I. INTRODUCTION

Opioids include both natural opiate alkaloids (*e.g.,* morphine, codeine) as well as their synthetic derivatives (*e.g,.* hydromorphone, oxycodone, fentanyl). These drugs exert their effects through binding at the mu, kappa, and delta opioid receptors producing sedation and analgesia. Opioids are widely prescribed for pain management, but there is a high potential for abuse and dependence with continued use. In addition, their increasing presence in illicit drug markets makes these drugs of important forensic significance.

Samples that require identification/confirmation by LC-MS/MS are extracted from a buffered, diluted sample aliquot by adsorption onto a solid phase extraction column. Drugs that may be present are then eluted from the SPE column, dried, and reconstituted before injection onto the LC-MS/MS system.

The detection of each specific analyte is determined by single point calibration cut off for urines. Bloods are quantitated with a multipoint calibration using deuterated opioid internal standard(s).

Matrix-specific (blood and/or urine as needed) positive and negative controls are extracted and analyzed in each analytical batch. The presence of opioids or other pain management drugs may be confirmed in urine, blood or fluids.

A. Method Targets

Codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, 6-monoacetyl morphine (6-MAM), buprenorphine, norbuprenorphine, naloxone, fentanyl, and norfentanyl, tramadol, N-desmethyltramadol, and O-desmethyltramadol. Analytes may be added or removed, as necessary.

Compounds may be added or eliminated from this list upon toxicologist review.

B. Safety

This procedure is carried out in a laboratory environment and standard safety procedures should be utilized, including (minimally) safety glasses and lab coat when deemed necessary. Biological specimens subject to the analytical procedure should be handled using universal precautions. Potentially contaminated items and surfaces should be disinfected prior to and after use.

D. Specimen Requirements

1. 0.5 mL blood or urine

II. MATERIALS

A. Equipment

1. General laboratory glassware
2. Vortex, Thermolyne Maxi Mix 1 or equivalent
3. Sonicator, Fisher-Scientific or equivalent (for blood samples)
4. Centrifuge, Beckman TJ-6 or equivalent
5. Trace B Extraction Columns - SPEWARE (Baldwin Park, CA) ; (or equivalent)
6. SPEWARE CEREX System-48 Solid phase extraction manifold ; (or equivalent)
7. SPEWARE CEREX System-48 Sample Concentrator ; (or equivalent)
8. Shimadzu LC/MS/MS System (or equivalent):
    a. Degasser: Shimadzu DGU-20A
    b. Pumps: 2 Shimadzu LC-20AD Prominence
    c. Autosampler: Shimadzu SIL 20AC Prominence
    d. Column Oven: CTO-20A
    e. Pre-Column: SecurityGuard ULTRA Cartridge UHPLC Phenyl for 4.6mmID Columns (Phenomenex)
    f. Column: Kinetex Phenyl Hexyl (Phenomenex)
    g. Detector: Shimadzu LCMS-8030 Mass Spectrometer
    h. Controller: Shimadzu CBM-20A
    i. Data Station: Shimadzu LabSolutions software

B. Reagents available as stock items:

Sigma or J.T. Baker reagent grade or equivalent unless specified

1. Methanol (CH_3OH): Fisher Optimum LCMS Grade, Burdick Jackson pesticide grade, or equivalent
2. Deionized water (DIW): Milli-Q, LCMS grade, or equivalent
3. Drug-free urine or blood (Drug free blood or equivalent)

4. Formic Acid (HCOOH) ; Reagent Grade or equivalent
5. Ammonium formate (NH_4HCO_2) ; Reagent Grade or equivalent
6. Glacial acetic acid (CH_3COOH) ; Reagent Grade or equivalent
7. Sodium acetate trihydrate ($NaCH_3COO{\cdot}3H_2O$) ; Reagent Grade or equivalent
8. Sodium carbonate (Na_2CO_3) ; Reagent Grade or equivalent
9. Sodium bicarbonate ($NaHCO_3$) ; Reagent Grade or equivalent
10. UTAK LC-2 Control – UTAK Laboratories (Valencia, CA), or equivalent
11. UTAK Whole Blood Pain Management 100 Control – UTAK Laboratories (Valencia, CA), or equivalent
12. β-Glucuronidase (*P. vulgata* ; Sigma or equivalent)

