CONFIRMATION OF 6-ACETYLMORPHINE, CODEINE, HYDROCODONE, HYDROMORPHONE, MORPHINE AND OXYCODONE BY LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

6.1 POLICY

This test method may be used to confirm the presence of 6-acetylmorphine (6AM), codeine (COD), hydrocodone (HYC), hydromorphone (HYM), morphine (MOR) and oxycodone (OXC) 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.

6.2 PURPOSE

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

6.3 PRINCIPLE

The targeted compounds 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 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 opiate identified in a sample is determined from its calibration curve.

6.4 SPECIMENS

6.4.1 The specimen volume is 1mL.

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

6.4.3 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.

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

6.5 REAGENTS, MATERIALS AND EQUIPMENT

6.5.1 REAGENTS

6.5.1.1 Acetic acid (glacial)

6.5.1.2 1% Acetic acid in acetonitrile

Add 10 mL of glacial acetic acid to 800 mL acetonitrile in a 1 L flask. Dilute to 1 L with acetonitrile 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 month. Adjustments to final volume are permitted as long as the proportions are maintained.

6.5.1.3 1% Acetic acid in DI H_2O

Add 10 mL of glacial acetic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O 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 month. Adjustments to final volume are permitted as long as the proportions are maintained.

6.5.1.4 Acetonitrile

6.5.1.5 Ammonium hydroxide (concentrated)

6.5.1.6 Certified blank blood

6.5.1.7 Deionized water (DI H_2O)

6.5.1.8 Elution solvent

To 26 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 72 mL methylene chloride and mix. Store in glass 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.

6.5.1.9 Hydrochloric acid (HCl, ~11.7N)

6.5.1.10 0.1N Hydrochloric acid

Add 4.2 mL of concentrated HCl to 400 mL DI H_2O. Dilute to 0.5 L with DI H_2O and mix. 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.

6.5.1.11 Isopropanol (IPA)

6.5.1.12 Methanol

6.5.1.13 Methylene chloride (dichloromethane, CH_2Cl_2)

6.5.1.14 0.1M Phosphate buffer (pH5.5):

Dissolve 13.6 g KH_2PO_4 in 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Check the pH and, if necessary, adjust to 5.5 ±0.1 using potassium hydroxide. 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.

6.5.1.15 Potassium hydroxide (10N, purchased or prepared)

Exothermic reaction, use caution when preparing!! Carefully dissolve 56.1 g of potassium hydroxide in 90 mL of DI H_2O. Dilute to 0.1 L with DI H_2O and mix. 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.

6.5.1.16 Potassium phosphate, monobasic (KH_2PO_4)

6.5.2 MATERIALS

6.5.2.1 Autosampler vials, inserts and caps

6.5.2.2 Disposable glass pipettes

6.5.2.3 Disposable 16 x 100mm or 16 x 125 mm tubes

6.5.2.4 Disposable glass centrifuge tubes

6.5.2.5 Disposable pipette tips

6.5.2.6 Disposable safety closures for 16mm tubes

6.5.2.7 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CCDAU206, 200mg/6mL), or equivalent

6.5.2.8 HPLC column (Agilent Zorbax Eclipse Plus-C18; 150 x 4.6 mm ID, d_p=5μm, or equivalent)

6.5.2.9 Laboratory glassware (beakers, flasks, graduated cylinders, etc.)

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

6.5.2.11 Volumetric glassware (flasks)

6.5.3 EQUIPMENT

6.5.3.1 Agilent HPLC (1100/1200 series or equivalent)

6.5.3.2 Agilent MS with API-ES source (1100 or equivalent)

6.5.3.3 Agilent ChemStation software

6.5.3.4 Calibrated, adjustable air-displacement pipettes

6.5.3.5 Centrifuge

6.5.3.6 Evaporator (Biotage, formerly Zymark, TurboVap)

6.5.3.7 pH Meter and/or indicating pH paper

6.5.3.8 Solvent filtration apparatus

6.5.3.9 Vortex mixer

6.5.3.10 Vacuum manifold

6.6 STANDARDS, CALIBRATORS AND CONTROLS

6.6.1 STANDARDS

6.6.1.1 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.

