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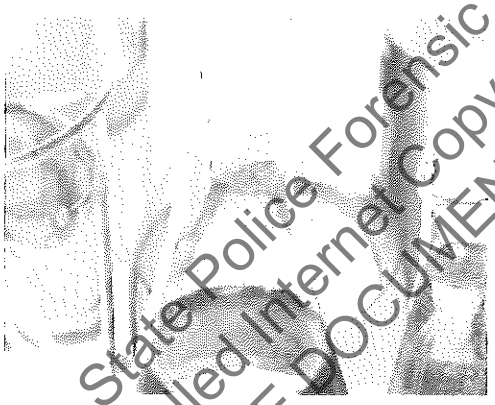

Discipline/Name of Document: Toxicology
3.10.4 – Extraction and Quantitation of Cocaine and Cocaine Metabolites in Blood Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column (**FOR QUALITATIVE USE ONLY)**

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Quality Manager

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Date Signed

Section Three
Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods

3.10.4 Extraction and Quantitation of Cocaine and Cocaine Metabolites in Blood Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column

3.10.4.1 BACKGROUND

The major metabolites of Cocaine (Methylbenzoylecgonine (Figure 1)), are benzoylecgonine, ecgonine and ecgonine methyl ester, all of which are inactive. When cocaine is ingested with ethanol, the methyl ester portion undergoes transesterification to form the active compound Cocaethylene (ethyl benzoylecgonine) that in turn adds the inactive metabolite, ecgonine ethyl ester. Refer to qualitative urine cocaine analytical method 2.3.6 and provided references and current literature for information regarding the background and pharmacology of these compounds.[2-8]

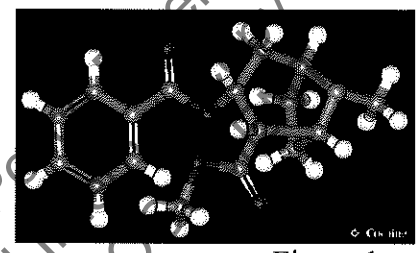

Figure 1.

3.10.4.2 PRINCIPLE

This procedure is based on a method developed by United Chemical Technology (UCT) which applies the UCT 200 mg CLEAN SCREEN® extraction column for the extraction of blood for cocaine and cocaine metabolites.[1] The CLEAN SCREEN® DAU column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest and minimally with interfering substances in the blood sample. The cation exchanger utilizes an anionic sorbent (-) to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption.

For this extraction method, the blood sample is diluted and adjusted with a pH 6 phosphate buffer and any necessary tweaks with 100mM Monobasic sodium phosphate of 100 mM Dibasic sodium phosphate. When the pH is optimal, the supernatant is loaded onto a pre-conditioned SPE column. The blood pH is adjusted to maximize the ionic character of the analyte. The conditioning creates an environment, which allows for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the amine functional groups present on the drug and the

anionic sulfonic acid exchanger on the sorbent. The column is subsequently washed with water, 100mM hydrochloric acid, and methanol to selectively remove matrix components and interfering substances from the column. The wash also disrupts the hydrophobic and adsorption interactions but not the ionically bound material. Next, the column is dried to remove traces of aqueous and organic solvents. After drying the sorbent the analytes of interest are eluted from the column with a basic organic solvent mixture. Following the elution and evaporation of the solvent, the extract is derivatized for confirmation on the GC/MSD. Quantitation is accomplished with a 5 to 6 point calibration curve using the corresponding deuterated internal standard to establish a response factor.

