SOP 408 - Opiates Extraction by Solid Phase Extraction (SPE) for Quantification by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC-ES/MS/MS)

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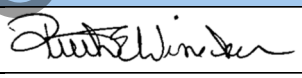
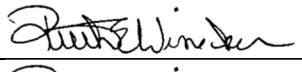
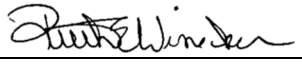
Ruth E. Winecker, Ph.D.		04/17/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 408 - Opiates Extraction by Solid Phase Extraction (SPE) for Quantification by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC-ES/MS/MS)

1. Principle of Assay

1.1. This method is designed to confirm and quantitate morphine, oxymorphone, hydromorphone, codeine, oxycodone, hydrocodone, and 6-acetylmorphine (via heroin) in biological specimens by Liquid Chromatography tandem-electrospray Mass Spectrometry (LC/MS/MS). The drugs are extracted from their biological matrix by solid-phase extraction and identified by the retention time and ion ratio of product ions. The target opiates are subject to matrix effects, thus stable isotopically labeled internal standards for each are used (1).

1.2. Opiates and opioids are one of the most prescribed classes of drugs in the United States, led by oxycodone (Oxycontin) and hydrocodone (Vicodin), for the management of moderate to severe, acute or chronic pain. Both drugs, as well as oxymorphone and hydromorphone, are semi-synthetic derivatives of morphine and codeine, found in opium poppy latex. In an attempt to discover a more potent and potentially less addictive opiate, diactetylmorphine was developed and marketed in 1827. What was once a common drug given for pain is now an illicit narcotic known as heroin. After administration, diacetylmorphine is quickly hydrolyzed to 6-acetylmorhine and morphine, leaving 6-acetylmorphine a marker for the source of morphine.

Pharmacologically, opiates and opioids act on opioid receptors, a group of G-protein coupled receptors widely distributed in the brain, spinal cord, and gut. Each of the three main receptors mu, delta, and kappa opioid receptors, plus their subtypes, contributes to the efficacy and side-effects of opioid drugs. Activation of the mu-opioid receptor (MOR), the main receptor targeted by opioid drugs, induces analgesia, but also respiratory depression, constipation, and physical dependence. Activation of the delta-opioid receptor (DOR) also produces analgesia and respiratory depression, albeit to a less significant degree than MOR. The kappa-opioid receptor (KOR), upon activation, leads to depression and dysphoria. Overall, chronic use or abuse can lead to severe psychological and physical dependence, with overdoses leading to respiratory depression and death. (2)

In this laboratory, screening for the above mentioned opiates and opioids is typically done in central blood specimens (*e.g.* aorta, inferior vena cava) via the multi-drug LC/MS targeted screen (SOP 120). These target drugs have low volumes of distribution (Vd <5 L/kg) and while not thought to exhibit classic postmortem redistribution can be subject to pre-analytical issues or site dependence, especially in circumstances involving aspiration or leakage from GI (3). Regardless, confirmation and quantitation is typically done in peripheral blood specimens (*e.g.* femoral, iliac) to more accurately reflect drug concentration at the time of death. Additionally, urine or vitreous specimens may be used to confirm 6-monoacetylmorphine (4).

2. Specimens

2.1. This procedure is applicable to blood, urine, serum, vitreous humor, *bile, properly prepared tissue specimens (typically 1:4 homogenates), and *gastric contents. A duplicate sample size of 0.5 mL and 0.1mL for blood, serum, and vitreous humor, and 0.5 mL(g) and 0.05 mL(g) of urine, bile, or tissue homogenate is generally employed - unless otherwise noted on the load worksheet - so that the calibration curve encompasses the expected range of unknown specimens.

