CONFIRMATION OF OPIATES BY LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRY

16.1 METHOD

This test method may be used to confirm the presence of morphine (MOR), oxymorphone (OXM), hydromorphone (HYM), codeine (COD), oxycodone (OXC), 6-acetylmorphine (6AM), and hydrocodone (HYC) in biological samples. Target compounds and their deuterated analog internal standards are isolated from biological matrices by solid phase extraction (SPE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

16.2 SPECIMENS

The specimen volume is 1 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.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in liver (tissue) homogenate or serum/plasma specimens (see 16.4.3.2).

16.3 REAGENTS, MATERIALS AND EQUIPMENT

16.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H_2O) is specified, laboratory general-use DI H_2O is used in reagent preparation. Organic solvents are reagent grade unless otherwise specified.

- Acetic acid (glacial)
- Acetonitrile (ACN), reagent grade and LC-MS grade
- Ammonium hydroxide (concentrated)
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade (or equivalent from a high purity filtration system)
- 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.
- Formic acid (concentrated)

- 0.1% Formic acid

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

0.1% Formic acid in acetonitrile (LCMSMS 5 solvent B) prepared as described here, with LC-MS grade acetonitrile in place of LC-MS grade DI H2O. AB 10/14/19

- Isopropanol (IPA)
- Methanol (MeOH)
- Methylene chloride (dichloromethane, CH_2Cl_2)
- 0.1M sodium acetate buffer (pH 4.5)

  Dissolve 2.93 g sodium acetate trihydrate in 400 mL DI H_2O. Add 1.62 mL glacial acetic acid. Dilute to 500 mL with DI H_2O and mix. Check pH and, if necessary, adjust to 4.5 ±0.2 with glacial acetic acid. Store the buffer in a glass bottle at room temperature for up to one year.

- Sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$)
- Sodium hydroxide (concentrated, NaOH)
- Sodium phosphate, dibasic anhydrous (Na_2HPO_4)
- Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)

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

16.3.2 MATERIALS

- Disposable extraction tubes (16 x 100mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206200 mg/6 mL), or equivalent
- HPLC column: Agilent Zorbax Eclipse Plus C18 100 mm x 2.1 mm ID, d_p=3.5 µm, or equivalent)
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

16.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Agilent MS-MS with API-ES source (6410 or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter or pH paper, solvent filtration apparatus, vacuum manifold, vortex mixer)

16.4 STANDARDS, CALIBRATORS AND CONTROLS

16.4.1 STANDARDS

- Working standard: 2, 10 ng/µL
- Working control standard: 2, 10 ng/µL
- Working internal standard: 4 ng/µL

16.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 16.5 SAMPLE PREPARATION.

16.4.3 CONTROLS

16.4.3.1 At least one negative whole blood control and two positive whole blood controls are tested with every batch, prepared as described in 16.5.

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

16.4.3.3 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.

16.4.3.4 Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both whole blood and alternate matrix controls apply towards 10% of the batch.

16.5 SAMPLE PREPARATION

NOTE: Laboratory general-use DI H_2O is used in sample preparation. 0.1% Formic acid used in reconstitution (16.5.22) is prepared using LC-MS grade DI H_2O (or equivalent). Organic solvents used in sample preparation are reagent grade.

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

16.5.2 Add 2 mL DI H_2O to each tube.

16.5.3 Add 2 mL of 0.1M phosphate buffer pH6 to each tube.

16.5.4 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).

16.5.5 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.

16.5.6 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 ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

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

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 (2/10 ng/mL)	100	0.1 ng/µL	1:100
Calibrator 2 (5/25 ng/mL)	25	1 ng/µL	1:10
Calibrator 3 (10/50 ng/mL)	50	1 ng/µL	1:10
Calibrator 4 (20/100 ng/mL)	100	1 ng/µL	1:10
Calibrator 5 (100/500 ng/mL)	50	10 ng/µL	WS
Calibrator 6 (200/1000 ng/mL)	100	10 ng/µL	WS

NOTE: HYM and 6AM standard/standard dilution concentrations are 2 ng/µL (WS), 0.2 ng/µL (1:10) and 0.02 ng/µL (1:100).

16.5.8 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.

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

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 – 6/30 ng/mL	30	1 ng/µL	1:10
Control 2 – 150/750 ng/mL	75	10 ng/µL	QC

NOTE: HYM and 6AM QC standard/QC standard dilution concentrations are 2 ng/µL (QC) and 0.2 ng/µL (1:10).

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

16.5.11 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 200 ng/mL.

16.5.12 Cap the tubes and briefly vortex mix.

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

16.5.14 Place new SPE columns into the vacuum manifold.

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

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

Do not let columns dry out between each conditioning step.