3.10.4.3 EQUIPMENT AND SUPPLIES

- 3.10.4.3.1 200mg CLEAN SCREEN® Extraction Column (ZSDAU020 or ZCDAU020 or equivalent)
- 3.10.4.3.2 Drybath or laboratory oven
- 3.10.4.3.3 Evaporative concentrator equipped with nitrogen tank.
- 3.10.4.3.4 Vortex mixer
- 3.10.4.3.5 Vacuum manifold/pump
- 3.10.4.3.6 Laboratory centrifuge capable of 3400rpm
- 3.10.4.3.7 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.
- 3.10.4.3.8 pH indicator strips
- 3.10.4.3.9 16 x 100mm round bottom glass tube
- 3.10.4.3.10 Screw Cap for 16mm O.D. tube
- 3.10.4.3.11 GC/MS Automated Liquid Sample (ALS) vials
- 3.10.4.3.12 GC/MS Vial Microinsert
- 3.10.4.3.13 Gas Chromatograph (GC) equipped with a mass selective detector (MSD) (HP 6890 GC/5973 MSD or equivalent) and a nonpolar capillary column with a phase composition comparable to 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%-diphenyl.

3.10.4.4 REAGENTS

Refer to manual section 5.12 for solution preparation instructions.

- 3.10.4.4.1 Deionized/distilled (DI) water
- 3.10.4.4.2 Methanol (Certified ACS Grade)
- 3.10.4.4.3 Methylene Chloride (Certified ACS Grade)
- 3.10.4.4.4 Ethyl Acetate (Certified ACS Grade)
- 3.10.4.4.5 Isopropanol (Certified ACS Grade)
- 3.10.4.4.6 Ammonium Hydroxide (Certified ACS Grade)
- 3.10.4.4.7 100mM Phosphate Buffer (pH 6.0)
- 3.10.4.4.8 100mM HCl

3.10.4.4.9 100mM Monobasic sodium phosphate
3.10.4.4.10 100mM Dibasic sodium phosphate
3.10.4.4.11 Elution Solvent
Mix 20mL Isopropanol and 2mL Ammonium Hydroxide QS to 100mL with methylene chloride. pH should be 11-12. ***Make fresh.***
3.10.4.4.12 BSTFA + 1% TMCS

3.10.4.5 QUALITY ASSURANCE MATERIAL

3.10.4.5.1 Calibrator and Control Solutions

3.10.4.5.1.1 **Stock Solutions**
Whenever possible, the source of a corresponding calibrator and control should be obtained from a different vendor.

Benzoylecgonine
Concentration: 1mg/mL

Cocaine
Concentration: 1mg /mL

Cocaethylene (Ethylcocaine)
Concentration: 1mg /mL

3.10.4.5.1.2 **Working Solutions**
Store remaining stock solution in ALS vial in freezer. Working solutions are stable for 6 months when stored at 4°C.

10ng/mL
Add 100µL each Benzoylecgonine, Cocaine and Cocaethylene Stock Solutions to ≅9mL Methanol in a 10mL volumetric class A flask . QS to 10mL. Store remaining stock solution in ALS vial in freezer.

1ng/µL
Add 1mL 10ng/µL working drug solution to ≅5mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.10.4.5.2 Internal Standard Stock Solutions
Benzoylecgonine-D_3
Concentration: 100µg /mL (100ng/µL)

Cocaine-D_3
Concentration: 100µg /mL

Cocaethylene-D_3
Concentration: 100µg /mL

3.10.4.5.3 1ng/µL Working Internal Standard Solution
Add 100µL Benzoylecgonine-D_3, Cocaine-D_3, and Cocaethylene-D_3 stock solutions to 9800µL Methanol.

Working solution is stable for 6 months when stored at 4 °C.

3.10.4.5.4 Whole Blood Controls
Negative Whole Blood

Positive Whole Blood
Vendor: Utak or comparable
Catalog No: 98818
Utak Control contains Benzoylecgonine, Cocaine and Cocaethylene each at a target of 100ng/mL. Refer to package insert for verified value and expected range.

3.10.4.6 PROCEDURE

3.10.4.6.1 Initial set-up
For calibrators, controls and case samples label extraction tubes (two per sample), a 200mg CLEAN SCREEN® extraction column, eluate collection tube and a GC/MSD vial with microinsert.

3.10.4.6.2 Calibrator Preparation
Use the same lot of negative blood used to prepare the negative control to prepare calibrators.

