

CONFIRMATION OF OPIATES BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

16.1 POLICY

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. 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 a member of TLD Management, and appropriately documented in the batch file.

16.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of MOR, OXM, HYM, COD, OXC, 6AM and HYC 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.

16.3 PRINCIPLE

The targeted compound and internal standard are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of solid-phase extraction (SPE). Following SPE, 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 tandem mass spectrometer (MS-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 and multiple-reaction 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 MOR, OXM, HYM, COD, OXC, 6AM or HYC identified in a sample is determined from its calibration curve.

16.4 SPECIMENS

- 16.4.1 The specimen volume is 1 mL.
- 16.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.
- 16.4.3 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.
- 16.4.4 Analysis of larger specimen volumes must be approved and documented.

16.5 REAGENTS, MATERIALS AND EQUIPMENT

16.5.1 REAGENTS

16.5.1.1 0.1M acetate buffer (pH4.5)

Dissolve 2.93 g sodium acetate trihydrate in 400 mL DI H₂O. Add 1.62 mL glacial acetic acid. Dilute to 500 mL with DI H₂O 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. Adjustments to final volume are permitted as long as the proportions are maintained.

16.5.1.2 Acetic acid (Glacial)

16.5.1.3 Acetonitrile

16.5.1.4 Ammonium hydroxide (concentrated)

16.5.1.5 Certified blank blood

16.5.1.6 Deionized water (DI H₂O)

16.5.1.7 Elution solvent

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

16.5.1.8 Formic acid (concentrated)

16.5.1.9 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H₂O in a 1 L flask. Dilute to 1 L with DI H₂O and mix. Filter this solution prior to use on the HPLC. Store the solution in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

16.5.1.10 Isopropanol (IPA)

16.5.1.11 Methanol

16.5.1.12 Methylene chloride (dichloromethane, CH₂Cl₂)

16.5.1.13 0.1M phosphate buffer (pH6)

Dissolve 1.7 g Na₂HPO₄ and 12.14 g NaH₂PO₄ in 800 mL DI H₂O. Dilute to 1 L with DI H₂O and mix. Check the pH and, if necessary, adjust to 6 ±0.5 with concentrated NaOH. Store the solution in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

16.5.1.14 Sodium acetate trihydrate (NaC₂H₃O₂ • 3H₂O)

16.5.1.15 Sodium hydroxide (concentrated, NaOH)

16.5.1.16 Sodium phosphate, dibasic anhydrous (Na₂HPO₄)

16.5.1.17 Sodium phosphate, monobasic monohydrate (NaH₂PO₄ • H₂O)

16.5.2 MATERIALS

- 16.5.2.1 Autosampler vials, inserts and caps
- 16.5.2.2 Disposable 16 x 100mm tubes with closures
- 16.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures
- 16.5.2.4 Disposable pipette tips
- 16.5.2.5 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent
- 16.5.2.6 HPLC column (Agilent Zorbax Eclipse Plus C18 100 mm x 2.1 mm ID, $d_p=3.5 \mu\text{m}$, or equivalent)
- 16.5.2.7 Laboratory glassware (graduated cylinders, flasks)
- 16.5.2.8 Solvent filters (0.45 μm pore size; reduced cellulose, other)
- 16.5.2.9 Volumetric glassware (flasks)

16.5.3 EQUIPMENT

- 16.5.3.1 Agilent HPLC (1100/1200 series or equivalent)
- 16.5.3.2 Agilent MS-MS with API-ES source (6410 or equivalent)
- 16.5.3.3 Calibrated, adjustable piston pipettes
- 16.5.3.4 Centrifuge
- 16.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)
- 16.5.3.6 pH Meter and/or indicating pH paper
- 16.5.3.7 Solvent filtration apparatus
- 16.5.3.8 Vortex mixer
- 16.5.3.9 Vacuum manifold

16.6 STANDARDS, CALIBRATORS AND CONTROLS

16.6.1 STANDARDS

- 16.6.1.1 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.
- 16.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:
 - a. Morphine: 1.0 mg/mL
 - b. Morphine- d_6 : 1.0 mg/mL
 - c. Oxymorphone: 1.0 mg/mL

