1. PROTOCOL FOR THE ANALYSIS OF COCAINE AND ITS METABOLITES IN URINE

PURPOSE

The following method describes the confirmation of Cocaine, Cocaethylene and Benzoylecgonine 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 exchange (UCT-SSDBX033)

(4) Heating block

(5) Sample concentrator with UHP Nitrogen

(6) Centrifuge

(7) Vortex mixer/ Multi-tube vortex mixer

B. Reagents:

(1) **100 mM 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 5.5-6.0. 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 at room temperature in glass. Stable until consumed.

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

(4) **100 mM 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 Elution Solvent**. Prepare fresh daily. Add ammonium hydroxide to isopropanol, followed by methylene chloride (i.e. per 10 mL of elution solvent add approximately 200 µl ammonium hydroxide). Mix thoroughly, elution solvent should have a turbid appearance when thoroughly mixed.

(6) **HFIPA. (1,1,1,3,3,3 hexafluoroisopropanol)**. Label. Store at room temperature. Stable until consumed. Crimp cap with breakaway seal after use.

(7) **PFAA. (pentafluoropropionic acid anhydride) or PFPA (pentafluoropropionic anhydride)**. Label. Store at room temperature. Stable until consumed. Crimp cap with breakaway seal after use.

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

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

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

(1) **1 mg/ml cocaine stock standard**. Purchase a 1 mg/ml ampoule of cocaine (Cerilliant C-008).

(2) **1 mg/ml cocaethylene stock standard**. Weigh out 5 mg cocaethylene free base. Dilute to volume with acetonitrile in a 5 ml volumetric flask. Or, purchase a 1 mg/ml ampoule of cocaethylene (Cerilliant C-010).

(3) **1 mg/ml benzoylecgonine stock standard**. Purchase a 1 mg/ml ampoule of benzoylecgonine (Cerilliant B-004).

(4) **100 µg/ml D3-cocaine internal stock standard**. Purchase a 100 µg/ml ampoule of D3-cocaine (Cerilliant C-004).

(5) **100 µg/ml D3-benzoylecgonine internal stock standard** Purchase a 100 µg/ml ampoule of D3-benzoylecgonine (Cerilliant B-001).

D. Calibrators and Internal Standard: (Store refrigerated. Stable for 2 years)

(1) **10 ng/µl benzoylecgonine calibrator stock solution in methanol**. In a 10 ml volumetric flask add 100 µl of 1 mg/ml benzoylecgonine stock standard. Dilute to volume with methanol.

(2) **1 ng/µl cocaine/cocaethylene calibrator stock solution in acetonitrile**. In a 10 ml volumetric flask add 10 µl of 1 mg/ml cocaine stock standard and 10 µl of 1 mg/ml cocaethylene stock standard. Dilute to volume with acetonitrile.

(3) **2/10 ng/µl D3-cocaine/ D3-benzoylecgonine internal standard**. In a 10 ml vial add 200 µl of 100 µg/ml stock D3-cocaine internal standard and 1.0 ml of 100 µg/ml stock D3-benzoylecgonine internal standard and 8.8 ml of methanol.

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

COCN/CE/BE calibrator concentration (ng/mL)	1 ng/µl COCN/CE calibrator stock (µl)	10 ng/µl BE calibrator stock (µl)
50/50/150	50	15
100/100/500	100	50
200/200/1000	200	100

E. Quality Controls: (Store refrigerated.)

(1) **Positive Control**.**180/60/60 ng/ml benzoylecgonine/cocaine/cocaethylene** Prepared in house from a different lot of stock solution than that used to prepare calibrators or purchased from an external vendor. (Option to use in house preparation for control stock from high QC in TOX-SOP-35, using 25 µl will result in a control target value of 400/80/80 ng/ml benzoylecgonine/cocaine/cocaethylene.)

(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 calibrators above and pipette 1.0 ml each of the positive control, and negative control as well as case samples. Add 50 µL of working internal standard to each tube. (High concentration samples may be diluted, as an example x20 by adding 50 µL sample/ 950 µl H_2O.)

(b) Add 1.0 ml of phosphate buffer and vortex until thoroughly mixed.

(c) 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 by 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 100 mM 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) Add 25 µl of HFIPA and 50 µl of PFAA to each microvial, cap, crimp and vortex.

(c) Heat microvials at 70°C for at least 20 minutes.

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

(e) Reconstitute with 100 µl of ethyl acetate. Cap and crimp vials, 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. Subsequent injections to include positive and negative controls, and solvent blanks prior to each case sample. For samples requiring dilutions add the appropriate sample multiplier in the sequence table. Load samples onto autosampler according to sequence and have it verified by another analyst before or after analysis but prior to unloading.

(3) The ion ratios and retention times should be set by a mid level calibrator.

(4) Analyze using the appropriate 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 (≥ 50/50/150ng/ml cocaine/cocaethylene/benzoylecgonine)

(3) Negative control < 25% of area count of cutoff calibrator

(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) Blank prior to sample < 25% area count of cutoff calibrator

(8) Report cocaine/cocaethylene/benzoylecgonine as positive ≥ 50/50/150ng/ml, respectively