3.10.4.6.2.1 Add 1mL of negative whole blood to six screw-top tubes.

3.10.4.6.2.2 Add the volume of working 1ng/µL Benzoylecgonine, Cocaethylene and Cocaine mixed reference material as indicated in the following table.

Level	ng/mL	µL Working Reference Material
1	25	25
2	50	50
3	100	100

3.10.4.6.2.3 Add the volume of working 10ng/µL Benzoylecgonine, Cocaethylene and Cocaine mixed reference material as indicated in the following table.

Level	ng/mL	µL Working Reference Material
4	250	25
5	500	50
6	1000	100

3.10.4.6.2.4 Additional or alternative concentrations may be used as necessary as long as the requirements in 3.10.4.6.15.1 are met.

3.10.4.6.3 Positive Control Sample Preparation

Use the same lot of negative blood used to prepare the negative control for positive control preparation.

3.10.4.6.3.1 Add 1mL of negative whole blood to two screw top tubes.

3.10.4.6.3.2 Add indicated amount of 1ng/µL working mixed control solution.

Desired ng/mL	µL Working Control
75	75

3.10.4.6.3.3 Add indicated amount of 10ng/µL working mixed control solution.

Desired ng/mL	µL Working Control
750	75

3.10.4.6.3.4 Additional or alternative concentrations may be used at the discretion of the analyst as

long as the requirements in 3.10.4.10.2 are met.

3.10.4.6.4 Negative Control Sample Preparation
Add 1mL of negative whole blood to screw top tube.

3.10.4.6.5 Case Sample Preparation

3.10.4.6.5.1 Based on enzyme immunoassay screen results, samples may be diluted with negative whole blood for additional analysis.

3.10.4.6.5.2 The total volume of blood or diluted blood should be 1mL.

3.10.4.6.5.3 Add 1mL neat or diluted sample to labeled extraction tube.

3.10.4.6.6 Internal Standard Addition

3.10.4.6.6.1 Add 100µL of internal standard mix to calibrator, control and case samples. This results in an internal standard concentration of 100ng/mL.

3.10.4.6.6.2 Allow tubes to stand 30 minutes for sample equilibration.

3.10.4.6.7 Sample Preparation

3.10.4.6.7.1 Add 4mL DI water, vortex, let stand for 5 minutes.

3.10.4.6.7.2 Centrifuge for 10 minutes @ 3400rpm.

3.10.4.6.7.3 Transfer supernatant to second tube.

3.10.4.6.7.4 Add 4mL 100mM phosphate buffer (pH 6.0), vortex.

3.10.4.6.7.5 Sample pH should be 6.0 ±0.5. Adjust as necessary with 100mM Monobasic sodium phosphate or 100mM Dibasic sodium phosphate.

3.10.4.6.8 SPE Column Preparation

3.10.4.6.8.1 Insert labeled 200mg CLEAN SCREEN® Extraction column onto the vacuum manifold.

3.10.4.6.8.2 Add 3mL methanol to the SPE column. Aspirate at ≤ 3 in. Hg to prevent sorbent drying.

3.10.4.6.8.3 Add 3mL DI water to the SPE column. Aspirate at ≤ 3 in. Hg.

3.10.4.6.8.4 Add 1mL 100mM Phosphate buffer (pH 6.00) to the SPE column. Aspirate at ≤ 3 in. Hg.

3.10.4.6.9 Blood Extract Loading

Load buffered blood onto column and allow to gravity flow or apply minimal vacuum.

3.10.4.6.10 Column Clean-up

3.10.4.6.10.1 Add 2mL DI water to the column. Aspirate.

3.10.4.6.10.2 Add 2mL 100mM HCl to the column. Aspirate.

3.10.4.6.10.3 Add 3mL Methanol. Aspirate.

3.10.4.6.10.4 Increase vacuum to ≥10 in. Hg (≥34 kPa) for ≥5 minutes (disc should be dry).

3.10.4.6.11 Compound Elution

3.10.4.6.11.1 Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled tapered tip centrifuge tubes.

3.10.4.6.11.2 Add 3mL elution solvent (3.10.4.4.12) to the column.
Collect eluate with gravity flow or apply minimal vacuum.