2.1.1. *For non-typical matrices, an additional 0.5mL aliquot shall be taken (volume permitting), spiked with appropriate QC, and analyzed to help to identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

3. Reagents and Materials

3.1. DI water, HPLC grade
3.2. 100 mM Phosphate Buffer pH 6.0
3.3. Methanol, HPLC grade
3.4. Isopropanol, HPLC grade
3.5. Concentrated ammonium hydroxide
3.6. Methylene Chloride, HPLC grade
3.7. 100mM Acetic Acid
3.8. Deuterated mixed Opiate Internal Standard Mix
3.9. Mixed Opiate Standard Mix Standard and Control Worksheet.
3.10. Mixed Opiate QC Standard Mix
3.11. Drug Free Blood, Urine, Liver Homogenate
3.12. Water with 0.1% formic acid
3.13. Acetonitrile with 0.1% formic acid
3.14. Methanol with 0.1% formic acid

4. Standards, Controls, and Solutions

4.1. Opiate Internal Standard Stock Solution (2/10µg/mL)

4.1.1. Into a 10mL volumetric flask, add the following:
4.1.1.1. 1 ampule (~1mL) of Codeine-d3 (Cerilliant - 100µg/mL)
4.1.1.2. 1 ampule (~1mL) of Hydrocodone-d6 (Cerilliant - 100µg/mL)
4.1.1.3. 1 ampule (~1mL) of Hydromorphone-d3 (Cerilliant - 100µg/mL)
4.1.1.4. 1 ampule (~1mL) of Morphine-d3 (Cerilliant - 100µg/mL)
4.1.1.5. 1 ampule (~1mL) of Oxycodone-d3 (Cerilliant - 100µg/mL)
4.1.1.6. 1 ampule (~1mL) of Oxycodone-d3 (Cerilliant - 100µg/mL)
4.1.1.7. 20µL from an ampule of 6-acetylmorphine-d3 (Cerilliant - 100µg/mL).

4.1.1.8. Fill to the line with acetonitrile, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.2. **Opiate Internal Standard Working Solution (200/1000ng/mL)**

4.2.1. Into a 10mL volumetric flask, add 1mL of Opiate Internal Standard Stock Solution (2/10µg/mL) with a micropipette.

4.2.2. Fill to the line with acetonitrile, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.3. **Opiate Calibrators and Positive Controls –** these standards are to be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if calibration/control standards need to be made.

4.4. **100 mM Acetic Acid Solution**

4.4.1. To a 1000 mL Erlenmeyer flask, add 5.7 ml of glacial acetic acid, and fill to the 1000 mL mark with deionized water, and mix well. Alternatively, a stock reagent at 4 Liters can be prepared by making appropriate multiplier of 4 for all volumes used.

4.5. **100mM Phosphate Buffer pH 6.0**

4.5.1. To a newly opened 4L bottle of DI H_2O, add 48.8 grams of potassium phosphate (KH_2PO_4) and 1.64 grams sodium hydroxide (NaOH). Swirl until dissolved. Check pH. If necessary, adjust with NaOH or phosphoric acid (H_3PO_4) to a final pH of 5.5-6.5.

4.6. **Column Elution Solution (make fresh daily)**

4.6.1. To a 200 mL graduated cylinder add 40 mL of isopropyl alcohol, 4 mL of concentrated ammonium hydroxide, and fill to the 200 mL mark with dichloromethane.

4.7. **Water with 0.1% formic acid**

4.7.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid.

4.7.2. Label bottle as “LC/MS” and “with 0.1% formic acid”.

4.8. **Acetonitrile with 0.1% formic acid**

4.8.1. To a 4L bottle of HPLC grade acetonitrile, add 4 mL of formic acid.

4.8.2. Label bottle as “LC/MS” and “with 0.1% formic acid”.

4.9. **Methanol with 0.1% formic acid**

4.9.1. To a 4L bottle of HPLC grade methanol, add 4 mL of formic acid

4.9.2. Label bottle as "LC/MS" and "with 0.1% formic acid

5. **Equipment and Special Supplies**

5.1. Test tubes, 16 x 100mm (or equivalent)
5.2. Centrifuge 2000 x g
5.3. Vortex mixer
5.4. Nitrogen evaporator
5.5. Positive Pressure Extraction Manifold
5.6. UCT Clean Screen® extraction columns (CSDAU206)
5.7. LC autosampler vials, 12 x 32 mm
5.8. Polyspring inserts, 5 mm O.D.

