Section Three
Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods

3.10.3 Extraction and Quantitation of Free (Unbound) Codeine and Morphine in Blood Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® DAU Extraction Column

3.10.3.1 BACKGROUND

Refer to provided references and current literature for information regarding the background and pharmacology of Codeine (figure 1) and Morphine (figure 2).[2-5]

Figure 1

Figure 2

3.10.3.2 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 opiates from blood.[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 the extraction of opiate class drugs, the blood sample is diluted and adjusted to pH 6 with a phosphate buffer. After optional centrifugation, the sample 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, a weak aqueous buffer 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. When the column is dry the analytes of

interest are eluted from the column with a basic organic solvent mixture. Following elution from the SPE column and evaporation, the extract is derivatized for confirmation on the GC/MSD. Quantitation is accomplished using the corresponding deuterated standard to establish the response factor.

3.10.3.3 EQUIPMENT AND SUPPLIES

3.10.3.3.1 200mg CLEAN SCREEN® Extraction Column (ZSDAU020 or ZCDAU020 or equivalent)
3.10.3.3.2 Laboratory oven or drybath capable of 70°C.
3.10.3.3.3 Evaporative concentrator equipped with nitrogen tank.
3.10.3.3.4 Tube Rocker
3.10.3.3.5 Vortex Mixer
3.10.3.3.6 Laboratory centrifuge capable of 3400 - 3500rpm
3.10.3.3.7 Vacuum Manifold/pump
3.10.3.3.8 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.
3.10.3.3.9 pH indicator strips
3.10.3.3.10 16 x 100mm silanized glass tubes
3.10.3.3.11 Screw Cap for 16mm O.D. tube
3.10.3.3.12 GC/MS Automated Liquid Sample (ALS) vials
3.10.3.3.13 Silanized GC/MS Vial Microinsert
3.10.3.3.14 Gas Chromatograph (GC) equipped with a mass selective detector (MSD) and a nonpolar capillary column with a phase composition comparable to 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5%-diphenyl.

3.10.3.4 REAGENTS

Refer to analytical method 5.12 for solution preparation instructions.

3.10.3.4.1 Deionized/distilled (DI) water
3.10.3.4.2 Methanol (Certified ACS Grade)
3.10.3.4.3 Methylene Chloride (Certified ACS Grade)
3.10.3.4.4 Ethyl Acetate (Certified ACS Grade)
3.10.3.4.5 Isopropanol (Certified ACS Grade)
3.10.3.4.6 Ammonium Hydroxide (Certified ACS Grade)
3.10.3.4.7 100mM Phosphate Buffer (pH 6.0)
3.10.3.4.8 100mM Acetate Buffer (pH 4.5)
3.10.3.4.9 100mM Monobasic sodium phosphate
3.10.3.4.10 100mM Dibasic sodium phosphate
3.10.3.4.11 Elution Solvent
Mix 20mL Isopropanol and 2mL Ammonium Hydroxide.
QS to 100mL with methylene chloride. pH should be 11-12.
Make fresh.
3.10.3.4.12 BSTFA + 1% TMCS

3.10.3.5 QUALITY ASSURANCE MATERIAL

3.10.3.5.1 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.

3.10.3.5.1.1 **Reference Material Stock Solutions**

Compound	Concentration
Codeine	1 mg/mL
Morphine	1 mg/mL

Store remaining stock solution in freezer.

3.10.3.5.1.2 **Reference Material Working Solutions**

Working solutions are stable for 6 months when stored under refrigeration.

10ng/µL

Add 100µL each Codeine and Morphine Stock Solution 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.

3.10.3.5.2 Internal Standard Solutions

3.10.3.5.2.1 **Stock Solutions**

Compound	Concentration
Codeine-D_3 or -D_6	1 mg/mL or 100µL/mL
Morphine-D_3 or -D_6	1 mg/mL or 100µL/mL

Store remaining stock solution in freezer.

3.10.3.5.2.2 **Working Internal Standard Solution 1ng/µL**

Add 10µL each 1mg/mL or 100µL each 100µL/mL Codeine-D_3 or -D_6 and Morphine-D_3 or -D_6 Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. Working solution is stable for 6 months when stored under refrigeration.