C. Drug Standard Solutions – Cerilliant Corporation (Austin, TX)

Target Analytes	Concentration
Codeine	1.0 mg/mL
Morphine	1.0 mg/mL
Hydrocodone	1.0 mg/mL
Hydromorphone	1.0 mg/mL
Oxycodone	1.0 mg/mL
Oxymorphone	1.0 mg/mL
6-Monoacetylmorphine (6-MAM)	1.0 mg/mL
Buprenorphine	1.0 mg/mL
Norbuprenorphine	1.0 mg/mL
Naloxone	1.0 mg/mL
Fentanyl	1.0 mg/mL
Norfentanyl	1.0 mg/mL
Tramadol	1.0 mg/mL
N-desmethyltramadol	1.0 mg/mL
O-desmethyltramadol	1.0 mg/mL

D. Deuterated Drug Standard Solution – Cerilliant Corporation (Austin, TX)

Codeine-D3	1.0 mg/mL
Morphine-D3	1.0 mg/mL
Hydrocodone-D3	1.0 mg/mL
Hydromorphone-D3	1.0 mg/mL
Oxycodone-D3	1.0 mg/mL
Oxymorphone-D3	1.0 mg/mL
Buprenorphine-D4	0.1 mg/mL
Tramadol-C^{13}-D3	0.1 mg/mL

E. Drug Standard Solutions – Lipomed Inc. (Cambridge, MA)

Target Analytes	**Concentration**
Codeine	1.0 mg/mL
Morphine	1.0 mg/mL
Hydrocodone	1.0 mg/mL
Hydromorphone	1.0 mg/mL
Oxycodone	1.0 mg/mL
Oxymorphone	1.0 mg/mL
6-Monoacetylmorphine (6MAM)	1.0 mg/mL
Buprenorphine	1.0 mg/mL
Norbuprenorphine	1.0 mg/mL
Naloxone	1.0 mg/mL
Fentanyl	1.0 mg/mL
Norfentanyl	1.0 mg/mL
Tramadol	1.0 mg/mL
N-desmethyltramadol	1.0 mg/mL
O-desmethyltramadol	1.0 mg/mL

F. Reagents prepared in the Toxicology Laboratory

1. <u>5M Ammonium formate</u>:

a. Dissolve 3.15 g of ammonium formate in 10 mL volumetric flask.

b. Q.S. to 10 mL with DIW. Stable for one year.

2. **Mobile Phase A (H_20 with 5mM ammonium formate and 0.01% formic acid)**:
   a. Add 0.5 mL 5M Ammonium formate and 0.05 mL formic acid to a 500 mL volumetric flask.
   b. Q.S. to 500 mL with high purity water.
   c. Transfer solution to a glass bottle reserved for LC/MS use only. Stable for one week.
   d. The above instructions make 500 mL of mobile phase; adjust volumes of reagents accordingly if requiring a different final
   e. check pH when necessary

3. **0.1M Sodium Acetate buffer (pH ~4.5):**
   a. Combine 5.86 g of sodium acetate trihydrate and 3.24 mL glacial acetic acid in a 1000 mL stoppered graduated cylinder.
   b. Q.S. to 1000 mL with deionized water.
   c. Store in glass container at room temperature (25°C)
   d. check pH when necessary
   e. Stable for one year
   f. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

4. **1.0 M Acetate buffer (pH ~5.0)**
   a. To approximately 400 mL DIW in a graduated cylinder,
   b. Dissolve 42.9 g sodium acetate trihydrate in approximately 400 mL DIW
   c. Add 10.4 mL glacial acetic acid $C_2H_4O_2$
   d. Dilute to 500 mL with DIW
   e. Mix. Check pH, adjust pH to 5.0 ± 0.1 (if using pH meter- otherwise pH~5) with 1.0 M acetic acid
   f. Storage: room temperature in glass or plastic. Stability: 6 months
   g. Inspect daily for contamination.

5. **0.1 M Acetate Buffer (pH ~5.0)**

a. Dilute 20 mL 1.0 M acetate buffer to 200 mL with DIW

b. Mix. Store at: room temperature in glass or plastic. Stability: 6 months

6. <u>β -Glucuronidase, (5,000 F units/mL) in 0.1 M Acetate Buffer (pH ~5.0)</u>

Prepare daily for use, make slight excess for each batch, each 0.5 mL sample requires 1250 F units. Add 250 uL of β -Glucuronidase to each tube.

