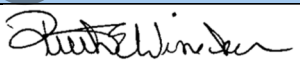
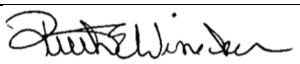
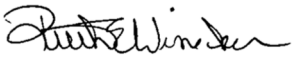
Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		04/17/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 409 - SSRI Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

1. **Principle of Assay**

1.1. This method is designed to confirm and quantitate Selective Serotonin Reuptake Inhibitors (SSRIs) in biological specimens by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC/MS/MS). The drugs are extracted from their biological matrix by protein precipitation with acetone and identified by the retention time and ion ratio of product ions. The analytes are subject to matrix effects, thus stable isotopically labeled internal standards are used (1).

1.2. In the United States antidepressants rank as the most frequently used drug by persons aged 18-44, with the rate increasing (among all ages) by 400% from 1994 through 2008. All told, over 10% of Americans aged 12 and older are currently taking an antidepressant, with 14% of those taking them long term, for over 10 years. (2)

Known for decades, serotonin deficiency has been implicated in clinical depressive symptoms, while stimulation of the serotonin system reduces them. In the body, serotonin inactivation is primarily accomplished by the reuptake of serotonin at the central synapse. Reuptake inhibitors, such as SSRIs, thus block the reuptake of serotonin, increasing the concentration of the neurotransmitter in the synapse, and reducing depressive symptoms. (3)

As a class, SSRIs are the most commonly prescribed antidepressants, accounting for 4 of the top 5 prescribed antidepressants (4). The most commonly prescribed SSRIs, and ones confirmed by this assay (plus metabolites), are sertraline (norsertraline), citalopram, fluoxetine (norfluoxetine), and paroxetine. Venlafaxine and O-desmethylvenlafaxine, at higher doses, also blocks norepinephrine, and is classed as a serotonin-norepinephrine reuptake inhibitor (SNRI).

In this laboratory, screening for SSRIs is typically done in central blood specimens (*e.g.* aorta, inferior vena cava) via the organic bases screen (SOP 102). SSRIs though, have high volumes of distribution (Vd >5 L/kg) and are readily distributed in perfused organs such as the liver, lung, heart, and kidneys. Along with a high volume of distribution, SSRIs are also subject to postmortem redistribution (PMR), in which drugs diffuse from areas of high drug concentration, such as organ tissue, into the blood. Because of these two features, confirmation and quantitation of SSRIs are done in peripheral blood specimens (*e.g.* femoral, iliac) and liver, to more accurately reflect SSRI concentration at the time of death and assist in interpretation. (5)

2. **Specimens**

2.1. This procedure is applicable to urine, blood, serum, vitreous humor, *bile, *gastric contents, and properly prepared tissue specimens (typically 1:4 homogenates). A 0.1 mL (g) sample size (in duplicate) is generally employed

unless a dilution is required so that the calibration curve encompasses the expected range of unknown specimens.

2.1.1. *For non-typical matrices, an additional 0.1mL aliquot shall be taken (volume permitting), spiked with appropriate QC, and analyzed to help to identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

3. **Reagents and Materials**
   - 3.1. DI water, HPLC grade
   - 3.2. Methanol, HPLC grade
   - 3.3. Acetone, HPLC grade
   - 3.4. Acetonitrile, HPLC grade
   - 3.5. Deuterated SSRI Internal Standard Mix
   - 3.6. SSRI Standard Mix
   - 3.7. SSRI QC Standard Mix (Standard & Control Worksheet)
   - 3.8. Drug Free Blood, Urine, Liver Homogenate
   - 3.9. Water with 0.1% formic acid
   - 3.10. Acetonitrile with 0.1% formic acid
   - 3.11. Methanol with 0.1% formic acid

4. **Standards, Controls, and Solutions**

4.1. Prepare Calibration, IS, and Control standards, as needed, according to SOP 010.

4.2. **Benzodiazepine Internal Standard Solution (10µg/mL)**

4.2.1. Into a 10mL volumetric flask, add the following:

- 4.2.1.1. 1 ampule (~1mL) of Paroxetine-d6 (Cerilliant - 100µg/mL)
- 4.2.1.2. 1 ampule (~1mL) of Venlafaxine-d6 (Cerilliant - 100µg/mL)
- 4.2.1.3. 1 ampule (~1mL) of Fluoxetine-d6 (Cerilliant - 100µg/mL)
- 4.2.1.4. 1 ampule (~1mL) of Citalopram-d6 (Cerilliant - 100µg/mL)
- 4.2.1.5. 1 ampule (~1mL) of Norfluoxetine-d6 (Cerilliant - 100µg/mL)
- 4.2.1.6. 1 ampule (~1mL) of Sertraline-d3 (Cerilliant - 100µg/mL)
- 4.2.1.7. 1 ampule (~1mL) of Norsertraline-$^{13}C_6$ (Cerilliant - 100µg/mL)
- 4.2.1.8. 1 ampule (~1mL) of O-desmethylvenlafaxine-d6 (Cerilliant - 100µg/mL)
- 4.2.1.9. Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.3. **Benzodiazepine Calibrators and Positive Controls –** these standards are to be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if calibration/control standards need to be made.