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

a.	6-acetylmorphine:	0.1 mg/mL
b.	Codeine:	1.0 mg/mL
c.	Hydrocodone:	1.0 mg/mL
d.	Hydromorphone:	1.0 mg/mL
e.	Morphine:	1.0 mg/mL
f.	Oxycodone:	1.0 mg/mL

6.6.1.3 Working standard (1 ng/µL, 0.1 ng/µL, 0.2 ng/µL)

a. Using calibrated pipettes, measure 50 µl each of the 6-AM, COD, HYC, MOR and OXC stock standard into a 50 mL class-A volumetric flask. Add 10 µL of the HYM stock standard to the flask.
b. Add acetonitrile to the flask to the designated volume.
c. The final concentration of the working standard is 1 ng/µl for all components except 6-AM (0.1 ng/µL) and HYM (0.2 ng/µL). The working standard is stored in the freezer in an amber bottle and expires one year from date of preparation.

6.6.1.4 Stock internal standard (1 mg/mL)

a. Weigh 5 mg of ethylmorphine and add to a 5 mL class-A volumetric flask.
b. Add methanol to the flask to designated volume.
c. The final concentration of the stock internal standard is 1 mg/mL. The stock internal standard is stored in the freezer in an amber bottle and expires two years from the date of preparation.

6.6.1.5 Working internal standard (2 ng/µL)

a. Using a calibrated pipette, measure 100 µl of the stock internal standard 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 internal standard is 2 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from date of preparation.

6.6.2 CALIBRATORS

6.6.2.1 Calibrators are prepared in certified blank whole blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 6.7 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.

6.6.3 CONTROLS

6.6.3.1 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.)

6.6.3.2 Positive Control

a. At least one positive whole blood control is tested with every batch. If the batch is in excess of twenty (20) case samples, an additional positive control is included for every 20-case increment.
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. Alternatively, the same manufacturer lot number may be used provided that the controls are prepared by an analyst who did not prepare the working standard.
d. Positive whole blood controls are produced by laboratory personnel.
e. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

6.7 SAMPLE PREPARATION

6.7.1 Label clean 16 x 100mm or 125mm tubes for each member of the test batch. (i.e. Calibrator, control, case sample).

6.7.2 Add the working standard to each labeled calibrator tube as described in the following table. Evaporate the working standard to dryness at 50°C.

Calibrator Description	Volume (µL) Added
Calibrator 1	20
Calibrator 2	100
Calibrator 3	250
Calibrator 4	500

6.7.3 Add 50 µL of the working internal standard to each tube. Final concentration of the internal standard is 100 ng/mL.

6.7.4 Add 1 mL of certified blank whole blood into each of the four calibrator tubes and the negative control tube(s).

6.7.5 Transfer 1 mL of each positive control into its respective tube.

6.7.6 Transfer 1 mL of each case sample into its respective tube.

6.7.7 Add 3 mL of 0.1M phosphate buffer to each tube. Cap the tubes and briefly vortex mix.

6.7.8 Centrifuge the tubes for 15 minutes at 2500rpm.

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

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

a. 3 mL methanol (remove the methanol to chemical waste)
b. 2 mL DI H_2O
c. 3 mL 0.1M phosphate buffer (pH5.5)

Do not let columns dry out between each conditioning step.

6.7.11 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.)

6.7.12 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. 3 mL 0.1M HCl
c. 3 mL methanol (collect the methanol for transfer to chemical waste)

Discard the wash solvents. Disinfect the manifold reservoir with dilute bleach then rinse with water and dry the reservoir.

6.7.13 Dry the columns for 10 minutes under vacuum.

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

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

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

6.7.17 Reconstitute the extracts by the addition of 100 µL of 1% acetic 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.

6.7.18 Transfer the extracts to labeled glass autosampler vials with inserts and cap.

6.8 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 OPI0911.M for each line.

6.9 DATA ANALYSIS

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

6.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

6.9.3 Generate reports for calibrators, controls and cases and submit them, along with the updated calibration curves for review.

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

6.10 CRITERIA FOR BATCH ACCEPTANCE

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

6.10.1 Calibrators and calibration curves

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

6.10.1.2 Retention times shall be within ±5% and ion ratios shall be within ±25% of those in calibrator 3. These are inclusive ranges.

6.10.1.3 Quantitative results for 6-AM, COD, HYC, HYM, OXC and MOR 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.

