CONFIRMATION OF METHADONE BY LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

5.1 METHOD

This test method may be used to confirm the presence of methadone in biological samples. Methadone (MDN) and internal standard (MDN-d_3) are isolated from biological matrices by liquid-liquid extraction (LLE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source.

5.2 SPECIMENS

The specimen volume is 0.2 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

The presence of fluoxetine/norfluoxetine has been shown to interfere with methadone qualifier ions. Where a specimen contains fluoxetine/norfluoxetine, an alternative test method must be used for methadone confirmation/quantification (see *Basic Drug Identification/Confirmation by Gas Chromatography – Mass Spectrometry/Nitrogen Phosphorus Detection*, TCb12714).

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in tissue homogenate or serum/plasma specimens (see 5.4.3.2).

5.3 REAGENTS, MATERIALS AND EQUIPMENT

5.3.1 REAGENTS

NOTE: Laboratory general-use deionized water (DI H_2O) and reagent-grade organic solvents are used in reagent preparation, unless otherwise specified.

- Acetonitrile (ACN), reagent grade and LC-MS grade
- n-Butyl chloride
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use, and LC-MS grade H_2O (or equivalent from a high-purity filtration system)
- Formic acid (concentrated)
- 0.1% Formic acid in LC-MS grade H_2O

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask and mix. Dilute to 1 L with LC-MS grade H_2O and mix. Store the acid in a glass bottle at room temperature for up to one year.

  NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H_2O must be used in place of LC-MS grade H_2O.
- Methanol (MeOH), reagent grade

- Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)
- 0.13M Sodium borate solution (saturated)

  In a 100 mL volumetric flask, dissolve 4.9 g $Na_2B_4O_7 \cdot 10H_2O$ in approximately 75 mL DI H_2O. Dilute to 100 mL with DI H_2O and mix thoroughly (may require low heating). The weighed contents may not go completely into solution – this is normal. Store the solution in a glass bottle at room temperature for up to 6 months.

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

5.3.2 MATERIALS

- Disposable extraction tubes (16 x 100mm recommended) and screw-cap or centrifuge tubes with closures
- HPLC Column, Agilent Zorbax Eclipse Plus C8, 50 mm x 2.1 mm ID, dp = 1.8 µM, or equivalent
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps

5.3.3 EQUIPMENT

- Agilent HPLC (1200 series, or equivalent)
- Agilent MS with API-ES source (6130 model, or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, rotary mixer, vortex mixer)

5.4 STANDARDS, CALIBRATORS AND CONTROLS

5.4.1 STANDARDS

- Working standard (WS): 10 ng/µL
- Working control standard (QC): 10 ng/µL
- Working internal standard (MDN-d_3): 1 ng/µL

5.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 5.5 SAMPLE PREPARATION.

5.4.3 CONTROLS

5.4.3.1 At least one negative blood control and two positive blood controls are tested with every batch, prepared as described in 5.5.

5.4.3.2 One positive and one negative control must be included for each alternate matrix type tested in the batch, for qualitative or quantitative analysis.

5.4.3.3 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. When the batch contains more than 20 specimens, a third positive control (low or high) must be extracted and analyzed mid-run.

5.5 SAMPLE PREPARATION

NOTE: Specimens containing fluoxetine/norfluoxetine must be analyzed using a different test method (see 5.2).

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

5.5.2 Add 2 mL sodium borate solution into each tube.

5.5.3 Using a calibrated pipette, add 0.2 mL of certified blank blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

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

a. Using a calibrated pipette, combine 100 µL of the working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

5.5.5 Prepare a 1:100 dilution of the working standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the 1:10 dilution with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

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

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 10 ng/mL	20	0.1 ng/µL	1:100
Calibrator 2 – 25 ng/mL	50	0.1 ng/µL	1:100
Calibrator 3 - 50 ng/mL	10	1 ng/µL	1:10
Calibrator 4 - 100 ng/mL	20	1 ng/µL	1:10
Calibrator 5 - 500 ng/mL	10	10 ng/µL	WS
Calibrator 6 - 1000 ng/mL	20	10 ng/µL	WS

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

a. Using a calibrated pipette, combine 100 µL of the control working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

5.5.8 Prepare a 1:100 dilution of the working control standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the 1:10 dilution with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

5.5.9 Using a calibrated pipette, spike the positive controls according to the following table, using the prepared dilutions of the working control standard.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC
Control 1 – 20 ng/mL	40	0.1 ng/µL	1:100
Control 2 - 400 ng/mL	80	1 ng/µL	1:10

5.5.10 Using a calibrated pipette, sample 0.2 mL of each case specimen into its respective tube.

5.5.11 Using a calibrated pipette or verified repeater-pipette, add 20 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 100 ng/mL.

