Solid Phase Extraction of Opioids from Biologicals with Analysis by LC-Tandem MS (High Resolution)

1 Introduction

Opioids are a class of substances that include natural, semi-synthetic and synthetic alkaloidal agents derived from opium or substances which have morphine-like activity. Naturally occurring opioids such as morphine and codeine are typically referred to as opiates. Heroin (diacetylmorphine) is a semi-synthetic opioid that is synthesized by the acetylation of morphine (MOR). In humans, heroin is rapidly metabolized to 6-monoacetylmorphine (6-MAM) and morphine. Morphine is further metabolized to N-desmethylmorphine (NorM). Codeine (COD) is the 3-methyl ether derivative of morphine, and is metabolized to morphine and N-desmethylcodeine (NorC). Among the more common synthetic opioids are oxycodone (OXYC; Oxycontin), hydrocodone (HC; Vicodin), hydromorphone (HM; Dilaudid), and dihydrocodeine (DHC; Drocode). Hydrocodone is biotransformed to hydromorphone, while oxycodone is metabolized to oxymorphone (OXYM) and N-desmethyloxycodone (NorOxyC), and dihydrocodeine is converted to dihydromorphine (DHM). The conversion of opioids to glucuronide conjugates is a common metabolic transformation. Conjugated opioids are difficult to extract and chromatograph in a single fraction with unconjugated opioids. Therefore, analysis of the total concentration of an opioid that is present in conjugated and unconjugated form requires a hydrolysis step to cleave the conjugates.

2 Scope

This procedure allows for the screening and confirmation of morphine, codeine, hydromorphone, hydrocodone, oxymorphone, oxycodone, 6-acetylmorphine, normorphine, norcodeine, noroxycodone, dihydromorphine, and dihydrocodeine in biological specimens. It also provides a method of quantitative analysis for the first seven of these compounds. This document applies to Chemistry Unit caseworking personnel who perform toxicology analyses.

3 Principle

Biological specimens are qualitatively screened and/or quantitated for opioids by this method. Since most opioids are biotransformed to form a glucuronide conjugate during metabolism, these conjugates need to be hydrolyzed to obtain "total" opioid concentrations. The hydrolysis occurs by cleaving the drug-conjugate with the enzyme β-glucuronidase. Analysis without hydrolysis yields "free" opioid concentrations. Analysis with hydrolysis yields "total" opioid concentrations. Specimens are mixed with internal standards, adjusted to a slightly acidic pH, and extracted using mixed mode hydrophobic/cation exchange solid phase extraction cartridges. Target drugs are eluted using a mixed solvent system of methylene chloride, isopropanol, and ammonium

hydroxide. The eluent is taken to dryness and reconstituted prior to analysis by Liquid chromatography-tandem mass spectrometry (LC-Tandem MS) High Resolution.

4 Specimens

This procedure can be used for assaying biological specimens such as blood, serum, plasma, urine, bile, gastric contents, vitreous humor, or a previously prepared tissue homogenate. When available, 1 mL of biofluid or 2 g of a prepared tissue homogenate (1:1) is used in the assay. Blood, bile, gastric content, and tissue homogenate samples are centrifuged prior to analysis. Urine, vitreous humor, plasma, or serum specimens can be directly extracted. Total opiate analysis requires that specimens such as blood, urine, and bile be enzymatically hydrolyzed prior to analysis. In instances where specimen volume is altered (e.g., to improve sensitivity or account for limited specimen volume), appropriate modifications may be made to this procedure.

5 Equipment/Materials/Reagents

a. Binary (or higher) liquid chromatograph coupled to an electrospray ion trap mass spectrometer capable of at least 15000 resolution (for example, Orbitrap)

b. Xterra Phenyl LC column: 150 x 2.1 mm. 5 µm d_p, with 2 µm titanium prefilter

c. Test tubes (16 x 125 mm screw-top, 16 x 100 mm and 12 x 75 mm culture, or comparable)

d. Centrifuge

e. Heating block

f. Vortex mixer

g. Solid phase extraction manifold (vacuum or positive pressure)

h. CLEAN SCREEN DAU solid phase extraction (SPE) cartridges (200 mg x 10 mL)

i. Evaporator with nitrogen

j. Homogenizer (for tissue or similar specimens)

k. β-Glucuronidase (Type H-2 from Helix Pomatia; 100,000+ units/mL)

l. Sodium acetate trihydrate (reagent grade)

m. Hydrochloric acid, concentrated (12 M) (ACS grade)

n. 1 N Hydrochloric Acid: To a 100-mL graduated cylinder, add 80 mL deionized water. Add 8 mL concentrated hydrochloric acid and mix well. Bring to 96 mL with deionized water. Store in glass at room temperature. Stable 6 months.