4.4. **Water with 0.1% formic acid**

4.4.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid

4.4.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

4.5. Acetonitrile with 0.1% formic acid

4.5.1. To a 4L bottle of HPLC grade acetonitrile, add 4 mL of formic acid

4.5.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

4.6. **Methanol with 0.1% formic acid**

4.6.1. To a 4L bottle of HPLC grade methanol, add 4 mL of formic acid

4.6.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

5. **Equipment and Special Supplies**
   - 5.1. Test Tubes, 13 x 100 mm
   - 5.2. LC autosampler vials, 12 x 32 mm
   - 5.3. Polyspring inserts, 5 mm O.D.
   - 5.4. Centrifuge 2000 x g
   - 5.5. Vortex mixer
   - 5.6. Nitrogen evaporator

6. **Instrumentation and Parameters**
   - 6.1. Windows PC with Thermo LCQuan and Xcaliber software
     - 6.1.1. Instrument method (TSQ02): "SSRI_EZ-Method"
     - 6.1.2. Click here for instrument parameters.
   - 6.2. Thermo Surveyor LC autosampler, or equivalent
   - 6.3. Thermo Surveyor LC pump, or equivalent
   - 6.4. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions** (± 1 nominal mass)

7.1.	Citalopram	(**325** 109 262)
7.2.	Citalopram-d6	(**331** 109 262)
7.3.	Fluoxetine	(**310** 44 148)
7.4.	Fluoxetine-d6	(**316** 44 154)
7.5.	Norfluoxetine	(**296** 134 ***)
7.6.	Norfluoxetine-d6	(**302** 140 ***)
7.7.	Paroxetine	(**330** 192 70)
7.8.	Paroxetine-d6	(**336** 198 76)
7.9.	Venlafaxine	(**278** 260 58)
7.10.	Venlafaxine-d6	(**284** 64 266)
7.11.	O-desmethylvenlafaxine	(**264** 246 58)

7.12. O-desmethylvenlafaxine-d6 (**270** 64 252)
7.13. Sertraline (**306** 275 159)
7.14. Norsertraline (**292** 275 159)
7.15. Norsertraline-13C6 (**298** 281 159)
7.16. Sertraline-d3 (**309** 159 275)

7.16.1. Note: The precursor ion of each analyte is listed first and bolded, the first product ion- used for quantification-is second, followed by the second product ion-used for qualification.
*** Second product ion not present.

8. **Procedure**

8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 13x100 mm test tubes.

8.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of Deuterated SSRI Internal Standard Mix to all the tubes.

8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of SSRI Standard Mix and SSRI QC Mix to the tubes labeled as standards and control, respectively, labeling test tubes as you go. Only internal standard should be present in the test tube labeled "Blank".

8.4. Add 0.1mL of blank blood to all standards, control, and blank test tubes (0.1 mL blank urine/0.1g blank liver homogenate to urine/liver blank and QC test tubes).

8.5. Add the appropriate amount of predetermined unknown specimen labeling test tubes as you go. (See Specimens section).

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 3.5mL acetone to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Decant the top acetone layer into clean and labeled 13x100 test tubes, place in nitrogen evaporator, and evaporate at 55° C to dryness.

8.10. Remove dried specimens from nitrogen evaporator and reconstitute with 500µL of methanol. Vortex for 10 seconds and centrifuge at 2000 x g for 5 minutes.

8.11. Transfer approximately 100µL of each extract to appropriately labeled autosampler vials fitted with 200 µL polyspring insert and place in the autosampler tray of the Thermo TSQ triple-quadrupole LC/MS/MS.

8.12. Build and initiate sequence as directed in SOP 053.

9. **Calculations**

9.1. Quantitative Ion ratios

9.1.1. The method for processing the data using the Thermo LCQuan software is "SSRI" (SOP 055). It is used to calculate the internal standard response ratios, raw amounts, concentration, and ion ratios.

9.1.2. These calculations are computed as follows:

9.1.2.1. Response Ratio:

9.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standard's quantifying product ion.

