1.0 PRINCIPLE

Cocaine is rapidly metabolized in the body by hydrolysis of the methyl ester group, to form benzoylecgonine (BE) with a cocaine half life of ~90 min. Because of the rapid breakdown, BE is usually the major detectable product of cocaine ingestion in biological fluids. In many cases, the parent compound, cocaine, will not be detected at all. This procedure details the process by which the presence of cocaine and/or BE may be confirmed in urine, blood or other aqueous fluids. Cocaine or BE present in the sample is extracted onto a solid phase extraction column. Co-extracting materials are washed from the column, and extracted cocaine and/or BE is subsequently eluted. The eluent is evaporated, and any BE present is derivatized with BSTFA (N,O-bis trimethylsilyl trifluoroacetamide)/1% TCMS (Trimethylchlorosilane). The presence of any cocaine and/or derivatized BE in the sample is qualitatively evaluated by 3-ion SIM, using retention time and 2 qualitative ions ratios as the basis for identification, in comparison to co-extracted calibrator and control samples. Quantitative evaluation is made by single point calibration, using deuterated internal standards, and two control samples within the quantitive range.

2.0 SPECIMEN

Specimens requiring confirmation for BE/Cocaine are listed by lab case number on the clip board marked "BE-Cocaine List" which is maintained in the Toxicology Instrument room. Analysts preparing a batch for analysis should generate their batch sample list from this document (see form 23.4, appended).

2.1 All evidence transfers, either between individuals or between an individual and a storage location must be documented on the Chain of Custody for the case, either in the LIMS, or on hard-copy COC document maintained in the Case Jacket.

2.2 When not in the sampling or aliquot process, samples in the Toxicology section must be stored in a the secure and locked Toxicology evidence storage room.

2.3 Samples must be maintained in such a manner so that they are protected from contamination or deleterious change. Depending on the nature of the sample, this may mean refrigeration or freezing when not in the analytical process.

2.4 When analysis of samples in the toxicology section is complete, they must be maintained "Under Proper Seal." This is interpreted to mean that the sample, or a container in which the sample is kept, is sealed with tamper-evident tape, with the initials and date of person placing the seal clearly marked on, or proximate to

that seal.

2.5 Samples are maintained in the Toxicology Section for 8 weeks after case is completed, in the absence of notification of any legal action, or other reason to maintain the samples. After this period, samples are discarded in the appropriate medical waste disposal container. Sample from fatalities, or cases with requests for retention are maintained by the laboratory.

3.0 EQUIPMENT:

GC/MS and associated data station/computer (HP6890/5973 or equivalent)
General laboratory glassware and equipment
Solid phase extraction manifold and associated vacuum equipment
Analytical evaporator Zymark Turbovap or equivalent.
UCT; Z5DAU020 "Clean Screen" extraction columns

4.0 REAGENTS:

4.1 Reagents available as stock items:
Methanol (MeOH; Baker HPLC grade or better)
Deionized water (DIW; 2-megohm In-House supply)
Hydrochloric Acid (HCl; Baker Reagent Grade, or equivalent)
Isopropanol (IPA) (Baker HPLC grade or better)
Methylene Chloride (Baker HPLC grade or better)
Ammonium Hydroxide(NH_4OH; Baker Reagent Grade, or equivalent)
Ethyl acetate (ETOAc; Baker HPLC grade or better)
BSTFA with 1% TMCS
Blank Blood (Received from Hartford Hospital)
Sodium Phosphate Dibasic (Na2HPO4).
Sodium Phosphate Monobasic (NaH2PO4).
Drug free urine

4.2 Reagents prepared in the Toxicology Laboratory:

4.2.1 Methylene chloride/isopropanol/ammonium hydroxide (39/10/1mL = 50 mL total; or equivalent ratio; Prepared fresh each day of use)
4.2.2 0.1M phosphate buffer pH 6.0 (24.38 g NaHPO4 + 3.44 g Na2HPO4/2000 mL H2O)
4.2.3 0.1 M HCl (8.4 ml conc. HCl/1000 mL H2O)

Note: Reagent Preparation and Validation is documented in the Toxicology "Reagent Preparation/Validation Logbook" maintained in the Toxicology section. Validation is addressed in section 5 below.