o. Sodium acetate buffer (1.1 M): To a 100-mL volumetric flask, add 14.95 g sodium acetate trihydrate, 60 mL deionized water, and 2.2 mL glacial acetic acid. Mix well to dissolve, and bring to volume with deionized water. Verify $5<pH<6$. Store refrigerated in glass. Stable 2 months.

p. Water (Optima grade and deionized)

q. 0.1 M, pH 6 Phosphate buffer: To a 500-mL volumetric flask, add 400 mL deionized water, 6.1 g sodium phosphate monobasic monohydrate, and 1.6 g sodium phosphate dibasic heptahydrate. Mix well to dissolve. Verify $5.8<pH<6.1$. Bring to volume with deionized water. Store refrigerated in glass. Stable 2 months.

r. 1:1 Methanol:Water: Combine 50 mL methanol with 50 mL water (both Optima grade) and mix well. Store in glass at room temperature. Stable 12 months.

s. Methanol (HPLC and Optima grades)

t. Acetic acid, glacial (17 M) (ACS grade)

u. 0.1 M Acetic acid: To a 100-mL graduated cylinder, add 80 mL deionized water and 0.5 mL glacial acetic acid. Mix well and bring to 85 mL with deionized water. Store in glass at room temperature. Stable 6 months.

v. Ammonium formate

w. Acetonitrile (Optima grade)

x. 0.5 µm PTFE membrane filter

y. Methylene chloride (HPLC grade)

z. Isopropanol (HPLC grade)

aa. Ammonium hydroxide (concentrated, reagent grade)

bb. Formic Acid (reagent grade)

cc. SPE elution solvent (78:20:2 methylene chloride:isopropanol:ammonia): Combine 20 mL HPLC grade isopropanol with 2 mL concentrated ammonium hydroxide and mix well. Add 78 mL HPLC grade methylene chloride and mix well. Store in glass at room temperature. To be prepared fresh.

dd. Reconstitution solvent (5:95 methanol:water): Combine 5 mL water with 95 mL methanol (both Optima grade) and mix well. Store in glass at room temperature. Stable for 6 months.

ee. LC mobile phase 1 (95:5:0.05 10 mM ammonium formate : acetonitrile : formic acid): Dissolve 630 mg of ammonium formate in 1 L of Optima grade water. Remove 50 mL of this solution, save for LC Mobile Phase #2, and add 50 mL of acetonitrile. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Add 500 µL formic acid and mix well. Store in glass at room temperature. Stable for 1 months.

ff. LC mobile phase 2 (5:95:0.05 10 mM ammonium formate : acetonitrile : formic acid): Add 25 mL of the aqueous formate solution from the preparation of LC mobile phase #1 to 475 mL of acetonitrile. Mix well and vacuum filter through a 0.5 µm PTFE membrane. Add 250 µL formic acid and mix well. Store in glass at room temperature. Stable for 1 months.

gg. Common laboratory supplies such as volumetric flasks, autosampler vials, pipette tips, etc.

6 Standards and Controls[1]

a. Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant or another approved supplier. Stability and storage conditions are determined by the manufacturer.

d_3-Morphine	d_6-Oxycodone
d_6-Codeine	d_3-Hydromorphone
d_3-Oxymorphone	d_3-Hydrocodone
d_3-6-MAM	

b. Internal Standards Working Solution (4 µg/mL or 1 µg/mL, depending on analyte): Mix 1 mL each of the d_3-Morphine and the d_6-Codeine Stock Solutions with 250 µL each of the d_3-Hydromorphone, d_3-Hydrocodone, d_3-Oxymorphone, and d_6-Oxycodone Stock Solutions. Dilute with 1:1 methanol:water to a final volume of 25 mL. Store at <0ºC in glass. Stable for at least 1 year.