Example: for 40 total tubes prepare 10 mL
Calculate activity for each lot of β-Glucuronidase as follows:
(Lot specific, value from bottle label)
e.g. 1,439,000 -glucuronidase units/g solid
5,000 Units/mL = 1,439,000
x mg 1000 mg
1. x = 3.47 mg/mL

5,000 Units/mL = 1,439,000
x mg 1000 mg
x = 3.47 mg/mL

To make 10 mL

Weigh out 34.7 mg β –Glucuronidase solid. Add to 10 mL of 0.1 M acetate buffer (pH ~5.0)
Dissolve before use by swirling gently.
Make fresh daily as needed for each batch

7. <u>0.1M Sodium Carbonate (pH ~8.0):</u>

a. Add 5.3 g of sodium carbonate to a 500 mL volumetric flask.

b. Q.S. to 500 mL with deionized water.

c. Check pH; should be 8.0 ±0.2 if using pH meter- otherwise pH~8

d. Store in glass container at room temperature (25^{o}C)

e. Stable for one year

f. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

8. 0.1M Sodium Bicarbonate (pH ~11.0):

a. Add 4.2 g of sodium bicarbonate to a **separate** 500 volumetric flask.

b. Q.S. to 500 mL with deionized water.

c. Check pH. Should be around 11.0 ±0.2 (if using pH meter- otherwise pH~11).

d. Store in glass container at room temperature (25^{o}C)

e. Stable for one year

f. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

g. check pH when necessary

9. Bicarbonate Buffer pH~9.0: Mixture of 0.1 M Sodium Carbonate and 0.1 M Bicarbonate Solutions

a. Into a beaker containing the 0.1M Sodium Carbonate solution (lower pH solution)

b. Check pH. Adjust with the 0.1M Sodium Bicarbonate solution (higher pH solution) until a pH of 9.0 ± 0.2 is reached (if using pH meter- otherwise pH~9).

c. Store in glass container at room temperature (25^{o}C)

d. Stable for one year

e. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

10. Reconstitution Mixture: 20% Methanol in Deionized Water

a. Into a 100 mL graduated cylinder with a cap, add 80 mL of deionized water.

b. Add 20 mL methanol.

c. Cap and shake. Store at room temperature; stable for 6 months.

d. Before use check for clarity; if cloudy, discard and prepare fresh.

III. PREPARATION OF STANDARDS

A. <u>Cerilliant Opiate Stock Standard Mix</u>

Note: Equivalent reference standards may substituted if needed. Prepared calibrators and controls should be made from standards from different manufacturers.

1. Into a borosilicate glass screw-top culture tube, **add 100 µL** of the following 1mg/mL Cerilliant reference standards:

Hydrocodone	Hydromorphone	Oxycodone	Oxymorphone	6-MAM

2. Into the same culture tube**, add 50 µL** of the following 1mg/mL Cerilliant reference standards:

Buprenorphine	Norbuprenorphine	Fentanyl	Norfentanyl

3. Add 300 µL methanol
4. Store in freezer (e.g., ≤ -10^{o}C)
5. Stable for 6 months when tightly capped.
7. Final concentrations:

100 µg/mL- Hydrocodone, Hydromorphone Oxycodone, Oxymorphone, 6-MAM

50 µg/mL - Buprenorphine, Norbuprenorphine, Fentanyl, Norfentanyl

B. <u>Cerilliant Opiate Working Standard</u>

1. Into a 10 mL volumetric flask, **add 20 µL** of the following 1 mg/mL Cerilliant reference standards:

Codeine	Morphine	Tramadol	N-desmethyl-tramadol	O-desmethyl-tramadol

2. Add 40 uL of mixed <u>Cerilliant Opiate Stock Standard</u>.

3. Q.S. to 10 mL with DI water. Protect from light, make fresh with each run.
4. *Final concentrations:*

2000 ng/mL – Codeine, Morphine, Tramadol, N-desmethyltramadol, O-desmethyltramadol

400 ng/mL – Oxycodone, Oxymorphone, Hydrocodone, Hydromorphone

200 ng/mL – Buprenorphine, Norbuprenorphine, Fentanyl, Norfentanyl

C. <u>Working Internal Standard Mix</u>

1. To a 50 mL volumetric flask, add 100 µL of each 1 mg/mL Cerilliant reference deuterated opiates.
2. Add 250 µL of each 100 µg/mL deuterated standard.
3. 3. Q.S. to 50 mL with methanol.
4. Store in freezer (e.g., ≤ -10°C)
5. Stable for 6 months when tightly capped.
6. Final concentrations:

2.0 µg/mL- Codeine-D3, Morphine D3, Oxycodone-D3, Oxymorphone-D3, Hydrocodone-D3, Hydromorphone-D3

500 ng/mL- Buprenorphine-D4, Tramadol-C^{13}-D3

IV. PREPARATION OF CONTROLS

Note: Alternative controls, both commercial and in-house, to those listed below may be employed at the analyst's discretion.