6.10.1.4 The calibration curves for 6-AM, COD, HYC, HYM, OXC and MOR shall have correlation coefficients ≥0.99.

6.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

6.10.2 Controls

6.10.2.1 The negative control(s) shall not identify 6-AM, COD, HYC, HYM, OXC or MOR. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

6.10.2.2 Positive control(s)

a. Chromatographic peaks for 6-AM, COD, HYC, HYM, OXC, MOR and the internal standard shall appear symmetrical.
b. Retention times shall be within ±5% and ion ratios shall be within ±25% of those in calibrator 3 for each compound in a positive control. These are inclusive ranges.
c. Quantitative results for 6-AM, COD, HYC, HYM, OXC and MOR 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. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
e. At least one positive control must meet these criteria for all compounds for the batch to be accepted.

6.11 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.

6.11.1 Any chromatographic peaks for 6-AM, COD, HYC, HYM, OXC and MOR shall appear symmetrical.

6.11.2 The retention times for any reportable compounds are ±5% and the ion ratios are within ±25% of those in calibrator 3. These are inclusive ranges.

6.11.3 The quantitative result for each identified compound must be within the dynamic range of the test method.

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

6.11.5 If any target compound in a given case sample is outside of the dynamic range it does not invalidate the result for other compounds.

6.12 REPORTING

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

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

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

a. For example: codeine is measured as 44.93 ng/mL.

b. The unit conversion step truncates the result to 44 ng/mL and then represents the result as 0.044 mg/L.

c. The result is truncated to 0.044 mg/L (two significant figures) and reported.

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

6.13 METHOD PERFORMANCE

6.13.1 Lower limit of quantification:

6-AM:	2 ng/mL (0.002 mg/L)
COD, HYC, MOR, OXC:	20 ng/mL (0.020 mg/L)
HYM:	4 ng/mL (0.004 mg/L)

6.13.2 Dynamic range:

6-AM:	2 – 50 ng/mL (0.002 – 0.050 mg/L)
COD, HYC, MOR, OXC:	20 – 500 ng/mL (0.020 – 0.50 mg/L)
HYM:	4 – 100 ng/mL (0.004 – 0.10 mg/L)

6.13.3 Upper limit of quantitation:

6-AM:	50 ng/mL (0.050 mg/L)
COD, HYC, MOR, OXC:	500 ng/mL (0.50 mg/L)
HYM:	100 ng/mL (0.10 mg/L)

Drug	Calibrator 1	Calibrator 2	Calibrator 3	Calibrator 4
6-acetylmorphine	2 ng/mL	10 ng/mL	25 ng/mL	50 ng/mL
Codeine	20 ng/mL	100 ng/mL	250 ng/mL	500 ng/mL
Hydrocodone	20 ng/mL	100 ng/mL	250 ng/mL	500 ng/mL
Hydromorphone	4 ng/mL	20 ng/mL	50 ng/mL	100 ng/mL
Morphine	20 ng/mL	100 ng/mL	250 ng/mL	500 ng/mL
Oxycodone	20 ng/mL	100 ng/mL	250 ng/mL	500 ng/mL

6.14 TRACEABILITY

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

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	1.00 mL/min
Solvent A	1% Acetic acid
Solvent B	Acetonitrile (w/ 1% Acetic acid)
Initial Composition	97% (A), 3% (B)
0 – 16.5 min	%B increased to 40%
16.5 – 17.0 min	%B increased to 50%
17.0 – 20.0 min	Hold %B at 50%
20.0 – 25.0 min	%B decreased to 3%
25.0 – 35.0 min	Hold %B at 3%
Column Temp	60° C
Autosampler	
Injection Volume	2.5 µL
Wash vial position	81
Wash vial contents	1% Acetic acid

MASS SPECTROMETER

Ion mode	(+) SIM	Nebulizer gas	Nitrogen
EM Gain	2.0	Nebulizer pressure	50 psi
Peakwidth	0.12 min	Drying gas	Nitrogen
		Drying gas flow	12 L/min
		Drying gas temp	350° C
		Capillary voltage	4kV
Signals	Ions	Ion Ratios	
Morphine	286, 227, 209	227/286, 209/286	
Hydromorphone	286, 227, 287	227/286, 287/286	
Codeine	300, 241, 181	241/300, 181/300	
Oxycodone	298, 316, 241	316/298, 241/298	
6-Acetylmorphine	328, 268, 329	268/328, 329/328	
Hydrocodone	300, 241, 301	241/300, 301/300	
Ethylmorphine (ISTD)	312, 227	227/312	

LIST OF CHANGES

Revision Date	Description	Page Number
09/01/11	Method approved by Washington State Toxicologist. See DRA dated 08/23/11. Method released for use in evidentiary testing on 09/01/11. Cocaine & metabolite confirmation removed from SOP.	All