4.3. Calibrators, Controls and Internal Standard:

Note: Preparation of all calibrator and control solutions is documented in the "Calibrator and Control Preparation Log" (maintained in the Toxicology Wet Laboratory)

4.3.1 BE internal standard (d3-benzoylecgonine) 10 ng/ul (Alltech; 1.0 mg/ml: 0.1 ml/10 ml MeOH)
4.3.2 BE Calibrator standard (benzoylecgonine) 10 ng/ul (Alltech; 1.0 mg/ml; 0.1 ml/10 ml MeOH)
4.3.3 BE Control standard (benzoylecgonine) 10 ng/ul (Alltech; 1.0 mg/ml; 0.1 ml/10 ml MeOH)
4.3.4 Cocaine Internal Standard (d3-cocaine) 10 ng/ul; (Alltech; 1.0 mg/ml; 0.1 ml/10 ml MeOH)
4.3.5 Cocaine Calibrator Standard 10 ng/ul (Alltech; 1.0 mg/ml; 0.1 ml/10 ml MeOH)
4.3.6 Cocaine Control Standard 10 ng/ul (Alltech; 1.0 mg/ml; 0.1 ml/10 ml MeOH)

4.4. Controls: Each run must incorporate a high and low control for each analyte. Blood matrix calibrators/controls may be used to validate urine results, but not the reverse. Calibrators and controls are prepared by addition of cocaine and BE from validated stock solutions to blank sample matrix aliquots as appropriate, prior to extraction (details in Procedure below). Acceptable control performance is target value +/- 20%.

4.5 Validation of Reagents: Acceptable performance of all batch control materials and overall batch acceptability (although individual samples may fail) is considered as validation of reagents. Validated reagents are marked with a green dot, detailing the specific procedure for which the reagent was validated, and the batch on which that process was documented. Newly prepared reagents may be evaluated for validity on an analytical batch, prior to any consideration of sample results. Reagents so validated are marked with a green sticker as noted above. Preparation of reagents, and their validation is documented in the Toxicology Section Reagent Preparation Validation Logbook, maintained in the Toxicology laboratory. See SOP #11.

5.0 PROCEDURE

Note: Departure from procedures as specified in this SOP is not anticipated. Should an issue arise that may require such a departure, the issue must be raised with the Section Supervisor, Quality Manager and/or the Director. If the proposed change will not present a change of such a magnitude so as to require validation, the change may be approved, and the Director will modify and re-issue the SOP accordingly.

Any such procedural changes would be subject to the review process afforded by the quality control measures of the analytical scheme described herein. Hence, any modification or change that produces an unexpected deleterious effect on the analytical procedure would be expected to trigger control or batch failure in the QC review stages.

5.1 Batch Format: Analytical batches for Cocaine/BE confirmation should follow the format indicated below: Note that samples are analyzed in duplicate.

Calibrator (250 ng/mL Cocaine, 500 ng/mL BE)
Matrix blank
Control High (500 ng/mL Cocaine, 1000 ng/mL BE)
Control Low (150 ng/mL Cocaine, 250 ng/mL BE)
Sample 1 rep 1
Sample 1 rep 2
Samples 2-10 (if present) Rep 1 and 2
Control High (500 ng/mL Cocaine, 1000 ng/mL BE)
(Final control, in the absence of additional samples)
Additional Samples (10 or fewer, as needed)
Final control (may be high or low)

5.2 Label a 13x100 test tube, or appropriate tube, for each sample replicate, blank, calibrator and control, similarly label a SPE tube, place a clean thru tip on each end and load in the manifold.

5.3 Using a validated dispensing pipette, place 1.0 ml aliquot of matrix blank (blood or urine) in each calibrator, control and matrix blank, and a 1.0 mL aliquot of sample replicate to the tube labeled in 5.2, above.