9.1.2.1.2. Response Ratio = QN_a / QN_{istd}

9.1.2.1.2.1. QN_a = response of the quantitative ion of the analyte

9.1.2.1.2.2. QN_{istd} = response of the quantitative ion of the internal standard amount

9.2. Calibration

9.2.1. A linear regression resulting from the 6 standards is used to quantitate the analytes in the load. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

9.3. Dilution Factor

9.3.1. D = Total volume/Sample volume

9.4. Multiplier for homogenates, dilutions, and non-standard volumes

9.4.1. $M = (V_{curve} / V_{samp}) \times D$

9.4.1.1. M = Multiplier

9.4.1.2. D = dilution factor

9.4.1.3. V_{curve} = matrix volume of calibration curve

9.4.1.4. V_{samp} = matrix volume of specimen

9.5. Concentration

9.5.1. $C = (A / V) * M$

9.5.1.1. C = Concentration (ng/mL) of the analyte in the unknown case.
9.5.1.2. A = Amount of drug in sample
9.5.1.3. V = Volume of sample
9.5.1.4. M = Multiplier

9.6. Max/Min

9.6.1. Percent Difference = $((R_h / R_l)-1) \times 100$

9.6.1.1. R_h = high result
9.6.1.2. R_l = low result

9.7. Average

9.7.1. Average = $(R_1 + R_2) / 2$

9.7.1.1. R_1 = first result
9.7.1.2. R_2 = second result

9.8. Qualifier Ion Ratios

9.8.1.1.1. Ratio 1 = QL_1/QN

9.8.2. QL_1 = response of the quantifying product ion
9.8.3. QN = response of the qualifying product ion

10. **Quality Control**

10.1. Acceptance criteria

10.1.1. Chromatogram

10.1.1.1. Peaks must be Gaussian shaped (symmetrical).

10.1.1.2. Peaks must not exhibit extreme fronting or tailing.

10.1.1.3. The internal standard (ISTD) in each case should be inspected for evidence of signal enhancement and suppression. The area of the quantifying ion should not be less than 50% or more than 200% of the average ISTD of the calibrators.

10.1.1.4. Peaks sharing parent/product ions must have baseline resolution.

10.1.1.5. Retention time must not deviate outside ± 3% (minimum window 7.2 seconds) of target, based upon the retention time of the calibrators and controls.

10.1.2. Mass spectroscopy

10.1.2.1. The ion ratio of all samples must not be greater than ± 20% of the target ratio as determined by a mid-level calibrator (CAL 4).

10.1.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

10.1.3. Calibrators

10.1.3.1. Analytical curves must have a coefficient of determination (R^2) of 0.992 or greater.

10.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value (± 25% at LOQ).

10.1.3.3. Refer to "Calibration curve point exclusion guidelines" section of the QA/QC Manual.

10.1.4. Controls

10.1.4.1. Controls must calculate within ± 20% of the target value.

10.1.5. Blanks

10.1.5.1. Blanks should not contain any target analyte signal with an internal standard response ratio greater than 10% that of the lowest calibrator for the same analyte.

10.1.6. Any deviation from the above criteria must be approved by a senior chemist.

11. **Validation of Method**

Parameter	Result - Blood	Result - Liver
Bias	**Citalopram -** Low: **-4.79%** High: **6.99%**	**Citalopram -** Low: **-1.95%** High: **-5.66%**
	Fluoxetine - Low: **-1.84%** High: **-6.52%**	**Fluoxetine -** Low: **1.04%** High: **-6.88%**
	Norfluoxetine - Low: **-1.37%** High: **-3.77%**	**Norfluoxetine -** Low: **-0.23%** High: **-2.79%**

	Norsertraline - Low: **6.49%** High: **-6.00%**	**Norsertraline -** Low: **7.88%** High: **-3.18%**
	O-Desmethylvenlafaxine - Low: **5.08%** High: **-0.67%**	**O-Desmethylvenlafaxine -** Low: **5.63%** High: **-0.31%**
	Paroxetine - Low: **-1.88%** High: **-5.19%**	**Paroxetine -** Low: **0.68%** High: **-3.45%**
	Sertraline - Low: **-4.31%** High: **-8.13%**	**Sertraline -** Low: **-0.44%** High: **-6.66%**
	Venlafaxine - Low: **-1.37%** High: **-4.02%**	**Venlafaxine -** Low: **1.40%** High: **-2.65%**
Precision	**Citalopram -** Low: **0.85%** High: **1.16%**	**Citalopram -** Low: **1.82%** High: **0.60%**
	Fluoxetine - Low: **4.36%** High: **1.90%**	**Fluoxetine -** Low: **3.76%** High: **1.60%**
	Norfluoxetine - Low: **5.55%** High: **2.04%**	**Norfluoxetine -** Low: **10.12%** High: **2.32%**
	Norsertraline - Low: **2.64%** High: **2.10%**	**Norsertraline -** Low: **3.94%** High: **1.75%**
	O-Desmethylvenlafaxine - Low: **1.05%** High: **0.61%**	**O-Desmethylvenlafaxine -** Low: **2.15%** High: **0.77%**