6. **Instrumentation and Parameters**

6.1. Windows PC with Thermo LCQuan and Xcaliber software

6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer

6.4.1. Click here for instrument method parameters: (TSQ02 - Opiates_EZmethod_).

7. **Target Ions (± 1 nominal mass)**

7.1.	Codeine	(**300** 165 215)
7.2.	Codeine-d3	(**303** 152 165)
7.3.	Morphine	(**286** 152 165)
7.4.	Morphine-d3	(**289** 128 157)
7.5.	6-MAM	(**328** 165 211)
7.6.	6-MAM-d3	(**331** 165 211)
7.7.	Hydrocodone	(**300** 199 128)
7.8.	Hydrocodone-d6	(**306** 202 174)
7.9.	Hydromorphone	(**286** 185 157)
7.10.	Hydromorphone-d3	(**289** 185 128)
7.11.	Oxycodone	(**316** 298 241)
7.12.	Oxycodone-d3	(**319** 244 259)
7.13.	Oxymorphone	(**302** 227 198)
7.14.	Oxymorphone-d3	(**305** 287 230)

7.14.1. Note: The precursor ion of each analyte is listed first and bolded, the product ion-used for quantification-is second, followed by the product ion-used for qualification/confirmation.

8. **Prepare Samples**

8.1.1. Add 50µL internal standard to appropriate number of 16X125mm test tubes.

8.1.2. To each tube add 1mL of 100mM phosphate buffer (pH=6).

8.1.3. Add the mixed opiate standards and mixed opiate quality control solution to appropriately labeled tubes according to Standard and Control Worksheet.

8.1.4. Add 0.5mL blank blood to each of the standards and controls. Include a urine blank and QC and/or a liver homogenate blank and QC by adding 0.5 mL(g) of blank urine or blank liver to appropriately labeled test tubes (as appropriate).

8.1.5. Aliquot specimens in duplicates described in the Specimens section (2.1).

8.1.6. Add an additional 2mL of 100mM phosphate buffer (pH=6) to each test tube, vortex for 20 seconds.

8.1.7. Centrifuge samples for 5 minutes at 2000 x g.

8.1.8. Load the appropriate number of labeled, new Clean Screen® extraction columns (CSDAU206) for each sample onto the positive pressure manifold.

8.2. Prepare SPE Columns:

8.2.1. Introduce 3mL of methanol into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.2.2. Introduce 3mL of deionized water into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.2.3. Introduce 2mL of 100mM phosphate buffer (pH=6) into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.3. Apply Samples:

8.3.1. Introduce samples into columns and allow to drip by gravity or under 2 PSI (positive pressure). If the matrix of the specimen prevents the sample from moving through the column by gravity alone, positive pressure (2-5 PSI) can be applied via manifold or pipette bulb.

8.4. Wash SPE Columns:

8.4.1. Introduce 2mL of deionized water into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.4.2. Introduce 2mL of 100mM acetic acid into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.4.3. Introduce 3mL of methanol into columns and allow to drip by gravity or under 2 PSI (positive pressure).

8.4.4. Dry for 5-10 minutes under full positive pressure.

8.4.5. Prepare extraction manifold for specimen collection. Place appropriately labeled 13X100mm test tubes into collection position. Wipe tips clean and place secured SPE columns onto collection rack, verifying that SPE tips are securely inside collection tubes.

8.4.6. Introduce 3mL of column elution solution (methylene chloride/IPA/Ammonium hydroxide 78:20:2) into columns and allow to drip by gravity.

8.4.6.1. Note: After column elution solution has completely eluted, apply full pressure to force through any remaining solution into the collection tubes.

8.4.7. Evaporate each specimen to dryness in a nitrogen evaporation apparatus at 40°C; 15 psi.

8.4.8. Reconstitute with 200µL 100mM phosphate buffer (pH=6), vortex and transfer at least100µL to LC autosampler vial.

8.4.9. Build and initiate sequence as directed in SOP 053.

9. **Calculations**

9.1. Quantification

9.1.1. The method for processing the data using the Thermo LCQuan software is "Opiates_EZ-Method" (SOP 055). It is used to calculate the internal standard response ratios, raw amounts, concentration, and ion ratios.

9.1.2. These calculations are computed as follows:

9.1.2.1. Response Ratio:

9.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standard's.