A. **<u>In-House Stock Control (Lipomed and/or Grace)</u>**

1. Into a borosilicate glass screw-top culture tube, **add 100 µL** of the following 1mg/mL Lipomed/Grace reference standards:

Hydrocodone	Hydromorphone	Oxycodone	Oxymorphone	6-MAM

2. Into the same culture tube, **add 50 µL** of the following 1mg/mL Lipomed/Grace reference standards:

Buprenorphine	Norbuprenorphine	Fentanyl	Norfentanyl

3. Add 300 µL methanol
4. Store in freezer (e.g., ≤ -10°C)
5. Stable for 6 months when tightly capped.
6. Final concentrations:

100 ug/mL - Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone, 6-MAM

50 µg/mL- Buprenorphine, Norbuprenorphine, Fentanyl, Norfentanyl

B. **Opiate Working Control**

1. Into a 10 mL volumetric flask, **add 20 µL** of the following 1mg/mL Lipomed/Grace reference standards:

Codeine	Morphine	Tramadol	N-desmethyl-tramadol	O-desmethyl-tramadol

2. Add 40 uL of mixed In-House Stock Control (Lipomed/Grace).
3. Q.S. to 10 mL with DI water. Make fresh with each run.
4. Store in freezer (e.g., ≤ -10°C)
5. Stable for 6 months when tightly capped.

6. Final concentrations:

2000 ng/mL- Codeine, Morphine, Tramadol, N-desmethyltramadol O-desmethyltramadol

400 ng/mL- Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone, 6-MAM

200 ng/mL- Buprenorphine, Norbuprenorphine, Fentanyl, Norfentanyl

C. **External Controls**

1. External controls will come from UTAK or equivalent commercially prepared samples. Reconstitute UTAK dried whole blood controls with 5 mL DI water using a volumetric pipette or equivalent.

    a. Cap and let sit 10-15 minutes

    b. Gently swirl 3-4 minutes or mix on rotator until all particles are dissolved into a homogeneous mixture, swirl gently each time an aliquot is removed to ensure a homogeneous mixture.

D. **Negative Controls**

1. Negative control: Drug-free human urine or drug-free human blood.

V. PROCEDURE

A. Label clean screw cap tubes appropriately with blank, calibrator, control and case number designations.

B. Prepare calibrator and control samples, according to tables below. Note a new calibration curve is not required for each batch. Historical calibration curves can be used, the appropriateness of the curve will be demonstrated through the application of controls extracted with each batch of

samples. New calibration curves should be extracted when there have been substantial instrument changes (i.e. a new column installed).

a. Blood Calibrators

Standard Concentrations (ng/mL)			**Opiate Working Standard Volume to Add:**	**Blank Blood Volume to Add:**
Buprenorphine Norbuprenorphine Fentanyl Norfentanyl	**Hydrocodone Hydromorphone Oxycodone Oxymorphone 6-MAM**	**Codeine Morphine Tramadol N-desmethyltramadol O-desmethyltramadol**		
Blank			**0 µL**	**500 µL**
1	**2**	**10**	**2.5 µL**	**500 µL**
2	**4**	**20**	**5 µL**	**500 µL**
5	**10**	**50**	**12.5 µL**	**500 µL**
10	**20**	**100**	**25 µL**	**500 µL**
20	**40**	**200**	**50 µL**	**500 µL**
50	**100**	**500**	**125 µL**	**500 µL**
100	**200**	**1000**	**250 µL**	**500 µL**
200	**400**	**2000**	**500 µL**	**500 µL**

b. In-House Blood Controls

Control Concentrations (ng/mL)			**Opiate Working Control Volume to Add:**	**Blank Blood Volume to Add:**
Buprenorphine Norbuprenorphine Fentanyl Norfentanyl	**Hydrocodone Hydromorphone Oxycodone Oxymorphone**	**Codeine Morphine Tramadol N-desmethyltramadol**		