[1] Working solutions may be made at different volumes by scaling components if necessary.

c. d_3-6-MAM Working Solution (2 µg/mL):
Dilute 500 µL of the d_3-6-MAM stock solution to 25 mL in acetonitrile. Store in glass at <0ºC. Stable for 6 months.

d. Standard Stock Solutions (1 mg/mL) may be purchased for Cerilliant (typically used for calibrators) and from Lipomed (typically used for controls) or another approved supplier. Stability and storage conditions are determined by the manufacturer.

Morphine	Oxymorphone	Norcodeine
Codeine	Oxycodone	Noroxycodone
Hydromorphone	6-MAM	Dihydromorphine
Hydrocodone	Normorphine	Dihydrocodeine
Mophine-3-β-glucuronide or Morhine-6-β-glucuronide (0.1 mg/mL)		

e. Column Performance Evaluation Mix (0.5 µg/mL each component)
Mix 50 µL each of the morphine, hydromorphone, oxycodone, dihydrocodeine, and norcodeine stock standards. Dilute to 100 mL with Reconstitution solvent (5:95 methanol:water) and mix well. Store refrigerated in glass. Stable for at least one year. A 5 µL portion of this solution is analyzed before each day's samples, in order to confirm acceptable instrument performance.

f. Control Working Solution #1 (3.5 or 0.7 µg/mL, depending on component):
Mix 175 µL each of the Morphine and Codeine Stock Solutions with 35 µL each of the Hydromorphone, Hydrocodone, Oxymorphone, and Oxycodone Stock Solutions. Dilute with 1:1 methanol:water to a final volume of 50 mL. Store in glass at <0ºC. Stable for at least 1 year.

g. Control Working Solution #2 (1 µg/mL):
Dilute 50 µL of the 6-MAM Stock Solution with acetonitrile to a final volume of 50 mL. Store in glass at <0ºC. Stable for 6 months.

h. Control Working Solution #3 (1 µg/mL each component):
Dilute 50 µL each of the Normorphine, Norcodeine, Noroxycodone, Dihydromorphine, and Dihydrocodeine Stock Solutions with 1:1 methanol:water to a final volume of 50 mL. Store in glass at <0ºC. Stable for at least 1 year.

i. Control Working Solution #4 (2.5 µg/mL):
Dilute 250 µL of the Mophine-β-glucuronide Stock Solution with 1:1 methanol:water to a final volume of 10 mL. Store in glass at <0ºC. Stable for 6 months.

j. Calibration Working Solution #1 (20 or 4 µg/mL, depending on component):
Mix 1.0 mL each of the Morphine and Codeine Stock Solutions with 200 µL each of the Hydromorphone, Hydrocodone, Oxymorphone, and Oxycodone Stock Solutions and

dilute with 1:1 methanol:water to a final volume of 50 mL. Store in glass at <0°C. Stable for at least 1 year.

k. Calibration Working Solution #2 (1 or 0.2 µg/mL, depending on component):
Dilute 2.5 mL of the Calibration Working Solution #1 to 50 mL with 1:1 methanol:water. Store in glass at <0°C. Stable for at least 1 year.

l. Calibration Working Solution #3 (2.5 µg/mL):
Dilute 125 µL of the 6-MAM Stock Solution with acetonitrile to a final volume of 50 mL. Store in glass at <0°C. Stable for 6 months.

m. Calibration Working Solution #4 (0.5 µg/mL):
Dilute 10 mL of the Calibration Working Solution #3 with acetonitrile to a final volume of 50 mL. Store in glass at <0°C. Stable for 6 months.

Table 1: Blood Calibrator Preparation

Volume of Matrix (mL)	Volume of Cal Solution #1 (µL)	Volume of Cal Solution #2 (µL)	Volume of Cal Solution #3 (µL)*	Volume of Cal Solution #4 (µL)*
Level 1 – 25 ng/mL morphine and codeine, 5 ng/mL for all others				
0.95	0	25**	0	10
Level 2 – 50 ng/mL morphine and codeine, 10 ng/mL for all others				
0.95	0	50	0	20
Level 3 – 100 ng/mL morphine and codeine, 20 ng/mL for all others				
0.85	0	100	0	40
Level 4 – 300 ng/mL morphine and codeine, 40 ng/mL 6-MAM, 60 ng/mL for all others				
0.95	15	0	16	0
Level 5 – 500 ng/mL morphine and codeine, 60 ng/mL 6-MAM, 100 ng/mL for all others				
0.95	25	0	24	0
Level 6 – 700 ng/mL morphine and codeine, 80 ng/mL 6-MAM, 140 ng/mL for all others				
0.95	35	0	32	0
Level 7 – 1000 ng/mL morphine and codeine, 100 ng/mL 6-MAM, 200 ng/mL for all others				
0.90	50	0	40	0

