1. PROTOCOL FOR THE ANALYSIS OF OPIATES IN URINE

PURPOSE

The following method describes the confirmation of methadone, codeine, morphine, 6-acetylmorphine, hydrocodone, hydromorphone, oxymorphone and oxycodone in urine by GC/MS. Samples which have been screened positive by a preliminary test, as well as special requests or retest requests will follow the following protocol. Additionally this protocol may be used as a screening method.

PLAN

A. Equipment:

(1) GC/MS with a 5% diphenylpolysiloxane, 95% dimethylpolysiloxane, 15/30 meter, 0.25 micron film thickness column

(2) Positive Pressure Manifold

(3) SPE Column - Polymeric bead-Copolymeric bonded phase with a hydrophobic cation exchanger (UCT-SSDBX033)

(4) Heating block

(5) Sample concentrator with UHP Nitrogen

(6) Centrifuge

(7) Water bath

B. Reagents:

(1) **100mM Phosphate buffer solution**. Dissolve 1.70 grams of Na_2HPO_4 and 12.14 g $NaH_2PO_4 \cdot H_2O$ in 800 ml of deionized water. Dilute to 1000 ml with deionized water. Mix well. pH should be 6.0. If necessary, adjust with 100 mM monobasic sodium phosphate (lowers pH) or 100 mM dibasic sodium phosphate (raises pH). Store refrigerated. Stable for six months.

(2) **Methanol**. Prepare a transfer bottle of ACS/HPLC grade methanol. Label accordingly. Store in glass at room temperature. Stable until consumed.

(3) **Acetonitrile.** Prepare a transfer bottle of ACS/HPLC grade acetonitrile. Label accordingly. Store in glass at room temperature. Stable until consumed.

(4) **100mM Hydrochloric Acid (HCL).** To 400 ml of deionized water, add 8.4 ml concentrated HCl. Dilute to 1L with deionized water. Mix well. Store at room temperature. Stable for 2 years.

(5) **78:20:2 methylene chloride: isopropanol: ammonium hydroxide solution**. Add ammonia hydroxide to isopropanol mix, followed by methylene chloride. Prepare fresh daily.

(6) **Pyridine**. Label. Store at room temperature. Stable until consumed.

(7) **Propionic anhydride**. Label. Store at room temperature. Stable until consumed.

(8) **Deionized Water** (DI Water). Stable until consumed.

(9) **Ethyl acetate**. Prepare a transfer bottle of ACS/HPLC grade ethyl acetate. Label accordingly. Store in glass at room temperature. Stable until consumed.

(10)**Abalone β-glucuronidase enzyme (>50,000 units/mL) and Hydrolysis Buffer solution.** Purchased from United Chemical Technologies (UCT) or equivalent. These solutions are kept separate and pipetted separately into case samples and quality controls on the day of use. Stable for one year.

C. Standards: (Store refrigerated. Stable 2 years if prepared in house or per manufacturer's recommendation)

(1) **1 mg/ml codeine stock standard**. Prepare by weighing out 10.0 mg codeine free base and dissolving in 10 ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant C-006).

(2) **1 mg/ml morphine stock standard**. Prepare by weighing out 10.0 mg morphine free base and dissolve in 10 ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant M-005).

(3) **1 mg/ml hydrocodone stock standard**. Prepare by weighing out 10.0 mg hydrocodone free base and dissolve in 10 ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant H-003).

(4) **1 mg/ml hydromorphone stock standard**. Prepare by weighing out 10.0 mg hydromorphone free base and dissolve in 10 ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant H-004).

(5) **1 mg/ml oxycodone stock standard**. Prepare by weighing out 10.0 mg oxycodone free base and dissolve in 10 ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant O-002).

(6) **1 mg/ml oxymorphone stock standard**. Prepare by weighing out 10.0 mg oxymorphone free base and dissolve in 10 ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant O-004).

(7) **1 mg/ml 6-acetylmorphine (6-AM) stock standard**. Purchase a 1mg/ml ampoule (Cerilliant A-009).

(8) **1 mg/ml methadone stock standard**. Prepare by weighing out 10.0 mg methadone free base and dissolve in 10ml of methanol. Or, purchase a 1mg/ml ampoule (Cerilliant M-007).

(9) **100 µg/ml D3-codeine stock internal standard**. Purchase an ampoule of D3-codeine (Cerilliant C-005).

(10)**100 µg/ml D3-morphine stock internal standard**. Purchase an ampoule of D3-morphine (Cerilliant M-003).

(11)**100 µg/ml D3-6-acetylmorphine stock internal standard**. Purchase an ampoule of D3-6-acetylmorphine (Cerilliant A-006).

(12)**100 µg/ml D6-hydrocodone stock internal standard**. Purchase an ampoule of D6-hydrocodone (Cerilliant H-047).

(13)**100 µg/ml D6-oxycodone stock internal standard**. Purchase an ampoule of D6-oxycodone (Cerilliant O-007).

(14)**100 µg/ml D3-hydromorphone stock internal standard**. Purchase an ampoule of D6-hydromorphone (Cerilliant H-006).

(15)**100 µg/ml D3-oxymorphone stock internal standard**. Purchase an ampoule of D6-oxymorphone (Cerilliant O-003).