5.5.12 Briefly vortex mix.

5.5.13 Add 2 mL n-butyl chloride to each tube.

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

5.5.15 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

5.5.16 Transfer the n-butyl chloride layer to a clean, labeled centrifuge or screwcap tube.

5.5.17 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

5.5.18 Reconstitute the extracts with the addition of 50 µL of 80:20 0.1% formic acid in LCMS grade H_2O:LC-MS grade ACN to each tube and briefly vortex mix. Centrifuge the tubes for 2 minutes at 2000 (recommended) rpm to collect the extracts at the bottom of the tubes.

5.5.19 Transfer the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

5.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – METHADONESIM (instrumental parameters in Appendix A)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±3%, ion ratios ±20%) – Cal 4
- Result comparisons – in units of ng/mL

  Cal 1: truncated to one decimal place (acceptable range 25%; 7.5 – 12.5 ng/mL)

  Cals 2-6, Ctls 1-2: truncated, whole integer values (acceptable range ±20%)

5.7 REPORTING

Results are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L), and truncated to two significant figures for reporting.

5.8 METHOD PERFORMANCE

- Limit of detection: 1 ng/mL (0.001 mg/L)
- Lower limit of quantification: 10 ng/mL (0.01 mg/L)
- Dynamic range: 10 – 1000 ng/mL (0.010 – 1.0 mg/L)
- Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)

5.9 REFERENCES

- A. Black, in-house method development.
- OCME Toxicology Laboratory, Washington D.C., Quantitation of Methadone by LC-MS (2007).

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.6 mL/min
Solvent A	0.1% Formic acid in LC-MS H_2O
Solvent B	LC-MS grade ACN
Initial composition	80% A, 20% B
0 – 1 min	% B increased to 50%
Hold time	5.0 min (50% B)
Re-equilibration	5.0 min
Column temp	30°C
Autosampler	
Injection volume	2.0 µL
Injection flush-port	Active
Flush-port time/volume	30 sec
Flush-port solvent	LC-MS grade ACN

MASS SPECTROMETER

Ion mode	(+) SIM	Nebulizer gas	Nitrogen
EM gain	1.0	Nebulizer pressure	40 psi
Peak width	0.08 min	Drying gas	Nitrogen
		Drying gas flow	13 L/min
		Drying gas temp	350 °C
		Capillary voltage	4 kV

Compound	Ions	Ion Ratios
Methadone	310, 265, 223	265/310, 223/310
Methadone-d_3	313, 268	268/313

LIST OF CHANGES

Revision Date	Description	Page Number
09/01/11	Method approved by Washington State Toxicologist. See DRA dated 8/25/11. Method released for use in evidentiary testing on 09/01/11.	All
2/01/14	HPLC column description in section 5.5.2.6 changed to Agilent Zorbax Eclipse Plus C8 (50 x 2.1 mm; 1.8um I.D.) or equivalent. Wording added to 5.7 and 5.11.5 to indicate the use of an alternative test method for case samples with fluoxetine/norfluoxetine.	2, 4, 7
3/16/16	Added wording for adjustment of prepared volumes in 5.5.1.6, 5.5.1.9, 5.6.1.3 and 5.6.1.4 and clarification to 5.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates in 5.6.1.3 and 5.6.1.4. Added option for use of a column rinse method in 5.8. Edited 5.12.3 to reflect that only two significant figures are used for reporting and added "Printed Copies are Uncontrolled" to footer. Other minor edits throughout.	All
5/8/17	Added note in 5.4.2 to indicate that analysis of tissue homogenate specimens does not require matrix-matching. Wording added to 5.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 5.7. Specified calibrator concentration criteria/ranges in 5.10.1.3. Edited 5.10.2.2.d to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-8
4/9/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 5.2 SPECIMENS. 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. Formatting and minor edits throughout.	All
6/15/20	NOTE added in section 5.3.1 regarding solvents and H_2O used; Removed requirement to filter 0.1% formic acid (no filtration required for prep with LC-MS grade H_2O). Specified LC-MS grade H_2O and LC-MS grade ACN, where applicable in 5.3.1 and 5.5. Use of mid-run control added in 5.4.3.3. Changed pipetted volumes in 5.5.4 – 5.5.5 and 5.5.7 – 5.5.8 from mL to µL. Changed retention time criteria in 5.6 to ±3%. Added references in 5.9. Other minor edits throughout.	All