* - Calibration solutions #3 and #4 should not be added to samples that will be subjected to hydrolysis.
** - This calibrator will be outside the linear range for hydromorphone.

n. Negative Control: Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Store refrigerated or obtain fresh. Stability determined by manufacturer. A Negative Control will be extracted and analyzed with every assay. When possible, the negative control will be matrix matched.

When samples are analyzed in a batch using hydrolysis, a Negative Control will be hydrolyzed, extracted and analyzed.

o. Positive Control: These are normally prepared in in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as circumstances dictate. Storage and stability determined by manufacturer. Normally prepared by adding the amounts of Control Working Solution to 1 mL matrix as directed in Table 1 below. Quantitative controls are typically prepared in duplicate. When possible, the Positive Control will be matrix matched. Additionally, deuterated analog internal standards serve as a qualitative positive control for each individual specimen.

Table 2: Opiate Control Preparation

Volume of Control Solution #1 (µL)	Volume of Control Solution #2 (µL)*	Volume of Control Solution #3 (µL)*	Volume of Control Solution #4 (µL)
Qualitative Blood or Urine Control (245 ng/mL morphine and codeine, 49 ng/mL for all other target analytes)			
70	49	49	0
Low Quantitative Blood Control (70 ng/mL morphine and codeine, 15 ng/mL for 6-MAM, and 14 ng/mL all other quantitated analytes)			
20	15	0	0
High Quantitative Blood Control (770 ng/mL morphine and codeine, 80 ng/mL 6-MAM, 154 ng/mL for all other quantitated analytes)			
220	80	0	0
Hydrolysis Control (250 ng/mL morphine-glucuronide = 154 ng/mL morphine)**			
0	0	0	100

* - Control solution #2 should not be added to samples that will be subjected to hydrolysis.
** - The Hydrolysis Control is analyzed whenever hydrolysis is performed on case specimens to ensure that the enzyme is working properly.

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

a. Measure out 1 mL of biofluid or 2 g of a 1:1 tissue homogenate into a labeled 16 x 125

mm screw-top test tube. For quantitation, case samples and Positive Controls are typically analyzed in duplicate. When performing hydrolysis on case samples, a set of hydrolyzed Negative and Positive Controls will be analyzed. (Ensure that no 6-MAM is added to Positive Controls when performing hydrolysis.)

b. Add 50 µL of the Internal Standards Working Solution to the specimen and vortex.[2]

c. For "total" opiate assays: Enzymatically hydrolyze the sample by adjusting the pH to approximately 5.2 with 1 mL of 1.1 M sodium acetate buffer coupled with the addition of 30 µL of β-glucuronidase. Vortex. Incubate overnight at approximately 37°C.

d. For "free" opiate assays: Add 25 µL of the d_3-6-MAM Working Solution and 1 mL of deionized water and vortex.

e. Add 4 mL of 0.1 M phosphate buffer and vortex. Verify that the pH is between 5.5 and 6.5.

f. For blood and tissue specimens: Centrifuge at high speed for 15 minutes. Transfer supernatant to a clean 16 x 100 mm culture tube, leaving solid material behind.

g. Pre-rinse SPE extraction cartridge by adding 3 mL of methanol (HPLC grade) at 1 mL/minute.

h. Condition cartridge with 3 mL of deionized water followed by 1 mL of 0.1 M phosphate buffer at 1 mL/minute. Do not allow sorbent to dry.

i. Load sample on SPE cartridge at 1-2 mL/minute. Do not allow sorbent to dry.

j. Wash cartridge with 3 mL of deionized water, 1 mL of 0.1 M acetic acid, and 3 mL of methanol (Optima grade) (each at 1-2 mL/minute).

k. Dry cartridge under full vacuum for 3 minutes.

l. Apply 3 mL of SPE Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm culture tubes.

m. Evaporate to dryness under nitrogen at 40EC.

n. Reconstitute the dry residue in 100 µL of reconstitution solvent (5:95 methanol:water) and analyze 5 µL portions by LC-electrospray-tandem MS with the conditions given in

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[2]Other internal standards may be substituted at relevant concentrations if deemed appropriate.

section 10. Be sure to analyze an injection of a solvent blank under the column wash conditions specified in Section 10.3 of this procedure at least every 15 analytical injections.