	6-MAM	O-desmethyltramadol		
5	**10**	**50**	**12.5 µL**	**500 µL**
20	**40**	**200**	**50 µL**	**500 µL**
100	**200**	**1000**	**250 µL**	**500 µL**

c. In-House Urine Control

Control Concentrations (ng/mL)			**Opiate Working Control Volume To Add:**	**Blank Urine Volume to Add:**
Buprenorphine Norbuprenorphine Fentanyl Norfentanyl	**Hydrocodone Hydromorphone Oxycodone Oxymorphone 6-MAM**	**Codeine Morphine Tramadol N-desmethyltramadol O-desmethyltramadol**		
5	**10**	**50**	**12.5 µL**	**500 µL**

d. Commercial Controls (optional)

Control Concentration	**Pipette Volume To Add:**	**Blank Matrix Volume to Add:**
UTAK PMWB dilution x4	125 µL	375 µL
UTAK PMWB	500 µL	0 µL

C. **Blood and Urine sample preparations**

1. Add 0.5 mL case specimen, blood or urine, to appropriate labeled tubes.
2. Add 100 µL of deuterated IS mix to each tube.

Urine Total conjugated and unconjugated

Note: All urines do not need hydrolysis, on a case by case basis the analyst may decide which samples to test for total and free analytes.

Add 250 µL of β –Glucuronidase in 0.1M acetate buffer, pH 5.0 to hydrolyze urine

Heat for 3 hours at ~60^{o}C in water bath. Cool tubes to room temperature.

3. Add 1 mL of 0.10 M sodium acetate buffer (pH ~4.5) to each tube.

a. **Blood sample preparations:** Add 500 µL DI water to each blood tube.

   Note: Adding 500uL DI water to urine samples is not detrimental.

4. Cap tubes, then vortex each tube for 10 seconds.

   Blood sample preparations: Sonicate blood samples for ~15 minutes.

   Note: Sonicating urine samples is not detrimental and may or may not be done.

5. Centrifuge **all** tubes for ~8 min at about 5200 rpm.

6. Label SPE columns to correspond with each screw-top culture tube.

7. Place labeled Trace B extraction columns in the SPE column rack in the appropriate order. Position plastic waste tray labeled “Methanol” underneath SPE column rack.

8. Condition each column sequentially with 1 mL methanol ; drain (≈3 psi) to Solvent “Hazardous Waste” stream

9. Remove plastic waste tray labeled “Methanol” and replace with plastic tray labeled “Biohazardous/Buffers” ; 1 mL DI water ; drain (≈ 3 psi) to “Non-Hazardous” regulated waste stream

10. Carefully transfer the sample to the center of the SPE column. Avoid splashing and/or transferring any sediment found at the bottom of the tube.

11. Wash each SPE column sequentially with:

    a. 1 mL bicarbonate buffer (pH ~9.0); drain (≈ 3 psi) to non-hazardous regulated waste stream
    b. 1 mL DI water; drain (≈ 3 psi) to non-hazardous regulated waste stream

12. Dry the columns for 10 minutes using maximum pressure, between 60-80 psi.

13. During this 10 minute window (or earlier), label collection tubes and place in the appropriate position in the SPE collection rack underneath the corresponding SPE column; prepare the elution solvent.

14. The elution solvent ratio is 80: 18: 2 Dichloromethane: IPA: NH_4OH. (Adjust volume prepared for the number of samples, minimize excessive hazardous waste, 2 mL needed for each tube)

    Add the IPA and NH_4OH together first before adding the dichloromethane portion (following this order prevents unsafe buildup of gases).

15. After ~10 minutes, replace plastic waste tray with SPE collection rack containing labeled tube in order corresponding to SPE columns.

16. Elute column with two 1.0 mL aliquots of 80:18:2 Dichloromethane:IPA:NH_4OH into the appropriate autosampler vial. Flow at 2-4 mL/min to optimize recovery.

17. Remove top SPE column rack and transfer collection rack from SPE manifold to sample concentrator.

18. Evaporate all tubes to dryness at <40^{o} C.

19. Reconstitute each slotted screw cap vial with 150 µL or less of the starting mobile phase of 20% methanol in DI water. All calibrators, controls and samples must be reconstituted with the same volume of starting mobile phase. If the volume is different from 150 ul note the volume.