3.10.4.6.12 Eluate Evaporation

Transfer centrifuge tube to Evaporative Concentrator. Evaporate solvent to dryness under a gentle stream of nitrogen at approximately 37°C .

3.10.4.6.13 Derivatization

3.10.4.6.13.1 In fume hood add 50µL ethyl acetate.

Vortex for ≅15 seconds.

3.10.4.6.13.2 Add 50.0µL BSTFA + 1% TMCS.

3.10.4.6.13.3 Cap tubes and vortex briefly.

3.10.4.6.13.4 Place tubes in 70°C dry bath or oven for 20 minutes.

3.10.4.6.13.5 Remove from heat and allow to cool to room temperature.

3.10.4.6.13.6 Transfer derivative to labeled GC/MSD ALS vial with microinsert.

3.10.4.6.14 Preparation for GC-MS Run

3.10.4.6.14.1 Perform an AUTOTUNE and TUNE EVALUATION. Evaluate applying acceptance criteria outlines in analytical method 5.3.1.

3.10.4.6.14.2 When tune values are acceptable, program SEQUENCE TABLE with sample, calibrator and control information.

3.10.4.6.14.3 Load ALS vials into quadrant racks as indicated in the SEQUENCE TABLE.

3.10.4.6.15 GC-MS Calibration Curve

3.10.4.6.15.1 The calibration curve should be established with a minimum of four data points.

3.10.4.6.15.2 All reported results must be bracketed by calibrators.

3.10.4.6.15.3 Calibrators should be analyzed in order of increasing concentration.

3.10.4.6.15.4 The least squares line resulting from the analysis of calibrators must have a coefficient of correlation of ≥0.98.

3.10.4.6.15.5 If calibrators are run in duplicate, it is not required that duplicate calibration points are included as long as the linearity requirement is met.

3.10.4.7 GC and MSD ACQUISITION PARAMETERS

Critical parameters are specified below. Parameters not specified are at the discretion of the analyst and should be optimized for the particular GC-MSD instrument. Each laboratory should maintain a centrally stored printed or electronic copy of current and past GC-MSD methods. The data supporting the GC-MSD method should be stored centrally.

3.10.4.7.1 GC Temperature Parameter

Injection Port: 250° or 260°C

3.10.4.7.2 MSD Instrument Parameters

Detector/Transfer Line: 280°C

3.10.4.7.3 ALS Parameters

Injection Volume: 1µL (1 stop)
Viscosity Delay: A minimum of 3 seconds
Solvent Washes (A & B): A minimum of 4 pre- and post-wash rinses.

3.10.4.7.4 MS SIM Parameters

Analyte	Target Ion	Qualifier Ion 1	Qualifier Ion 2
Benzoylecgonine-TMS	240	256	361
Benzoylecgonine-TMS-D3	243	259	364
Cocaine	182	198	303
Cocaine-D3	185	201	306
Cocaethylene	196	212	317
Cocaethylene-D3	199	215	320

3.10.4.8 REPORTING CRITERIA

3.10.4.8.1 Qualitative Chromatographic and SIM Criteria

3.10.4.8.1.1 Qualitative results can be accepted when the following two criteria are met.

1. The retention time falls within the ±0.2 minute window established by calibrators.

2. Ion ratios for the analyte and its corresponding internal standard,

established by calibrators for target and qualifier ions, do not differ by more than ±20%.

3.10.4.8.2 Quantitative Mass Spectral Criteria

3.10.4.8.2.1 Quantitative results can be accepted if the calculated concentration of all calibrator and control samples are within ±20% of their respective concentrations.

3.10.4.8.2.2 Quantitation is achieved through the plotting of the target ion response ratio versus the concentration for each calibrator.

3.10.4.8.2.3 Quantitative values for case samples, calibrators and controls will be truncated for reporting purposes.

3.10.4.8.2.4 Administrative limit of detection (LOD) for Benzoylecgonine, Cocaine and Cocaethylene is 25ng/mL. Results < this LOD should be reported as negative unless there are extenuating circumstances. The Toxicology Discipline Leader must be consulted to evaluate exceptions.