9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
2: 5:95:0.05 10 mM formate : acetonitrile : formic acid	total flow	0.25 mL/min		type	Phenyl (Xterra)
	time (min)	%2	%1	length	15 cm
1: 95:5 0.05 10 mM formate : acetonitrile : formic acid	0	0	100	internal diameter	2.1 mm
	2	0	100	particle size	5 µm
	6	20	80	temperature	30ºC
	10	20	80		
	11	60	40		
	16	60	40		
	17	0	100		
	25	0	100		
	total time	25 min			

9.2 Mass Spectrometer Parameters

<table>
<tr><th colspan="3">Source Parameters</th></tr>
<tr><td>Mode: Electrospray</td><td>Spray Voltage: +5 kV</td><td>Capillary Temperature: 225°C</td></tr>
<tr><td>Sheath Gas: 25 (arb units)</td><td>Aux Gas: 12 (arb units)</td><td>Sweep Gas: 0 (arb units)</td></tr>
<tr><td colspan="3">All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.</td></tr>
<tr><th colspan="3">Segment #1 (0-2 minutes) (1 scan event)</th></tr>
<tr><td>Event #1</td><td colspan="2">full scan m/z 200 – 370, 7500 resolution (minimum)</td></tr>
<tr><th colspan="3">Segment #2 (2-6.5 minutes) (3 scan events)</th></tr>
<tr><td>Event #1</td><td colspan="2">full scan m/z 200 - 370, 7500 resolution (minimum)</td></tr>
<tr><td rowspan="3">Event #2</td><td>MS/MS data dependant scan (unit resolution)</td><td>collision energy: 30% (rel)</td></tr>
<tr><td colspan="2">precursor: most intense of m/z 272.13, 284.13, 286.14, 288.16</td></tr>
<tr><td>isolation width: 2.0 AMU</td><td>scan range: software control</td></tr>
<tr><td rowspan="3">Event #3</td><td>MS^3 product scan (unit resolution)</td><td>collision energy: see below</td></tr>
<tr><td colspan="2">precursor: m/z 302.2 (CE = 30%) > m/z 284.2 (CE = 30%)</td></tr>
<tr><td>isolation width: 2.0 AMU</td><td>scan range: m/z 75-320</td></tr>
<tr><th colspan="3">Segment #3 (6.5-15 minutes) (4 scan events)</th></tr>
<tr><td>Event #1</td><td colspan="2">full scan m/z 200 - 370, 7500 resolution (minimum)</td></tr>
<tr><td rowspan="3">Event #2</td><td>MS/MS data dependant scan (unit resolution)</td><td>collision energy: 25% (rel)</td></tr>
<tr><td colspan="2">precursor: most intense of m/z 284.13, 286.14, 298.14, 300.16, 302.18, 328.15</td></tr>
<tr><td>isolation width: 2.0 AMU</td><td>scan range: software control</td></tr>
<tr><td rowspan="3">Event #3</td><td>MS^3 product scan (unit resolution)</td><td>collision energy: see below</td></tr>
<tr><td colspan="2">precursor: m/z 316.2 (CE = 25%) > m/z 298.2 (CE = 35%)</td></tr>
<tr><td>isolation width: 2.0 AMU</td><td>scan range: m/z 80-330</td></tr>
<tr><td rowspan="3">Event #4</td><td>MS^3 product scan (unit resolution)</td><td>collision energy: see below</td></tr>
<tr><td colspan="2">precursor: m/z 302.2 (CE = 30%) > m/z 284.2 (CE = 30%)</td></tr>
<tr><td>isolation width: 2.0 AMU</td><td>scan range: m/z 75-320</td></tr>
<tr><th colspan="3">Segment #4 (15-25 minutes) (1 scan event)</th></tr>
<tr><td>Event #1</td><td colspan="2">full scan m/z 200 – 370, 7500 resolution (minimum)</td></tr>
</table>

9.3 Column Washing – At least once every 15 injections, the column will be washed under the following conditions to keep the analytical column in good working order.