20. Individually transfer solutions to properly labeled auto sampler vials containing sample inserts.

21. Analyze by liquid chromatography/mass spectrometry (LC/MS).

VI. INSTRUMENT PARAMETERS (may need to be changed when necessary)

A. LC Parameters

1. Shimadzu Prominence LC-20 System

a. Flow	0.6 mL/min
b. Autosampler Temperature	15°C
c. Injection Volume	10 µL
d. Needle Wash	500 µL; before and after aspiration

e.	**Column**	Kinetex 2.6um Phenyl-Hexyl 100A 50 x 4.6 cm
		Manufactured by Phenomenex (Torrance, CA)
f.	**Oven Temperature**	40°C
g.	**Gradient**	

Time (min)	Mobile Phase B (Methanol)	Mobile Phase A (0.01% Formic Acid in Water)
Initial	5%	95%
2.50	45%	55%
4.50	45%	55%
5.50	95%	5%
7.50	95%	5%
7.51	5%	95%
9.00	STOP	STOP

B. M/S Acquisition Parameters: The following conditions can be adjusted if needed based on availability of gases.

Interface	DUIS (APCI and ESI)
DL Temperature	250°C
Nebulizing Gas	2 L/min
Drying Gas	15 L/min

C. Transition Ions Monitored and Retention Times (Times are approximate and may vary)

Analytes may be added or deleted as needed.

Using the LabSolutions optimization software**,** the following transitions were identified:

Drug/Metabolite	Precursor Ion	Quantification Ion	Reference Ion(s)	Retention Time (min)
Codeine	299.7	58.1	165.1	3.05
Codeine D3	303.0	61.0	128.0	3.22
Morphine	286.1	165.1	181.1	2.30
Morphine D3	288.7	201.1	165.0	2.40
6-MAM	328.0	164.95	58.1	3.31
Oxycodone	315.9	298.15	241.1	3.28
Oxycodone D3	319.0	301.15	129.1	3.41

Oxymorphone	301.9	284.15	227.1	2.43
Oxymorphone D3	305.0	287.1	230.1	2.53
Hydrocodone	299.8	199.1	171.0	3.40
Hydrocodone D3	302.75	199.05	170.9	3.50
Hydromorphone	285.9	185.0	157.0	2.47
Hydromorphone D3	289.0	185.05	157.0	2.68
Buprenorphine	468.2	55.2	84.5	6.55
Buprenorphine D4	472.0	59.1	88.15	6.62
Norbuprenorphine	414.1	57.1	101.1	5.30
Fentanyl	336.75	188.15	105.1	6.28
Norfentanyl	233.1	84.1	55.0	3.93
Tramadol	264.0	58.15	30.20, 246.20	4.07
N-desmethyltramadol	250.15	44.00	232.30	4.23
O-desmethyltramadol	250.15	58.10	30.15	3.29
Tramadol-C^{13}-D3	268.20	58.10		4.05

D. **Detection and Identification**:

All chromatography, peak integrations, and transition ion ratios used for identifications will be reviewed.

Determination of the presence of target analytes in the sample extract are identified by appearance and ratio of product ions that are characteristic of each drug at the appropriate retention time. In this manner, both retention time (an LC characteristic) and fragmentation pattern and ratio (an MS characteristic) are used as the basis for qualitative identification. For the identification of an analyte to be made, the retention time of the chromatographic peak should be within 0.1 minute of the corresponding analyte in the calibrator sample and the ion ratios should be within certain limits (see the following table). Initially the 100 ng/mL Cerilliant Standard sample can be utilized to set the expected ion ratios, however it is recognized that some ion ratios are concentration dependent. As such, ratios may be set on a case by case basis using a standard with a concentration close to the concentration of the analyte of interest in the case.

Expected (Set) Ion Ratio	**Allowance**
> 50%	20%

20-50%	25%
< 20%	30%

E. **Calibration**:

Calibration is accomplished by the addition of a known amount of analytes (in addition to the internal standard) into a blank sample of the matrix that is tested. The response of the system to this calibration defines the standard curve that is used as the basis for the quantitative calculations for controls and samples. The system for blood samples is "multi-point calibration, multi-point control."

While a calibration curve for each drug and metabolite may be analyzed with each batch, a previously established (or "historical") calibration curve may be used. The calibration correlation coefficient must be ≥ 0.990 when using deuterated internal standards. When using non-deuterated internal standards a correlation coefficient ≥ 0.98 is acceptable. A calibrator may be removed to attain a correlation coefficient of ≥ 0.990. Samples resulting in concentrations higher than the highest calibrator should be diluted (approval not to dilute must be obtained by the Unit Lead or higher). The lower limit of quantitation (LOQ) is the concentration of the lowest calibrator. The urine control will be quantitated based on the blood calibration curve.