9.1.2.1.2. Response Ratio = QN_a / QN_{istd}

9.1.2.1.2.1. QN_a = response of the quantitative ion of the analyte

9.1.2.1.2.2. QN_{istd} = response of the quantitative ion of the internal standard Amount

9.2. Calibration

9.2.1. A linear regression resulting from the 5 standards is used to quantitate the analytes in the case. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

9.3. Dilution Factor

9.3.1. D = Total volume/Sample volume

9.4. Multiplier for homogenates, dilutions, and non-standard volumes

9.4.1. $M = (V_{curve} / V_{samp}) \times D$

9.4.1.1. M = Multiplier

9.4.1.2. D = dilution factor

9.4.1.3. V_{curve} = matrix volume of calibration curve

9.4.1.4. V_{samp} = matrix volume of specimen

9.5. Concentration

9.5.1. C = (A / V) * M

9.5.1.1. C = Concentration (ng/mL) of the analyte in the unknown case.

9.5.1.2. A = Amount of drug in sample

9.5.1.3. V = Volume of sample

9.5.1.4. M = Multiplier

9.6. Max/Min

9.6.1. Percent Difference = $((R_h / R_l)-1) \times 100$

9.6.1.1. R_h = high result

9.6.1.2. R_l = low result

9.7. Average

9.7.1. Average = $(R_1 + R_2) / 2$

9.7.1.1. R_1 = first result

9.7.1.2. R_2 = second result

9.8. Qualifier Ion Ratios

9.8.1. Ratio 1 = QL_1/QN

9.8.1.1. QL_1 = response of the quantifying product ion

9.8.1.2. QN = response of the qualifying product ion

10. **Quality Control**

10.1. Acceptance criteria

10.1.1. Chromatogram

10.1.1.1. Peaks must be Gaussian shaped (symmetrical).

10.1.1.2. Peaks must not exhibit extreme fronting or tailing.

10.1.1.3. Peaks sharing parent/product ions must have baseline resolution.

10.1.1.4. The internal standard (ISTD) in each case should be inspected for evidence of signal enhancement and suppression. The area of the quantifying ion should not be less than 50% or more than 200% of the average ISTD of the calibrators.

10.1.1.5. Retention time must not deviate outside ± 3% (minimum window 7.2 seconds) of target, based upon the retention time of the calibrators and controls.

10.1.2. Mass spectroscopy

10.1.2.1. The ion ratio of all samples must not be greater than ± 20% of the target ratio as determined by a mid-level calibrator (CAL 3).

10.1.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

10.1.3. Calibrators

10.1.3.1. Analytical curves must have a coefficient of determination (R^2) of 0.992 or greater.

10.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value (± 25% at LOQ).

10.1.3.3. Refer to "Calibration curve point exclusion guidelines" section of the QA/QC Manual.

10.1.4. Controls

10.1.4.1. Controls must calculate to within ± 20% of the target value.

10.1.5. Blanks

10.1.5.1. Blanks should not contain any target analyte signal with an internal standard response ratio greater than 10% that of the lowest calibrator for the same analyte.

11. Validation of Method

Parameter	Result - Blood
Bias	**6-MAM - Report qualitativly** Low: 18.08% High: -11.12%
	codeine - Low: -2.10% High: 2.41%
	morphine - Low: -0.24% High: 6.76%
	hydrocodone - Low: 5.39% High: 5.67%
	hydromorphone - Low: 3.08% High: 18.47%
	oxycodone - Low: 7.93% High: 8.34%
	oxymorphone - Low: 4.21% High: 9.41%
Precision	**6-MAM -** Low: 12.31% High: 5.04%

	codeine - Low: 3.22% High: 5.16%
	morphine - Low: 7.48% High: 5.76%
	hydrocodone - Low: 2.98% High: 2.86%
	hydromorphone - Low: 4.64% High: 6.25%
	oxycodone - Low: 3.27% High: 3.42%
	oxymorphone - Low: 4.92% High: 7.55%
	Result - Urine
Bias	**6-MAM - Report qualitativly** Low: -20.76% High: -10.38%
	codeine - Low: -1.76% High: 3.51%
	morphine - Low: -1.25% High: 6.17%
	hydrocodone - Low: 5.50% High: 5.17%
	hydromorphone - Low: 5.95% High: 8.59%
	oxycodone - Low: 8.26% High: 11.25%
	oxymorphone - Low: 3.10% High: 7.09%
Precision	**6-MAM -** Low: 5.96% High: 4.01%
	codeine - Low: 5.85% High: 5.01%
	morphine - Low: 6.47% High: 4.37%