Mobile Phase Compositions	Flow Parameters			Column Parameters	
A: 5:95:0.05 10 mM formate : acetonitrile : formic acid	total flow	0.25 mL/min		type	Phenyl (Xterra)
	time (min)	%A	%B	length	15 cm
B: 95:5:0.05 10 mM formate : acetonitrile : formic acid	0	0	100	internal diameter	2.1 mm
	1	0	100	particle size	5 µm
Mass Spectrometer	4	90	10	temperature	30°C
As above, but only one segment with one scan event throughout the analysis: full scan from m/z 200 to m/z 370.	14	90	10		
	17	0	100		
	25	0	100		
	total time	25 min			

10 Decision Criteria

10.1 Batch Acceptance Criteria

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as those analytes that will be reported for this batch.

All intended analytes should be present in the Positive Control. Each Quantitative Positive Control shall quantitate within ±20% of the target value. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for more information.

10.2 Sample Acceptance Criteria

10.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.2.1.1 Retention Time

The retention time of the peak should be within ± 2% of the retention time (relative or absolute,

as appropriate) obtained from injection of a reference standard, calibrator, or Positive Control.

Table 3: Retention Time Data

Compound Name	RRT (to d_3-morphine)
d_3-Morphine	RT ~ 4 min
Morphine	1.0
Codeine	2.0
6-MAM	2.1
Oxycodone	2.1
Oxymorphone	1.1
Hydrocodone	2.2
Hydromorphone	1.4
Normorphine	0.8
Norcodeine	2.0
Noroxycodone	2.1
Dihydromorphine	0.9
Dihydrocodeine	2.0

Note: Norhydrocodone (M+H 286.144) elutes with a RRT of 2.3.

10.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

10.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance. Mass spectral fragments of commonly encountered opioids are listed in Table 4. Under the listing of preferred tandem MS product ions, the normal base peak is listed in bold text. Other significant ions may be substituted for preferred ions if uncorrectable interference exists for that ion.

Table 4: Mass Spectrometry Data:

Compound Name	Quantitation Ion(s) from Full Scan MS	Precursor Ion for MS^2 or Precursor Chain for MS^3	Preferred Tandem MS Product Ions
Morphine	286.144	286.14	183, **201**, 211
d_3-Morphine	289.163	NA	NA
Codeine	300.159	300.16	**215**, 225, 282
d_6-Codeine	306.197	NA	NA
6-MAM	328.154	328.15	193, **211**, 268
d_3-6-MAM	331.173	NA	NA
Oxycodone	316.154, 298.144*	316.2 >298.2*	187, 241, **256**
d_6-Oxycodone	322.192, 304.151*	NA	NA
Oxymorphone	302.139	302.2 > 284.2	199, 227, **242**
d_3-Oxymorphone	305.158	NA	NA
Hydrocodone	300.159	300.16	**199**, 241, 257
d_3-Hydrocodone	303.178	NA	NA
Hydromorphone	286.144	286.14	**185**, 227, 243
d_3-Hydromorphone	289.163	NA	NA
Normorphine	NA	272.13	201, 229, **254**
Norcodeine	NA	286.14	215, 225, **268**
Noroxycodone	NA	302.2 > 284.2*	187, **229** **
Dihydromorphine	NA	288.16	**187**, 213, 231
Dihydrocodeine	NA	302.18	**201**, 227, **245** ***

* Oxycodone and noroxycodone both show large (M-18) fragments in their full scan mass spectra, with significant variation in the ion ratio dependant upon concentration. The instrument method is set to acquire MS/MS spectra of these fragments in addition to the MS^3 spectra of the pseudomolecular ion in case the pseudomolecular precursor is too weak to provide good spectral fidelity. The MS/MS and MS^3 spectra are qualitatively similar, but show different ion ratios.
** Noroxycodone normally yields only two fragment ions of reasonable intensity in MS^3 analysis. A criterion of no other ions present at >15% of the base peak may be used as additional criteria for the presence of this compound.
*** Either m/z 201 or m/z 245 may be the base peak for MS/MS of dihydrocodeine, depending upon the specific sample.