F. Quantitation:

Quantitation is accomplished by the comparison of the response ratio of the analyte and the internal standard in a specific sample relative to the response ratios of the calibration curve. The concentration of the analyte in the sample is then extrapolated from the standard curve.

VII. RESULTS INTERPRETATION

Positive results will be reported only when:

1. Analyte identification is based on at least two transitions with relative abundances within +/- 20 % of the target, relative to a calibrator.
2. Retention times are within 0.1 min, or +/- 3% relative to a calibrator analyzed in same batch.
3. Qualitative results have at least a 3x signal to noise (S/N) ratio.
4. The integration of the analyte peak has acceptable symmetrical shape and chromatography.
5. All MRMs show peaks at the appropriate retention times.

6. Quality control sample results are acceptable.

VIII. QUALITY CONTROL

A. Criteria for Quantitative Results

1. Statistics will be maintained on all controls and evaluated.
2. Results of controls must fall within ±20% of an analyte's target concentration.
3. All results are recorded.

B. Verification of Vial Sequence: The vial sequence is checked prior to and following injection of samples. These checks will be documented.

C. Evaluation of Potential Carryover: Potential carryover will be determined by a blank sample being analyzed after the highest calibrator. In addition, a solvent blank will be run after each sample to ensure there is no carryover in-between samples.

D. Linearity: Linearity of the calibration curve is demonstrated as a function of the correlation coefficient (r^2) and based on the quantitative results of controls.

E. Sensitivity (LOD, LOQ): For the purposes of this procedure the limit of quantitation (LOQ) is defined as equal to the lowest concentration of the lowest calibrator. The Limit of Detection (LOD) must have a response of at least 3 times the signal to noise ratio and have acceptable ion ratios.

F. Specificity: Specificity is a function of both the resolution of target analyte during the analytical process and the mass spectral fragmentation that analyte molecules undergo during the instrumental analysis.

IX. QUALITY ASSURANCE

Quality Assurance is provided by the following multi-layer program:

1. The LC/MS analysis is thoroughly checked by the instrument operator, including vial position on the auto sampler, prior to and following the injection of samples.
2. The LC/MS data is reviewed and includes an evaluation of the following:
    a. Positive and Negative Controls

b. Chromatographic Characteristics

c. Transcription Errors

3. Reported results are checked against the supporting data.

X. SOURCES OF ERROR

It has been established that no known interferences are present in the calibrators/controls. Ion suppression or enhancement and potential interferences from other analytes have not been found for the common drugs and metabolites typically seen in casework. Both ante mortem and post mortem samples seem to provide comparable results, but matrix effects may occur within some samples and may be a source of error.

XI. References

A. A Comparison of the Validity of Gas Chromatography-Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry Analysis of Urine Samples for Morphine, Codeine, 6-Acetylmorphine, and Benzoylecgonine; Peter R. Stout, Nichole D. Bynum, John M. Mitchell, Michael R. Baylor and Jeri D. Ropero-Miller; J.Anal Toxicol (2009) 33 (8): 398-408

B. Opiates. Connecticut Division of Scientific Services: Toxicology Laboratory.

C. Solid-Phase Extraction Method for Trace B Columns. SPEWARE: Baldwin Park, CA.

Revision #	History
4	Slight grammatical changes throughout document. Added approximates throughout document where necessary Added to section 1.A. Added 'or equivalent' to 'Materials' section. Updated sections II.F.2, II.F.3, II.F.4, II.F.5, II.F.6, , II.F.7,, II.F.8, and II.F.9 regarding pH checks. Updated III.A.4, III.C.4, IV.A.4, IV.B.4, and V.C regarding temperatures. Updated table in V.B. regarding how much blank blood volume to add. Updated V.C.20 regarding injection volume. Updated VI.D regarding identification criteria. Updated VI.E, VII, VIII.C, VIII.D, IX, and X. Added History section to document. Updated section E. Calibration to remove the need to check calibrators against the generated calibration curve. Section V. changed auto sampler vial to collection tube in multiple locations in section. (V.19) Changed the volume of reconstitution solution from 520 ul to 150 ul. Added line 20 Individually transfer solutions to properly labeled auto sampler vials containing sample inserts.