- d. Oxymorphone-d₃: 1.0 mg/mL
- e. Hydromorphone: 1.0 mg/mL
- f. Hydromorphone-d₃: 1.0 mg/mL
- g. Codeine: 1.0 mg/mL
- h. Codeine-d₆: 1.0 mg/mL
- i. Oxycodone: 1.0 mg/mL
- j. Oxycodone-d₆: 1.0 mg/mL
- k. 6-acetylmorphine: 1.0 mg/mL
- l. 6-acetylmorphine-d₆: 1.0 mg/mL
- m. Hydrocodone: 1.0 mg/mL
- n. Hydrocodone-d₃: 1.0 mg/mL

16.6.1.3 Working standard (10 ng/μL)

- a. Using a calibrated pipette, measure 500 μl each of MOR, OXM, COD, OXC and HYC stock standards and 100 μl each of HYM and 6AM stock standards into a 50 mL class-A volumetric flask.
- b. Add acetonitrile to the flask to the designated volume.
- c. The final concentration of the working standard is 10 ng/μl (2 ng/μl HYM, 6AM). The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

16.6.1.4 Working internal standard (4 ng/μL)

- a. Using a calibrated pipette, measure 400 μl each of stock internal standards into a 100 mL class-A volumetric flask.
- b. Add acetonitrile to the flask to the designated volume.
- c. The final concentration of the working internal standard is 4 ng/μl. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

16.6.2 CALIBRATORS

- 16.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 16.7 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.

16.6.3 CONTROLS

- 16.6.3.1 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.)

16.6.3.2 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 must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.
- d. The control working standard (10 ng/ μ L) is prepared as described in 16.6.1.3.
- e. The preparation of the positive whole blood controls is detailed in 16.7 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.

16.7 SAMPLE PREPARATION

- 16.7.1 Label a clean 16 x 100mm tube for each member of the test batch. (i.e. Calibrator, control, case sample)
- 16.7.2 Add 2 mL DI H₂O to each tube.
- 16.7.3 Add 2 mL of 0.1M phosphate buffer pH6 to each tube.
- 16.7.4 Add 1 mL of certified blank whole blood into each of the six calibrator tubes, the two positive control tubes and the negative control tube(s).
- 16.7.5 Prepare a 1:10 dilution of the working standard. (1 ng/ μ L)
 - a. Using a calibrated pipette, 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.
- 16.7.6 Prepare a 1:100 dilution of the working standard. (0.1 ng/ μ L)
 - a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution 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.

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

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

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

16.7.9 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 (6/30 ng/mL)	30	1 ng/µl
Control 2 (150/750 ng/mL)	75	10 ng/µl

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

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

16.7.12 Add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 200 ng/mL.

16.7.13 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500rpm.

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

16.7.15 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 H₂O
- c. 2 mL 0.1M phosphate buffer (pH6)

Do not let columns dry out between each conditioning step.

16.7.16 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.)

16.7.17 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 H₂O
- b. 2 mL 0.1M acetate buffer (pH4.5)
- c. 3 mL methanol

- 16.7.18 Dry the columns for 10 minutes under vacuum.
- 16.7.19 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.
- 16.7.20 Pass 3 mL of elution solvent through each SPE column and collect the extracts.
- 16.7.21 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.
- 16.7.22 Reconstitute the extracts by the addition of 50 µl 0.1% formic acid 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.
- 16.7.23 Transfer the extracts to labeled autosampler vials and cap.

16.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a batch worklist and set the data path in MassHunter 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 OPIATES.M for each line. As needed, the sequence may conclude with an injection that rinses the column (e.g. using method RINSE.M), or this may be done manually.

16.9 DATA ANALYSIS

- 16.9.1 Analysis of the batch data is conducted using the MassHunter Quantitative instrumental data analysis software.
- 16.9.2 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.
- 16.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.
- 16.9.4 Technical review of the batch is conducted according to the criteria listed below.