	hydrocodone - Low: 3.49% High: 4.97%
	hydromorphone - Low: 5.12% High: 6.86%
	oxycodone - Low: 3.43% High: 4.20%
	oxymorphone - Low: 5.84% High: 6.05%
Calibration model	**6-MAM -** Linear 1/x weighting
	codeine - Linear 1/x weighting
	morphine - Linear 1/x weighting
	hydrocodone - Linear 1/x weighting
	hydromorphone - Linear 1/x weighting
	oxycodone - Linear $1/x^2$ weighting
	oxymorphone - Linear 1/x weighting
Carryover	Signal >10% low calibrator observed for hydromorphone and 6-MAM following injections of 1000ng/mL and 500ng/mL respectively. High calibrator shall not exceed 500ng/mL and 200ng/mL respectively and any specimen injected directly after a case with one of these analytes approaching/exceeding the carryover limit shall be scrutinized accordingly..
Interference Studies	No interfering signal from matrix, internal standard, common drugs of abuse (including metabolites), OTC drugs, and Prescription medications was observed.
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	Not evaluated - IS coelute within 0.05 minutes of associated analytes.
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	**6-MAM -** 1.4ng/mL
	codeine - 1.5ng/mL
	morphine - 2.65ng/mL
	hydrocodone - 2.44ng/mL
	hydromorphone - 2.14ng/mL
	oxycodone - 3.03ng/mL
	oxymorphone - 2.34ng/mL

LOQ (Set to lowest calibrator with acceptable Bias/Precision).	**6-MAM -** = 2ng/mL
	codeine - = 10ng/mL
	morphine - = 10ng/mL
	hydrocodone - = 10ng/mL
	hydromorphone - = 5ng/mL
	oxycodone - = 10ng/mL
	oxymorphone - = 5ng/mL
Dilution Integrity	Evaluated case-by-case. (All cases injected in duplicate, @ 1:1 and 1:5 dilutions (blood/vitreous) and 1:1 and 1:10 dilutions (urine/gastric/tissue homogenates). Results from both injections must fall within 25% of each other to be reported.
Processed Sample Stability - (re-analyze after 8 days)	Sample extracts remain stable for 8 days (recapped) and 3 days (not recapped).

11.1.

12. **Reporting**

12.1. The percent difference of duplicate analysis for an analyte must be less than or equal to 25% (see Max/Min in Calculations section).

12.2. Reporting of duplicate analysis should be done according to the table below:

Reporting Duplicates

- Dilution factors of 1 and 5 (or other)

Dil / Scenario	1	5	REPORT
A	In curve	In curve	Average
B	In curve	BQL	"In" value
C	AQL	In curve	"In" value
D	In curve	ND (should be in)	Repeat
E	AQL/BQL	AQL/BQL	Less than/Greater than
***F**	BQL	ND	ND
G	In curve	ND (should be BQL)	"In" value

12.2.1.

12.2.1.1. *** Exception – 6-Monoacetylmorphine shall be reported as "Present" when detected as "BQL" in one aliquot and ND in the other. This designation may be altered on a case-by-case**

basis during final review if case reporting criteria are not met (see section 1.4 – Guidelines for Confirming Positive Results of the QA/QC Manual)

12.2.1.2. In Curve = Measured concentration (pre-multiplier) falls within the calibration range

12.2.1.3. AQL = Measured concentration (pre-multiplier) falls Above Quantitation Limit

12.2.1.4. BQL = Measured concentration (pre-multiplier) falls Below Quantitation Limit

12.2.1.5. ND = None Detected

12.3. Averaging reportable values

12.3.1. Results for duplicate analysis (both falling within calibration curve) shall be truncated prior to averaging.

12.3.2. Enter calculated concentration for each specimen into toxlog.

12.4. Significant figures

12.4.1. Concentrations are truncated and reported with two significant figures in mg/L.

12.4.2. Analyte concentrations entered into Toxlog shall not exceed 3 decimal places (e.g. a result of 7.4ng/mL shall be reported as 0.007mg/L)

13. **Preparation of Load**

13.1. The load paperwork and data is to be arranged in the following order:

13.1.1. Assignment sheet

13.1.2. Comments or note to file if applicable

13.1.3. Load summary

13.1.4. Specimen worklist

13.1.5. Chain of custody (Specimen)

13.1.6. Aliquot chain of custody

13.1.7. Standard and control worksheet

13.1.8. Sequence summaries/calibration reports – paper clipped

13.1.9. Calibrator data - paper clipped

13.1.10. Blank matrix data - paper clipped

13.1.11. Control data - paper clipped

13.1.12. Specimen data – stapled

14. **References**

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