3.10.3.5.3 Commercial Whole Blood Controls

3.10.3.5.3.1 **Negative Whole Blood**

3.10.3.5.3.2 **Positive Whole Blood**

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

3.10.3.6 PROCEDURE

3.10.3.6.1 Initial set-up

Label extraction tubes, 200mg CLEAN SCREEN® extraction columns, eluate collection tubes and GC/MSD vials with microinserts for calibrators, controls and case samples.

3.10.3.6.2 Calibrator Preparation

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

3.10.3.6.2.1 Add 1mL of negative whole blood into six screw-top extraction tubes.

3.10.3.6.2.2 Add the volume of working Codeine-Morphine 1ng/µL mixed calibrator solution to appropriate tube as indicated in the chart below.

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

3.10.3.6.2.3 Add the volume of working Codeine and Morphine 10ng/µL mixed calibrator solution to the appropriate tube as indicated in the chart below.

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

3.10.3.6.2.4 Additional or alternative concentrations may be used as necessary as long as the requirements in 3.10.3.6.15.1 are met.

3.10.3.6.3 Positive Control Sample Preparation

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

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

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

ng/mL	µL Working Control
75	75

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

ng/mL	µL Working Control
750	75

3.10.3.6.3.4 Additional or alternative concentrations at the discretion of the analyst may be used as long as the requirements in 3.10.3.10.2 are met.

3.10.3.6.4 Negative Control Sample Preparation

Add 1mL of negative whole blood to a screw top tube.

3.10.3.6.5 Case Sample Preparation

3.10.3.6.5.1 Based on enzyme immunoassay screen results, samples may be diluted with negative whole blood prior to analysis.

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

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

3.10.3.6.5.4 Add 1mL neat or diluted sample into labeled screw top tube.

3.10.3.6.6 Internal Standard Addition

3.10.3.6.6.1 To prepare 100ng/mL internal standard add 10µL of 1mg/mL (1000ng/µL) or 100µL of (100ng/µL) 1ng/µL of internal standard mix to calibrators, controls and casework samples.

3.10.3.6.6.2 Vortex tube briefly and allow to stand 15 - 30 minutes for sample equilibration.

3.10.3.6.7 Sample Preparation

3.10.3.6.7.1 Add 4mL DI water, vortex.

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

3.10.3.6.7.3 Sample pH should be 6.0 ± 0.5. Adjust as necessary with 100mM monobasic sodium phosphate or 100mM dibasic sodium phosphate.

3.10.3.6.7.4 Centrifuge for about 10 minutes at approximately 3400 – 3500 rpm. Again, supernatant step missing

3.10.3.6.8 SPE Column Preparation

No vacuum is necessary except for drying step, however, if desired, aspirate at ≤ 3 in. Hg to prevent sorbent drying.

3.10.3.6.8.1 Insert labeled 200mg CLEAN SCREEN® extraction column onto the vacuum manifold.

3.10.3.6.8.2 Add **3**mL **methanol** to the column.

3.10.3.6.8.3 Add **3**mL **DI water** to the column.

3.10.3.6.8.4 Add **1**mL **100mM phosphate buffer** (pH 6.00) to the column.

3.10.3.6.9 Blood Extract Loading

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

3.10.3.6.10 Column Clean-up

3.10.3.6.10.1 Add **2**mL **DI water** to the column.

3.10.3.6.10.2 Add **2**mL **100mM acetate buffer (pH 4.5)** to the column. Aspirate.

3.10.3.6.10.3 Add **3**mL **methanol**.

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

3.10.3.6.11 Compound Elution

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

3.10.3.6.11.2 Add **3**mL elution solvent (3.10.3.4.12) to the column. Collect eluate with gravity flow or apply minimal vacuum.

3.10.3.6.12 Eluate Evaporation

Transfer centrifuge tube to Evaporative Concentrator. Evaporate solvent to dryness under a gentle stream of nitrogen at approximately 40°C.