Note: Samples requiring dilution as a function of concentration greater than the high control, should be diluted with 0.1M pH 6.0 Phosphate buffer as

appropriate. The initial quantitative values may be used as a guide for the dilution process. The final dilution volume should be greated than the 1.0 ml aliquot that will be taken. The dilution process shall be documented on the batch worksheet, including the pipette(s) used in the process:

Example: Blood sample; 1st run result, ~ 850 ng/mL cocaine, ~ 3.2 mg/L benzylecgonine. Since a 1:1 dilution would be inadequate for the BE (final result would be expected to be ~ 1.6 ug/mL, still in excess of the 1.0 ug/mL high control), a 1: 4 dilution is selected. Therefore, 300 uL sample is added to a 13 x 100 test tube using a 0 – 1 mL adjustable volume pipette, along with 3 x 300 uL 0.1 M pH 6.0 Phosphate buffer. (Note; because equal volumes of sample were utilized, pipette precision determines the accuracy of the dilution). The solution is mixed, and aliquotted as per step 5.3. The dilution details, including the dilution factor (Volume sample: Volume Diluent + Volume Sample) are documented on the batch worksheet.

5.4 Using a validated dispensing pipette, add 50 ul of deuterated-BE internal standard stock solution and 20 ul of deuterated –Cocaine internal stock solution to each sample replicate, blank, calibrator and control tubes.

5.5 Using a validated dispensing pipette, add 50 ul of BE calibrator standard stock solution and 25 ul of Cocaine calibrator standard stock solution to tube labeled Calibrator.

5.6 Using a validated dispensing pipette, add 100 ul of BE control standard stock solution and 50 ul of Cocaine control standard stock solution to tube labeled High Control.

5.7 Using a validated dispensing pipette, add 20 ul of BE control standard stock solution and 15 ul of Cocaine control standard stock solution to tube labeled Low Control.

5.8 Using an automatic dispensing pipette, add 3.0 ml of 0.1M pH 6.0 Phosphate buffer to each tube and mix gently.

5.9 Label SPE tubes to correspond with each culture tube, place a clean thru tip on each end and load in the manifold.

5.10 Condition the columns:

1 x 3 ml methanol; aspirate
1 x 3 ml DIW; aspirate
1 x 1 ml 0.1 M phosphate buffer pH 6; aspirate
DO NOT LET COLUMN GO DRY!

5.11 Transfer contents of each tube to the appropriately labeled SPE tube, and allow gentle drop wise flow until the level reaches the top if the column bed.

5.12 Wash column:

1 x 2 ml DIW; drain (vacuum assist)
1 x 2 ml 0.1 M HCL; drain (vacuum assist)
1 x 3 ml methanol; drain (vacuum assist)
Dry column (10 minutes at > 10 inches Hg)

5.13 Position appropriately labeled 13x100 test tubes under each SPE column, with clean thru tip inside collection test tube.

5.14 Elute analytes from SPE columns by adding 3 ml methylene chloride/ Isopropanol/ammonium hydroxide (39/10/1) to each SPE tube. Collect at 1-2 ml/minute.

Note: Elution solvent must be prepared fresh the day of use.

5.15 Remove receiver tubes and evaporate to dryness at < 40 C using a gentle flow of Nitrogen (Turbovap).

5.16 Reconstitute residue with 150 ul of ethyl acetate and transfer to gc/ms vial with limited volume insert. Add 25 micro liters BSTFA (with 1% TMCS), cap and reserve for GCMS analysis (no evaporation).