	Paroxetine - Low: **1.44%** High: **0.923%**	**Paroxetine -** Low: **1.35%** High: **0.56%**
	Sertraline - Low: **2.63%** High: **0.59%**	**Sertraline -** Low: **2.03%** High: **1.09%**
	Venlafaxine - Low: **2.14%** High: **0.84%**	**Venlafaxine -** Low: **2.13%** High: **0.67%**
Calibration model	**Citalopram -** Linear 1/x weighting	
	Fluoxetine - Linear 1/x weighting	
	Norfluoxetine - Linear 1/x weighting	
	Norsertraline - Linear 1/x weighting	
	O-Desmethylvenlafaxine - Linear 1/x weighting	
	Paroxetine - Linear 1/x weighting	
	Sertraline - Linear 1/x weighting	
	Venlafaxine - Linear 1/x weighting	
Carryover	Signal >10% not observed in blank following sample spiked with 100ug/mL (all analytes).	
Interference Studies	No interfering signal from matrix, internal standard, common drugs of abuse (including metabolites), OTC drugs, and Prescription medications was observed.	
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	Not evaluated - IS coelute within 0.05 minutes of associated analytes.	
	Blood	**Liver**
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	**Citalopram -** 14ng/mL	**Citalopram -** 31ng/g
	Fluoxetine - 41ng/mL	**Fluoxetine -** 46ng/g
	Norfluoxetine - 133ng/mL	**Norfluoxetine -** 77ng/g
	Norsertraline - 42ng/mL	**Norsertraline -** 102ng/g

	O-Desmethylvenlafaxine - 26ng/mL	**O-Desmethylvenlafaxine -** 31ng/g
	Paroxetine - 29ng/mL	**Paroxetine -** 31ng/g
	Sertraline - 42ng/mL	**Sertraline -** 32ng/g
	Venlafaxine - 21ng/mL	**Venlafaxine -** 88ng/g
LOQ (Set to lowest calibrator with acceptable Bias/Precision).	**Citalopram -** 200ng/mL	
	Fluoxetine - 200ng/mL	
	Norfluoxetine - 200ng/mL	
	Norsertraline - 200ng/mL	
	O-Desmethylvenlafaxine - 200ng/mL	
	Paroxetine - 200ng/mL	
	Sertraline - 200ng/mL	
	Venlafaxine - 200ng/mL	
Processed Sample Stability - (re-analyze after 8 days)	Sample extracts remain stable for 10 days (recapped) and 3 days (not recapped).	

12. **Reporting**

12.1. The percent difference of duplicate analysis for an analyte must be less than or equal to 25% (see Max/Min in Calculations section)

12.2. Reporting of duplicate analysis should be done according to the table below:

Reporting Duplicates

- Dilution factors of 1 and 1

Dil / Scenario	1	1	REPORT
A	In curve	In curve	Average
B	In curve	AQL or BQL	"In" value
C	In curve	ND *	Repeat
D	AQL/BQL	AQL/BQL	Less than/ Greater than
E	BQL	ND	ND

* ND = None Detected, due to IRC, S/N threshold, r.t., or other

12.2.1.1. In Curve = Measured concentration (pre-multiplier) falls within the calibration range

12.2.1.2. AQL = Measured concentration (pre-multiplier) falls Above Upper Quantitation Limit

12.2.1.3. BQL = Measured concentration (pre-multiplier) falls Below Lower Quantitation Limit

12.2.1.4. ND = None Detected

12.3. Averaging reportable values

12.3.1. Results for duplicate analysis (both falling within calibration curve) shall be truncated prior to averaging.

12.3.2. Enter calculated concentration for each specimen into toxlog.

12.4. Significant figures

12.4.1. Concentrations are truncated and reported with two significant figures in milligrams per liter (mg/L).

13. **Preparation of Load**

13.1. The load paperwork and data is to be arranged in the following order:

13.1.1. Assignment sheet

13.1.2. Comments or note to file if applicable

13.1.3. Load summary

13.1.4. Specimen worklist

13.1.5. Chain of custody (Specimen)

13.1.6. Aliquot chain of custody

13.1.7. Standard and control worksheet

13.1.8. Sequence summaries/calibration reports – paper clipped

13.1.9. Calibrator data - paper clipped

13.1.10. Blank matrix data - paper clipped

13.1.11. Control data - paper clipped

13.1.12. Specimen data – stapled

14. **References**

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