3.10.3.6.13 Derivatization

3.10.3.6.13.1 In fume hood add 50µL ethyl acetate. Vortex for ≅15 seconds.

3.10.3.6.13.2 Add 50.0µL BSTFA + 1% TMCS.

3.10.3.6.13.3 Cap tubes and vortex briefly.

3.10.3.6.13.4 Heat tubes at 70°C for 20 minutes.

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

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

3.10.3.6.14 Preparation for GC-MS Run

3.10.3.6.14.1 Perform an AUTOTUNE and TUNE EVALUATION.

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

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

3.10.3.6.15 GC-MS Calibration Curve

3.10.3.6.15.1 The calibration curve must be established with a minimum of four data points.

3.10.3.6.15.2 Calibrators should be analyzed in order of increasing concentration.

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

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

3.10.3.7 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.

3.10.3.7.1 GC Temperature Parameter

Injection Port: 250° or 260°C

3.10.3.7.2 MSD Instrument Parameters

Detector/Transfer Line: 280°C

3.10.3.7.3 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.

3.10.3.7.4 MS SIM Parameters

Analyte	Target Ion	Qualifier Ion 1	Qualifier Ion 2
Morphine	429	287	324
Morphine-D_3	432	290	327
Morphine-D_6	435	293	330
Codeine	371	234	343
Codeine-D_3	374	237	346
Codeine-D_6	377	237	349

3.10.3.8 REPORTING CRITERIA

3.10.3.8.1 Qualitative Chromatographic and SIM Criteria

3.10.3.8.1.1 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).

3.10.3.8.2 Quantitative Mass Spectral Criteria

3.10.3.8.2.1 Quantitative results can be accepted if the calculated concentrations of all calibrator and control samples are within ±20% of their respective concentrations (relative).

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

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

3.10.3.8.2.4 Administrative limit of detection (LOD) for Codeine and Morphine is 25ng/mL, the lowest calibrator level. Results < this LOD should be reported as negative unless there are extenuating circumstances. The Toxicology Discipline Leader must be consulted to evaluate exceptions.

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

3.10.3.9 REPORTING OF RESULTS

3.10.3.9.1 Quantitative Value

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.

3.10.3.10 QUALITY ASSURANCE REQUIREMENTS

3.10.3.10.1 General

3.10.3.10.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

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

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

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

3.10.3.10.1.5 Refer to toxicology manual section 5.10 for reference material authentication

requirements.

3.10.3.10.2 Per Analysis Run Quality Requirements

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

3.10.3.10.2.2 A minimum of the spiked blood controls described in section 3.10.3.6.3 must be run per batch of samples.

3.10.3.10.2.3 If the number of case samples exceeds 10, in addition to the two spiked described in 3.10.3.6.3, one blood spiked or commercially obtained control must be run for each additional 10 case samples,. Additional concentrations may be used.

3.10.3.10.3 Monitoring of Control Values

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

3.10.3.11 ANALYSIS DOCUMENTATION

3.10.3.11.1 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.

3.10.3.11.2 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.

3.10.3.12 REFERENCES AND RECOMMENDED READING

3.10.3.12.1 Telepchak, M.J., August, T.F. and Chaney, G., Drug Methods for the Toxicology Lab, pp. 227 - 230. *in:* Forensic and Clinical Applications of Solid Phase Extraction, Humana Press: New Jersey, 2004.

3.10.3.12.2 Stout, P.R. and Farrell, L.J., *Opioids - Effects on Human Performance and Behavior*, Forensic Science Rev. 15(1): 29 - 60, 2003.

3.10.3.12.3 Drummer, O.H., *Opioids* pp. 219 - 265. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

3.10.3.12.4 Kerrigan, S. and Goldberger, B.A., *Opioids*. Refer to index for page numbers, *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, Third ed., 2010 or more recent version.

3.10.3.12.5 Baselt, R.C., *Codeine*, pp. 355 - 360 and *Morphine*, pp. 1057 - 1061. *in:* Disposition of Toxic Drugs and Chemicals in Man, Biomedical Publications: Foster City, CA. Eighth ed., 2008 or more recent version.

Revision History

Section Three
Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods
3.10.3 Extraction and Quantitation of Free (Unbound) Codeine and Morphine 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.