16.10 CRITERIA FOR BATCH ACCEPTANCE

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

- 16.10.1 Calibrators and calibration curves
 - 16.10.1.1 Chromatographic peaks for MOR, OXM, HYM, COD, OXC, 6AM, HYC and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

- 16.10.1.2 Retention times shall be within $\pm 5\%$ and ion ratios shall be within $\pm 20\%$ of those in calibrator 4. These are inclusive ranges.
- 16.10.1.3 Quantitative results for MOR, OXM, HYM, COD, OXC, 6AM and HYC in each calibrator shall be within $\pm 20\%$ of their target values with the exception of calibrator 1 which shall be within $\pm 25\%$ of their targets. These are inclusive ranges. Result comparisons for MOR, OXM, COD, OXC and HYC will use whole integer, truncated results in units of ng/mL. Result comparisons for HYM and 6AM will use results truncated to one decimal place in units of ng/mL.
- 16.10.1.4 The calibration curves for MOR, OXM, HYM, COD, OXC, 6AM and HYC shall have a correlation coefficient ≥ 0.99 .
- 16.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

16.10.2 Controls

- 16.10.2.1 The negative control(s) shall not identify MOR, OXM, HYM, COD, OXC, 6AM or HYC 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.
- 16.10.2.2 Positive controls
 - a. Chromatographic peaks for MOR, OXM, HYM, COD, OXC, 6AM and HYC and internal standards shall appear symmetrical.
 - b. Retention times shall be within $\pm 5\%$ and ion ratios shall be within $\pm 20\%$ of those in calibrator 4. These are inclusive ranges.
 - c. Quantitative results for MOR, OXM, HYM, COD, OXC, 6AM and HYC in each control shall be within $\pm 20\%$ of their target values. These are inclusive ranges. Result comparison for MOR, OXM, COD, OXC and HYC will use whole integer, truncated results in units of ng/mL. Result comparison for HYM and 6AM will use results truncated to one decimal place in units of ng/mL.
 - d. At least one positive control must meet these criteria for all compounds for the batch to be accepted.
- 16.10.2.3 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

16.11 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.

- 16.11.1 Any chromatographic peak for MOR, OXM, HYM, COD, OXC, 6AM or HYC shall appear symmetrical.
- 16.11.2 The retention times for MOR, OXM, HYM, COD, OXC, 6AM or HYC are $\pm 5\%$ and the ion ratios are within $\pm 20\%$ of those in calibrator 4. These are inclusive ranges.

16.11.3 The quantitative results for MOR, OXM, HYM, COD, OXC, 6AM or HYC must be within the dynamic range of the test method.

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

16.12 REPORTING

16.12.1 Results for MOR, OXM, COD, OXC and HYC are reported in units of milligrams per liter (mg/L).

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

16.12.1.2 Converted results are truncated to two significant figures for reporting.

- a. For example: morphine 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.

16.12.2 Results for HYM and 6AM are truncated after the first decimal place and reported in units of nanograms per milliliter (ng/mL).

16.12.2.1 Results are truncated to two significant figures for reporting.

- a. Example 1: HYM is measured as 4.82 ng/mL.
- b. The result is truncated to 4.8 ng/mL (two significant figures) and reported.
- c. Example 2: 6AM is measured as 16.18 ng/mL.
- d. The result is truncated to 16.1 ng/mL, but reported as 16 ng/mL (two significant figures).

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

16.13 METHOD PERFORMANCE

16.13.1 Limit of detection:

MOR, OXM, COD, OXC, HYC	5.0 ng/mL
HYM, 6AM	1.0 ng/mL

16.13.2 Lower limit of quantification:

MOR, OXM, COD, OXC, HYC	10 ng/mL (0.01 mg/L)
HYM, 6AM	2.0 ng/mL

16.13.3 Dynamic range:

MOR, OXM, COD, OXC, HYC	10 ng/mL to 1000 ng/mL
HYM, 6AM	2.0 ng/mL to 200 ng/mL

16.13.4 Upper limit of quantitation:

MOR, OXM, COD, OXC, HYC	1000 ng/mL (1.0 mg/L)
HYM, 6AM	200 ng/mL

16.14 TRACEABILITY

16.14.1 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	0.50 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 ₂ O

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
Signals	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		