11 Calculations

Linear regression analysis with equal or 1/x weighting is performed for all analytes except codeine using a ±20 mmu extracted ion mass window in the full scan high resolution data. For codeine, calibration is performed using the quadratic log-log fit. See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

12 Measurement Uncertainty

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- precision of the pipette used to deliver the sample
- precision of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

13 Limitations

a. Method Performance Parameters:
LOD = Limit of Detection; LLOQ = Lower Limit of Quantitation

Compound	LOD in Blood (ng/mL)	LOD in Urine (ng/mL)	LLOQ (ng/mL)	Linear Range (ng/mL)	Accuracy (% bias)	Precision (% intermed)
Morphine	10	25	25	25-1000	-0.8	3.5 to 8.6
Codeine	5	10	25	25-1000	+16.5	4.5 to 10.6
Hydromorphone	5	10	10	10-200	-2.8	5.0 to 9.0
Hydrocodone	2	5	5	5-200	+1.6	4.8 to 7.2
Oxymorphone	2	5	5	5-200	-2.0	9.7 to 12.7
Oxycodone	1	2	5	5-200	-1.1	4.9 to 10.2
6-MAM	2	10	5	5-100	-13.5	4.3 to 6.8
Normorphine	5	10	*Not evaluated.*			
Norcodeine	10	10				
Noroxycodone	5	10				
Dihydromorphine	10	10				
Dihydrocodeine	5	5				

b. Interferences: Grossly decomposed or putrefied samples may affect both detection and quantitation limits. Very high levels of codeine (>1 µg/mL) may interfere with accurate quantitation of oxycodone, and very high levels of naltrexone may interfere with accurate quantitation of oxycodone. In none of these cases will qualitative identification be compromised. High levels of naloxone may interfere with detection and quantitation of hydromorphone, but would not yield false positive results. A compound that is present in many blank blood samples has shown to interfere with

the quantitation of oxycodone and oxymorphone at unit mass resolution, but this compound can be resolved using high resolution.

c. Other Considerations: The enzymatic hydrolysis procedure will convert a large fraction of any 6-MAM in a sample to free morphine. Appropriate care should be taken in interpreting total morphine concentration in any sample for which 6-MAM was detected in the free opioid analysis.

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

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Al-Asmari, A. I., Anderson, R. A., "Method for Quantification of Opioids and Their Metabolites in Autopsy Blood by Liquid Chromatography-Tandem Mass Spectrometry", *Journal of Analytical Toxicology*, v. 31, pp. 394-408, 2007.

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Guidelines for Toxicological Quantitations (Tox 101)**;** FBI Laboratory Chemistry Unit - Toxicology SOP Manual.

Instrument Support SOP Manual; FBI Laboratory Chemistry Unit.

Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology SOP Manual.

Rev. #	Issue Date	History
4	10/01/14	In sections 5 and 15, removed references to Tox 103. In 5.b, updated precolumn to prefilter. In Section 5, included recipes for reagents. In Section 5 (ee and ff) and Section 9.1, changed mobile phase designators from letters to numbers. In Section 6, combined standards into tables and renumbered rest of Section. Updated Positive Control scheme in Section 6 to cover far ends of the calibration curve and to include a Hydrolysis Control. Removed Calibration Section (Section 7) and renumbered subsequent sections. Moved calibrator preparation instructions to Section 6 (Table 1) and renames old Table 1 as Table 2. In 8.a and bench sheet, specified duplicate analysis for quantitation. In Section 11, added option for 1/x weighting. Reformatted Appendix 2 to include all pertinent instrumental parameters.
5	09/11/19	Updated Scope language. In section 5 (ee and ff) changed stability to 1 month. Added footnote to allow for preparation of different volumes of working solutions in Section 6. In 6.n., clarified when a Negative Control must be analyzed hydrolyzed. Updated qualitative control preparation instructions in Table 2. In Section 8 and bench sheet, clarified hydrolysis control analysis requirement. Updated codeine linearity calculations in Section 11. Removed references to “Subunit”.

Approval

Redacted - Signatures on File

Acting Toxicology Technical Leader: Date: 09/09/2019

Chemistry Unit Chief: Date: 09/09/2019

QA Approval

Quality Manager: Date: 09/09/2019

Appendix 1: Abbreviated version of the Solid Phase Extraction of Opioids from Biologicals with Analysis by LC-Tandem MS for bench use.

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Appendix 2: Abbreviated version of the Opioids LC-Tandem MS Instrumental Parameters for bench use.

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Appendix 2: Abbreviated version of the Opioids LC-Tandem MS Instrumental Parameters for bench use. (continued)

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