3.10.4.8.2.5 If the concentration exceeds the calibration range, the sample must be appropriately diluted with negative whole blood for reanalysis.

3.10.4.9 REPORTING OF RESULTS

3.10.4.9.1 Quantitative Value

Analysis results should be truncated and reported out without decimal places.

3.10.4.9.2 Uncertainty Value

Based on the current uncertainty assessment, the +/– range should be included on the analysis report. Refer to method quality monitoring spreadsheet for current uncertainty figure.

3.10.4.10 QUALITY ASSURANCE REQUIREMENTS

3.10.4.10.1 General

3.10.4.10.1.1 Blood samples are to be stored under

refrigeration after aliquots are removed for analysis.

3.10.2.10.1.2 Refer to toxicology manual section 5.1 for pipette calibration and intermediate check options.

3.10.2.10.1.3 Refer to toxicology manual section 5.2 for balance calibration and intermediate check requirements.

3.10.2.10.1.4 Refer to toxicology manual section 5.8 for additional GC-MSD quality assurance requirements.

3.10.2.10.1.5 Refer to toxicology manual section 5.10 for reference material authentication requirements.

3.10.4.10.2 Per Analysis Run Quality Requirements

3.10.4.10.2.1 Solvent blank should follow the highest calibrator as well as each case sample.

3.10.4.10.2.2 A minimum of two blood commercially obtained controls and the spiked controls described in section 3.10.3.6.3 must be run per batch of samples. Bracket

3.10.4.10.2.3 In addition to the four blood controls indicated above, for each additional 10 case samples, one control must be run. The preparation of controls is outlined in section 3.10.4.6.3. If desired, additional concentrations may be used.

3.10.4.10.3 Monitoring of Control Values

Upon the completion of analysis, input blood control values on spreadsheet used to assess uncertainty for this method.

3.10.4.11 ANALYSIS DOCUMENTATION

3.10.4.11.1 A packet containing original data for controls and standards will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed until archiving.

3.10.4.11.2 A copy of controls and standards need not be included in

individual case files. When necessary, a copy of the control and standard printouts can be prepared from the centrally stored document.

3.10.4.12 REFERENCES AND RECOMMENDED READING

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3.10.4.12.2 Crouch, D.J., Alburges, M.E., Spanbauer, A.C., Rollins, D.E. and Moody; D.E., Analysis of Cocaine and Its Metabolites from Biological Specimens Using Solid-Phase Extraction and Positive Ion Chemical Ionization Mass Spectrometry, J. Anal. Toxicol. 19(6): 352-358, 1995.

3.10.4.12.3 Cone, E.J., Hillsgrove, M. and Darwin, W.D., Simultaneous measurement of Cocaine, Cocaethylene, Their Metabolites, and "Crack" Pyrolysis Products by Gas Chromatography - Mass Spectrometry, Clin Chem 40(7):1299-1305, 1994.

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3.10.4.12.5 Drummer, O.H., *Stimulants* pp. 49-96. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

3.10.4.12.6 Isenschmid, D.S., *Cocaine,* pp. 207-228. *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, 2nd ed, 2003.

3.10.4.12.7 Baselt, R.C., *Cocaine*, pp. 256-262. *in:* Disposition of Toxic Drugs and Chemicals in Man, Biomedical Publications: Foster City, CA. 7th ed., 2004.

3.10.4.12.8 *Cocaine*, pp. 842-845. *in:* Clarke's Analysis of Drugs and Poisons. Pharmaceutical Press: London, 3rd ed., 2004.

Revision History

Section Three
Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods

3.10.4 Extraction and Quantitation of Cocaine and Cocaine Metabolites in Blood Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column

Revision No.	Issue Date	Revision/Comments
0	11-21-2006	Original Issue
1	07-28-2008	Clarified that negative blood used to prepare calibrators and positive controls is the same lot as used for negative control.