16.5.16 Transfer the contents of each extraction tube to its respective SPE column and allow to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

16.5.17 Wash the SPE columns by passing each of the following reagents/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 acetate buffer (pH4.5)
c. 3 mL MeOH

16.5.18 Dry the columns for 10 minutes under vacuum.

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

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

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

16.5.22 Reconstitute the extracts with the addition of 50 µL 0.1% formic acid to each tube and briefly vortex mix. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

16.5.23 Transfer the extracts to labeled polypropylene autosampler vials and cap.

16.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – OPIATES (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±5%, ion ratios ±20%) – Cal 4
- Result comparisons –

  Cal 1: truncated to one decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL; 1.5 – 2.5 ng/mL for HYM and 6AM)

Cals 2-3, Ctl 1: truncated, whole integer values in units of ng/mL; truncated to one decimal place for HYM and 6AM (acceptable ranges: 4.0 – 6.0 ng/mL for Cal 2, 8.0 – 12.0 for Cal 3 and 4.8 – 7.2 for Ctl 1)

Cals 4-6, Ctl 2: truncated, whole integer values in units of ng/mL

16.7 REPORTING

Results for MOR, OXM, COD, OXC and HYC are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L). Results for HYM and 6AM are reported in units of ng/mL. All results are truncated to two significant figures for reporting.

16.8 METHOD PERFORMANCE

- Limit of detection: MOR, OXM, COD, OXC, HYC – 5 ng/mL (0.005 mg/L)
  HYM, 6AM – 1.0 ng/mL
- Lower limit of quantification: MOR, OXM, COD, OXC, HYC – 10 ng/mL (0.01 mg/L)
  HYM, 6AM – 2.0 ng/mL
- Dynamic range: MOR, OXM, COD, OXC, HYC 10 – 1000 ng/mL (0.010 – 1.0 mg/L)
  HYM, 6AM – 2.0- 200 ng/mL
- Upper limit of quantitation: MOR, OXM, COD, OXC, HYC – 1000 ng/mL (1.0 mg/L)
  HYM, 6AM – 200 ng/mL

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Morphine
Morphine-d_6 (MOR-d_6)
Oxymorphone
Oxymorphone-d_3 (OXM-d_3)
Hydromorphone
Hydromorphone-d_3 (HYM-d_3)
Codeine
Codeine-d_6 (COD-d_6)
Oxycodone
Oxycodone-d_6 (OXC-d_6)
6-acetylmorphine
6-acetylmorphine-d_6 (6AM-d_6)
Hydrocodone
Hydrocodone-d_3 (HYC-d_3)

APPENDIX B
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.50 mL/min
Solvent A	0.1% Formic Acid
Solvent B	Acetonitrile
Initial Composition	95% (A), 5% (B)
0 – 3.5 min	%B increased to 40%
Hold time	0.5 min (40%B)
4.0 – 5.0 min	%B increased to 75%
Hold time	1.0 min (75%B)
Re-equilibration	10.0 min
Column Temp	30° C
Autosampler	
Injection Volume	5.0 µL
Injection flush-port	Active
Flush-port time	15 sec
Flush-port solvent	75:25/ Methanol:DI H_2O

Solvent B: 0.1% Formic Acid in Acetonitrile on LCMSMS 5. AB 10/14/19

MASS SPECTROMETER

Ion mode	(+) MRM	Nebulizer gas	Nitrogen
Peakwidth	0.05 min	Nebulizer pressure	50 psi
Dwell time	50 msec	Drying gas	Nitrogen
Time segment 1 (Time 0 min)	To waste	Drying gas flow	12 L/min
Time segment 2 (Time 0.3 min)	To MS	Drying gas temp	350° C
Time segment 3 (Time 6.0 min)	To waste	Capillary voltage	4kV
Compound	MRM Transitions		
Morphine	286→152/128		
Morphine-d_6	292→155		
Oxymorphone	302→227/198		
Oxymorphone-d_3	305→157		
Hydromorphone	286→185/157		
Hydromorphone-d_3	289→157		
Codeine	300→152/115		
Codeine-d_6	306→155		
Oxycodone	316→241/256		
Oxycodone-d_6	322→247		
6AM	328→165/211		
6AM-d_6	334→165		
Hydrocodone	300→199/128		
Hydrocodone-d_3	303→199		

LIST OF CHANGES

Revision Date	Description	Page Number
02/25/13	Method approved by Washington State Toxicologist. See DRA dated 02/22/13. Method released for use in evidentiary testing on 02/25/13.	All
02/01/14	Wording changed in sections 16.10.1.3 and 16.10.2.2 to reflect that 6AM and HYM are in units of ng/mL to one decimal place. Number format changed to reflect the procedure as confirmation test method number 16.	All
8/12/15	Sections 16.6.1.2, 16.6.1.4 and 16.7.12 changed to reflect use of 1.0 mg/mL CRMs for preparation of working internal standard and addition of 50 µL to each tube in sample preparation.	3, 4, 6
3/16/16	Added wording for solution storage in 16.5.1.9 and clarification to 16.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates in 16.6.1.3 and 16.6.1.4. Edited 16.12.1 and 16.12.2 and removed example in 16.12.2.1.c-f to reflect that only two significant figures are used for reporting. Other minor edits throughout.	1-5, 9
5/8/17	Wording added to 16.4.3 regarding dilution and standard volume testing. Changed prepared volume of internal standard to 250 mL in 16.6.1.4.a. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 16.7. Specified calibrator concentration criteria/ranges in 16.10.1.3 and described control result comparisons for HYM and 6AM in 16.10.2.2.c. Edited 16.10.2.2.d to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-9
6/11/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix matching in 16.2 SPECIMENS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 16.3.1. 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. Target compound/internal standard list added in APPENDIX A, with test method parameters moved to APPENDIX B. Formatting and minor edits throughout.	All