Section Three
Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods

3.10.4 Extraction and Confirmation 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]

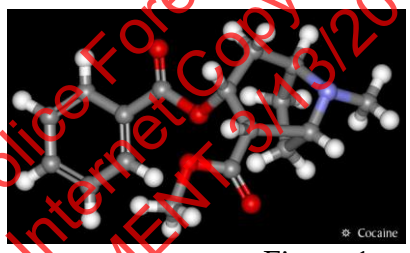

Figure 1.

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 100mM 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 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
Corresponding calibrator and control reference materials must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. The addition of Cocaethylene is optional.

3.10.4.5.1.1 **Reference Material Stock Solutions**

Compound	Concentration
Benzoylecgonine	1 mg/mL
Cocaine	1 mg/mL
Cocaethylene	1 mg/mL

Store remaining stock solution in freezer.

3.10.4.5.1.2 **Reference Material Working Solutions**
Working solutions are stable for 6 months when stored under refrigeration.

10ng/mL
Add 100µL each Benzoylecgonine, Cocaine and Cocaethylene (optional) Stock Solutions to ≅9mL Methanol in a 10mL volumetric class A flask . QS to 10mL.

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

Compound	Concentration
Benzoylecgonine–D_3 or –D_6	100µg/mL (100ng/µL)
Cocaine–D3	100µg/mL
Cocaethylene–D3*	100µg/mL

*Use if Cocaethylene will be included.

3.10.4.5.3 1ng/µL Working Internal Standard Solution

Add 100µL Benzoylecgonine–D_3 or –D_6, Cocaine–D_3, and Cocaethylene–D_3 stock solutions to 9800µL Methanol. Working solution is stable for 6 months when stored under refrigeration.

3.10.4.5.4 Commercial Whole Blood Controls

Negative Whole Blood

Positive Whole Blood

Positive control must contain a minimum of Benzoylecgonine and Cocaine each at a target of 100ng/mL. Refer to package insert for verified value and expected range. Additional concentrations may also be utilized.

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 Place sample container on tube rocker for a minimum of five minutes. If sample is clotted, homogenize as necessary.

3.10.4.6.5.4 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 calibrators, controls and case samples. This results in an internal standard concentration of 100ng/mL.

3.10.4.6.6.2 Vortex and allow tubes to stand 15 - 30 minutes for sample equilibration.

3.10.4.6.7 Sample Preparation

3.10.4.6.7.1 Add 4mL DI water, vortex.

3.10.4.6.7.2 Add 2mL 100mM phosphate buffer (pH 6.0), vortex, allow to stand for 5-10 minutes.

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

3.10.4.6.7.4 Centrifuge for about 10 minutes at approximately 3400 - 3500rpm. Shouldn't something be transferred here, or removed.

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 40°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.

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 must be established with a minimum of four data points.

3.10.4.6.15.2 Calibrators should be analyzed in order of increasing concentration.

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

3.10.4.6.15.4 If calibrators are run in duplicate, it is not required that duplicate calibration points be 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
Benzoylecgonine-TMS-D6	243	354	369
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% (relative).

3.10.4.8.2 Qualitative Mass Spectral Full Scan Criteria

Analytes may be confirmed from full scan data if there are no significant differences in the mass spectral data as compared to the appropriate reference material

3.10.4.8.3 Quantitative Mass Spectral Criteria

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

respective concentrations (relative).

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

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

3.10.4.8.3.4 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.

3.10.4.8.3.5 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 This method is currently only approved for the qualitative identification of drugs. Quantitative values are not to be reported or expressed. They are currently being used to establish an administrative cut off. Once the uncertainty of measurement is established for this method it will be evaluated for quantitative reporting.

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 A solvent blank must follow the highest calibrator, as well as precede each case sample.

3.10.4.10.2.2 A minimum of the spiked blood controls described in section 3.10.4.6.3 must be run per batch of samples.

3.10.4.10.2.3 If the number of case samples exceeds 10, in addition to the two spiked described in 3.10.4.6.3, one blood spiked or commercially obtained control must be run for each additional 10 case samples,. 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 Case results are to be recorded in the LIMS system.

3.10.4.11.2 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.

3.10.4.11.3 A copy of controls and standards may be stored electronically in a central location and 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

3.10.4.12.1 Telepchak, M.J., August, T.F. and Chaney, G., Drug Methods for the Toxicology Lab, pp. 209-211. *in:* Forensic

and Clinical Applications of Solid Phase Extraction, Humana Press: New Jersey, 2004.

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.

3.10.4.12.4 Isenschmid, D.S., *Cocaine - Effects on Human Performance and Behavior*, Forensic Science Rev. 14(1&2): 62-100, 2002.

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 Confirmationof 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.
2	03-07-2011	Storage condition specifications updated, emphasized need for sample homogeneity, updated nomenclature, minor reformatting.
3	11-28-2012	Amended sample preparation, time to stand after water addition was removed and the centrifuge step was moved to after the pH adjustment. Clarified current reporting limitations.
4	1-16-2014	Removed reference to quantitation in titles, added option for confirmation by full scan. Amendment to 3.10.4.11 in accordance with new LIMS system. Minor formatting changes