6.0 CHROMATOGRAPHY AND MASS SPECTROSCOPY

6.1 Instrument and Setup:

GCMS/Autosampler: (Hewlett-Packard 6890/5973, or equivalent)
Column: 30M RTX-5MS (0.25 mm ID; 0.25 micron film)
Inj. Temp. 250 ^{o}
Det. Temp. 160 ^{o}

Oven (init.): 160°, 25 °/min to 280 °, (4.50 min hold).
9.30 min total run time
1 ul inj
Method: cocbe20.M
Method Details Appended (Appendix I)

6.2 Injection sequence: Samples are injected on the GC/MS in the following sequence:

Calibrator
Matrix blank
Control High
Control Low
Sample 1 rep 1
Sample 1 rep 2
Samples 2-10 Rep 1 and 2
Control High
Additional Samples (if present)
Final control (if present)

6.3 DETECTION AND IDENTIFICATION:
Determination of the presence of Cocaine and/or BE in the sample extract are identified by appearance and ratio of the 3 ions characteristic of each species at the appropriate retention time, hence both retention time (a GC characteristic) and fragmentation pattern and ratio (MS characteristics) are used as the basis of qualitative identification. For a positive identification of either cocaine or BE, the retention time must be within 5% of the corresponding analyte in the calibrator injection, and the ion ratios for both qualitative ions must be within 20% of the corresponding ratio calibrator sample. Qualitative identification of each analyte is independent.

6.4 CALIBRATION AND QUANTITATION

6.4.1 Calibration for each batch is done independently. Hence, no sample analysis conducted under CTDESPP guidelines is quantitated based on an historical calibration curve. Calibration is accomplished by the incorporation into the sample procedures of a blank sample of the matrix being analyzed, that has known quantities (500 ng/ml for BE and 250 ng/ml for Cocaine) of BE and cocaine (in addition to the deuterated internal standards). The response of the system to these calibrators, and the assumption of a 0 response to a 0 concentration, defines a run-specific standard curve that is used as the basis for the quantitative calculation in all controls and samples. The system is therefore

"single-point calibration, multi-point control".

6.4.2 Quantitation is accomplished by the comparison of the response ratio of the analyte in a specific sample, to the response ratios of the calibrators as expressed as a "standard curve". The concentration of the analyte in the sample is then extrapolated, from the standard curve, and corrected for any dilution that may have been performed to facilitate the analysis of relatively concentrated samples.

Note: The available quantitative range of this procedure is (in terms of amount of drug injected on the instrument (thereby allowing for appropriate calculation of diluted samples) is defined for , and validated on each batch by the high and low control, and the acceptable performance of each.

7.0 QUALITY CONTROL AND RUN EVALUATION

7.1 Verification of Vial Sequence: The vial sequence is checked both prior to and after the injection of samples when the auto injector is used. The check after the injection of samples is documented on the run summary sheet.

7.2 Chromatography - Evaluation and Acceptance Criteria: Chromatographic quality is evaluated for each peak. While general guidelines are that a peak should be symmetrical, and be resolved to baseline on at least one side, with 90% resolution on the other side, significant departures from those guidelines may be experienced with forensic samples. In many cases, chromatographic quality will warrant rejection of the chromatographic run, or specific samples, by the operator. Any such action should be clearly documented on the batch summary sheet. Questionable chromatographic peak shape, resolution, or other problems with chromatography can be discussed with the Director, Section Supervisor or Quality Manager.

7.3 Evaluation of Potential Carryover: Carryover in the chromatographic quality is evaluated by injection of a blank sample immediately following the calibrator. Carryover of greater than 2% of cocaine or BE requires batch rejection, and remedial action for the instrument (e.g. replacement of injector insert, new septum and perhaps column trim or even replacement. Demonstrated carryover, if less than 2%, will require operator consideration with regard to the potential for effects on specific samples, and may require re-extraction of specific samples. Carryover is further evaluated on a per sample basis by the requirement that quantitative results between replicates agree within 20%. Any

significant carryover effect should cause the first of the two replicate samples to exceed the second by an amount in excess of the 20% differential. If not, any carryover may be considered inconsequential. In practice, when a question of potential carryover exists, the potentially affected sample replicates may be repeated at the end of the batch.

7.4 Control Results: For the batch to be considered acceptable for any particular analyte, qualitative results for both controls for that analyte must be within 20% of the target value.

7.5 Internal Standard: Minimal acceptable internal standard abundance is 500K for cocaine, and 1.5M for BE For any individual injection to be acceptable, the internal standard abundance must be at least 20% of the corresponding abundance in the calibrator, and greater than the minimal abundance noted above.