D. Calibrators and Internal Standard: (Store refrigerated. Stable for two years.)

(1) **10 ng/µl mix opiate calibrator stock solution in methanol**. In a 10 ml volumetric flask, add 100µl each of 1.0 mg/ml methadone, hydrocodone, hydromorphone, codeine, morphine, oxycodone, and oxymorphone stock standards. Dilute to volume with methanol.

(2) **2 ng/µl 6-acetylmorphine calibrator stock solution in methanol**. In a 10 ml volumetric flask, add 20µl of 1.0 mg/ml 6-acetylmorphine (6-AM) stock standard. Dilute to volume with acetonitrile.

(3) **10 ng/µl D3-codeine, D6-hydrocodone, D6-oxycodone, D3-morphine / 5 ng/µl D3-hydromorphone, D3-oxymorphone / 2 ng/µl D3-6-acetylmorphine internal standard**. In a 10 ml volumetric flask add 1.0 ml each of 100µg/ml D3-codeine, D6-hydrocodone, D6-oxycodone, and D3-morphine stock internal standards. Add 500 µl each of 100µg/ml D3-hydromorphone and D3-oxymorphone. Add 200 µl of 100µg/ml D3-6-acetylmorphine stock internal standard. Dilute to volume with methanol.

(4) Calibrators to be prepared by adding the appropriate volume of each calibrator stock solution as indicated below to 1mL negative urine:

OPIA/6-AM calibrator concentration (ng/mL)	10 ng/µl mix opiate calibrator stock (µl)	2 ng/µl 6-AM calibrator stock (µl)
150/50	15	25
300/100	30	50
1000/300	100	150

E. Quality Controls. (Store refrigerated.)

(1) **Positive Control. 360 ng/ml mixed opiate/ 120 ng/ml 6-AM control**. Prepared in house from a different lot of stock solution than that used to prepare calibrators or purchased from external vendor.

(2) **Negative Control**. Urine produced in house will be used as negative control.

F. Solid Phase Extraction (SPE):

(1) ***Sample Preparation***.
Prepare in appropriately labeled culture tubes as follows:

(a) Prepare a set of opiate calibrators above and pipette 1 ml of the positive control, negative control as well as samples. High samples may be diluted, as an example x10 by adding 100µl sample/ 900µl H_2O.

(OPTIONAL) Perform enzymatic hydrolysis on preliminary positive samples, positive hydrolysis control, and negative control by adding 100 µl of Abalone β-glucuronidase/ 400 µl of Hydrolysis Buffer solution to 1.0 ml of urine in labeled culture tubes, mix and incubate samples in a water bath at 50°C for a minimum of three hours. Remove from water bath and allow to cool.

(b) Add 50 µl of working internal standard to each tube.

(c) Add 1.0ml of 100mM phosphate buffer (pH 6.0) and vortex until thoroughly mixed.

Turbid samples should be centrifuged at 3500 rpm for 5 minutes prior to application.

(2) ***Sample Application***

Apply sample to column, being careful to not allow the sediment, if present, which will be in the base of the centrifuge tube to pass. Flow rate should be 1 - 2 ml / minute or under gravity

(3) ***Column Rinse and Elution***

Pass through the column sequentially the following reagents at <1.0 ml/min or by gravity only:

(a) 1 ml of deionized water

(b) 1 ml of 100mM HCl

(c) 1 ml methanol

(d) Dry column under full pressure for (≥15 inches Hg) for 5 minutes.

(e) Elute two times with 0.5 ml of freshly prepared 78:20:2 methylene chloride: isopropanol: ammonium hydroxide solution directly into appropriately labeled microvials.

(4) ***Derivatization***

(a) Evaporate samples to dryness under nitrogen.

(b) To the microvials add 25 µl of pyridine and 25 µl of propionic anhydride. Crimp using the red PTFE crimp caps. Vortex.

(c) Derivatize at 70°C for at least 20 min.

(d) When derivatization is complete, Remove cap and dry down under nitrogen.

(e) Reconstitute with 100 µl of ethyl acetate. Crimp cap microvials. Vortex.

G. Data Acquisition and Analysis:

(1) Make sure the Autotune was performed, rinse vials filled, etc.

(2) Set up a sequence with the calibrator(s) injected first in order to calibrate the instrument used. The ion ratios and retention times should be set by a mid-level calibrator. Subsequent injections to include positive and negative controls, and solvent blanks between case samples. For samples requiring dilutions add the appropriate sample multiplier in the sequence table.

(3) Analyze using the appropiate method on GC/MS.

H. Results and Acceptability (Qualitative):

(1) Calibration $R^2 \geq 0.97$ and calibrators within 20% of set value

(2) Positive control is positive (≥ 50 ng/ml for 6–acetylmorphine, ≥ 150 ng/ml for all other analytes)

(3) Negative control <LOD

(4) Retention time within 2% as set or stored from calibrator

(5) Qualifier ion ratios within 20% as set or stored from calibrator

(6) Chromatographically acceptable i.e. peak purity ≥90% for target ion

(7) Report 6–acetylmorphine as positive ≥ 50 ng/ml, report all other analytes as positive ≥ 150 ng/ml