CONFIRMATION OF METHADONE BY LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

5.1 POLICY

This test method may be used to confirm the presence of methadone 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 either the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch file.

5.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of methadone 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.

5.3 PRINCIPLE

The targeted compound and internal standard are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, the specimens, now termed extracts, are injected into 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 mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion-monitoring 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 methadone identified in a sample is determined from its calibration curve.

5.4 SPECIMENS

5.4.1 The specimen volume is 0.2 mL.

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

5.4.3 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.

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

5.5 REAGENTS, MATERIALS AND EQUIPMENT

5.5.1 REAGENTS

5.5.1.1 Acetonitrile (Filter this solvent prior to use on the HPLC.)

5.5.1.2 n-Butyl chloride

5.5.1.3 Certified blank blood

5.5.1.4 Deionized water (DI H_2O)

5.5.1.5 Formic acid (concentrated)

5.5.1.6 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H2O and mix. Filter this solution prior to use on the HPLC.

5.5.1.7 Methanol

5.5.1.8 Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)

5.5.1.9 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.

5.5.2 MATERIALS

5.5.2.1 Autosampler vials, inserts and caps

5.5.2.2 Disposable 16 x 100mm tubes

5.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

5.5.2.4 Disposable pipette tips

5.5.2.5 Disposable safety closures for 16 x 100mm tubes

5.5.2.6 HPLC column (Agilent Zorbax SB-18 RR; 50 mm x 2.1 mm ID, d_p=1.8 µm, or equivalent)

5.5.2.7 Laboratory glassware (graduated cylinders, flasks)

5.5.2.8 Solvent filters (0.45 µm pore size; nylon, reduced cellulose, other)

5.5.2.9 Volumetric glassware (flasks)

5.5.3 EQUIPMENT

5.5.3.1 Agilent HPLC (1100/1200 series or equivalent)

5.5.3.2 Agilent MS with API-ES source (6130 or equivalent)

5.5.3.3 Calibrated, adjustable air-displacement pipettes

5.5.3.4 Centrifuge

5.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

5.5.3.6 pH Meter and/or indicating pH paper

5.5.3.7 Rotary mixer

5.5.3.8 Solvent filtration apparatus

5.5.3.9 Vortex mixer

5.6 STANDARDS, CALIBRATORS AND CONTROLS

5.6.1 STANDARDS

5.6.1.1 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.

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

a. Methadone: 1.0 mg/mL
b. Methadone-d_3: 0.1 mg/mL

5.6.1.3 Working standard (10 ng/µL)

a. Using a calibrated pipette, measure 100 µl of methadone stock standard into a 10 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.

5.6.1.4 Working internal standard (1 ng/µL)

a. Using a calibrated pipette, measure 100 µl of methadone-d_3 stock internal standard into a 10 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 1 ng/µl. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation.

5.6.2 CALIBRATORS

5.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 5.7 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.

5.6.3 CONTROLS

5.6.3.1 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.)

5.6.3.2 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.
d. The control working standard (10 ng/μL) is prepared as described in 5.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 5.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

5.7 SAMPLE PREPARATION

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

5.7.2 Place 2 mL of sodium borate solution into each tube.

5.7.3 Add 0.2 mL of certified blank whole blood into each of the six calibrator tubes, the two positive control tubes and the negative control tube(s).

5.7.4 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 methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

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

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

5.7.6 Using the working standard and the prepared dilutions, spike the calibrators according to the following table.

Calibrator	Volume (μL)	Working

Description	Added	Standard
Calibrator 1 (10 ng/mL)	20	0.1 ng/µl
Calibrator 2 (25 ng/mL)	50	0.1 ng/µl
Calibrator 3 (50 ng/mL)	10	1 ng/µl
Calibrator 4 (100 ng/mL)	20	1 ng/µl
Calibrator 5 (500 ng/mL)	10	10 ng/µl
Calibrator 6 (1000 ng/mL)	20	10 ng/µl

5.7.7 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 methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

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

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

5.7.9 Using the control working standard dilutions, spike the positive controls according to the following table.

Control Description	Volume (µL) Added	Control Working Standard
Control 1 (20 ng/mL)	40	0.1 ng/µl
Control 2 (400 ng/mL)	80	1 ng/µl

5.7.10 If in-house positive controls are being used, transfer 0.2 mL of each into their labeled tubes.

5.7.11 Sample 0.2 mL of each case sample into its respective tube.

5.7.12 Add 20 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 100 ng/mL.

5.7.13 Cap the tubes and briefly vortex mix.

5.7.14 Add 2 mL of n-butyl chloride to each tube.

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

5.7.16 Centrifuge the tubes for 10 minutes at 3500 rpm.

5.7.17 Transfer the n-butyl chloride layer to clean, labeled 10 mL centrifuge or screw cap tubes.

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

5.7.19 Reconstitute the extracts by the addition of 50 µL of mobile phase 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.

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

5.8 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 METHADONESIM.M for each line.

5.9 DATA ANALYSIS

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

5.9.2 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.

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

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

5.10 CRITERIA FOR BATCH ACCEPTANCE

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

5.10.1 Calibrators and calibration curves

5.10.1.1 Chromatographic peaks for methadone and internal standard shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

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

5.10.1.3 Quantitative results for methadone 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.

5.10.1.4 The calibration curve for methadone shall have a correlation coefficient ≥0.99.

5.10.2 Controls

5.10.2.1 The negative control(s) shall not identify methadone above its limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

5.10.2.2 Positive controls

a. Chromatographic peaks for methadone and internal standard shall appear symmetrical.

b. Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4 for methadone in the positive control. These are inclusive ranges.
c. Quantitative results for methadone 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. At least one positive control must meet these criteria for methadone for the batch to be accepted.

5.11 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.

5.11.1 Any chromatographic peak for methadone shall appear symmetrical.

5.11.2 The retention time for methadone is ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

5.11.3 The quantitative result for methadone must be within the dynamic range of the test method.

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

5.12 REPORTING

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

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

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

a. For example: methadone is measured as 107.9 ng/mL.

b. The unit conversion step truncates the result to 107 ng/mL and then represents the result as 0.107 mg/L.

c. The result is truncated to 0.10 mg/L (two significant figures) and reported.

5.12.4 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

5.13 METHOD PERFORMANCE

5.13.1 Limit of detection: 1 ng/mL (0.001 mg/L)

5.13.2 Lower limit of quantification: 10 ng/mL (0.010 mg/L)

5.13.3 Dynamic range: 10 ng/mL to 1000 ng/mL (0.010-1.0 mg/L)

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

5.14 TRACEABILITY

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

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.60 mL/min
Solvent A	0.1% Formic Acid
Solvent B	Acetonitrile
Initial Composition	80% (A), 20% (B)
0 – 1 min	%B increased to 50%
Hold time	5.0 min at 50% (B)
Re-equilibration	5.0 min
Column Temp	30° C
Autosampler	
Injection Volume	2.0 μL
Injection flush-port	Active
Flush-port time	30 sec
Flush-port solvent	Acetonitrile

MASS SPECTROMETER

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