7.6 LINEARITY:

Linearity of the calibration curve is demonstrable in each batch, for each analyte as a function of quantitative results of control materials.

Note; Blood, or body fluid quantitative analysis with quantitative results of either cocaine or benzoylecgonine concentrations greater than the corresponding calibrator will be re-analyzed with an appropriate dilution (see section 5.3, above) such that the target analyte concentration will be above the LOQ, but below the calibrator concentration. Because of the lack of correlation between blood and urine concentrations, urine samples will be subject to consideration for such re-analysis on a case-by case basis, as determined by the analyst.

7.7 SENSITIVITY- LIMITS OF DETECTION (LOD) and QUANTITATION (LOQ):
For the purposes of this procedure, the LOD and LOQ are defined as equal to the lowest concentration of the lowest control. Qualitative Identification and/or Quantitative analysis below the concentration of the low control may be accepted on a case by case basis with the concurrence of the analyst, technical reviewer, Director and/or Quality Manager.

7.8 ACCURACY AND PRECISION: Precision of the procedure is evaluated on a yearly basis, by repeat analyses of control or PT materials. Accuracy is expressed as a mean (absolute value) percentage difference between mean quantitative value of 10 reps of the specific control, and the target value. Precision is expressed as the CV of that value

7.9 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. There has been no report of any material either than BE and cocaine that elute within 5% if the retention time of known standard materials, and produce the same fragmentation ions and ratios.

8.0 REPORTING OF RESULTS:

BE/Cocaine runs are performed as part of GC/MS batches, containing controls and calibrators. The complete batch packets are in the Toxicology Laboratory. This packet contains all run evaluation documentation. Specific chromatograms for each case are filed in the appropriate case file. Results are documented on the "Batch Summary" sticker on each case file and include a reference number for the batch as a whole. A Batch summary sheet will be produced with each batch. Data on each batch should include fields such as: Sample name, Batch ID (Date of Batch), analysts who generated data, matrix, analyte found (and concentration if applicable), controls run with the batch and results obtained. If controls do not meet the criteria, the batch can be rejected as a whole or by a case by case basis. The supervisor is notified and proper action is taken to correct any problem. Batches and/or cases shall be repeated as needed.

Procedural Uncertainty is reported with all quantitative results, and is calculated and tabulated annually for each analytical method, (See SOP TX-19 section 6.3).

9.0 QUALITY ASSURANCE:

Quality Assurance is provided by the multiple layers of checks that are performed both during and after analysis. Specifically:

9.1 The GC/MS run is thoroughly checked by the operator, including vial position on the autosampler, both prior to and following the injection of samples.

9.2 The GC/MS run is reviewed and signed off by a reviewer distinct from the operator, with this review including an evaluation of qualitative and quantitative (where applicable) results, including:

a. Control Results
b. Chromatographic Characteristics

9.3 The results, as transcribed in the Case Summary Form are checked against the original run summary sheet during the process of report preparation, and during the administrative review of case results.

9.4 The original run is compared to the Final Report during the Final Director's review, prior to case sign-off.

10.0 SOURCES OF ERROR: The utilization of 3-ion SIM methodology, with reference to procedural, controls and calibrators yields qualitative drug identification with essentially no uncertainty. Urine drug analyses are reported only as qualitative results.

11.0 REFERENCES

Clarke's Isolation and Identification of Drugs. 2nd Edition

UCT United Chemical Technologies: Solid phase extraction methods

Appendix I:

GC/MS temperature program specifications

Temperature program	COCBE20.M		
Parameter			
Initial temp	160° C		
Initial Time	0.00min		
Ramps Rate/final temp/final time	rate	temp	time
	25.0	280	4.5
Post temp	280°c		
Post time	0.50		
Run Time	9.3min		
Front inlet			
Mode	Pulsed splitless		
Initial temp	250°c		
Pressure	14.26psi		
Pulse pressure	30psi		
Pulse time	1.0 min		
Total flow	53.4mL/min		
Gas type	Helium		
Injection volume	1 microliter		
Post injection washes Solvent A / Solvent B	Solvent A -3 Solvent B - 3		
Tune file	ATUNE		
Acquisition mode	SIM		
Solvent delay	5.3 minutes		
M/Zs	182, 185, 198, 201, 303, 306		
M/Zs	240, 243, 256, 259, 361, 364		
Dwell	20		
MS Quad	150°c max 200		
MS Source	230°c max 250		

Example of controlled Coc-BE batch document TX COC-BE-1. Batch documents can vary based on nature of

CT DPS; Toxicology Laboratory Batch ID: 03-26-09

Cocaine/Benzoylecgonine GCMS Batch Summary Page 1

Autosampler vial position	Sample (Case Number)	Specimen (Matrix)	Specimen Volume, (ml)	Cal. Stock Vol. (ul)	Con. Stock Vol. (ul)	Int. Std. Volume (ul)	Analyte	Abund I.S. (x 1 E 4)	Theoretical Concentration (ng/ml)	Observed Concentration (ng/ml)	Percent Recovery (Acceptable: 80 - 120)	Acceptable*** Analyst	Technical Review**
34	Calibrator	Urine	1.0	50		20	Cocaine	276	500	500			
				100		50	Benzoylecgonine	127	1000	1000			
35	Blank	Urine	1.0			20	Cocaine	304	ND	ND			
						50	Benzoylecgonine	137	ND	ND			
36	High Control	Urine	1.0		25	20	Cocaine	324	250	255	102		
					50	50	Benzoylecgonine	163	500	463	92.6		
37	Low Control	Urine	1.0		15	20	Cocaine	321	150	159	98.6		
					20	50	Benzoylecgonine	159	200	171	95.5		
38	ID-09-864-5	Urine	0.1			20	Cocaine	189		ND			
						50	Benzoylecgonine	136		8660			
39	ID-09-864-5	Urine	0.1			20	Cocaine	194		ND			
	Duplicate					50	Benzoylecgonine	151		8430			
40	ID-09-864-5	Urine	0.02			20	Cocaine	290		ND			
						50	Benzoylecgonine	147		9800			
41	ID-09-864-5	Urine	0.02			20	Cocaine	297		ND			
	Duplicate					50	Benzoylecgonine	152		9980			
36	High Control	Urine	1.0		25	20	Cocaine	304	250	264	105		
					50	50	Benzoylecgonine	158	500	480	96.0		

Samples Extracted by: _____ Date: _____ GCMS Run Date: _____
GCMS # _____

Vial position verified prior to sample removal _____

Elution solvent valid? _____ Yes _____ No

Derivitizing agent valid? _____ Yes _____ No

Carryover check: Cocaine < 5 ng/ml ? _____ Yes ___ No Benzoylecgonine < 10 ng/ml ? ___ Yes ___ No

*** Acceptability; Peak Shape, Retention Time, IS Area, Fragment Ratio all must be acceptable.

Analyst Review by: _____ Date: _____ Run Accepted? ___ Yes ___ No

Acccepted quantitative results have internal standard abundances of at least 20% of internal standard abundance observed in calibrator.

Analyst Comments: ND = not detected or value below lowest control.
*1: re-inject sample for possible carryover
*2: will re-extract sample for BE only

Technical Review by: _____ Date: _____ Run Accepted? ___ Yes ___ No

Reviewer Comments:

Toxicology Batch Worklist (example)

Batch Type: _____
Extraction/Prep Date: _____
Analyst: _____

Sample Number	Case Number	Submission Number	Dilution Factor	Comments/Dilution Details* [Cal & Con Theoretical Conc.]
1	Calibrator		1	
2	Blank		1	
3	High Con.		1	
4	Low Con.		1	
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

Cal Stock Exp. Date: _____ Cntl Stock Exp. Date: _____

* Note; Dilution details should include sample and diluent volumes, composition of diluent, and pipette indentification number(s).
(e.g. "100 uL sample/400 uL 0.1 M PO_4; pH 7.5; 100 uL Eppendorf; #7")

Notes: _____