Section Three
Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods

3.10.3 Extraction and Confirmation 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 centrifugation, the sample is loaded onto a pre-conditioned SPE column. The blood pH is adjusted to maximize the ionic character of the analyte. Column 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 Disposable inserts for SPE manifold ports
3.10.3.3.3 Laboratory oven or drybath capable of 70°C
3.10.3.3.4 Evaporative concentrator equipped with nitrogen tank
3.10.3.3.5 Tube Rocker
3.10.3.3.6 Vortex Mixer
3.10.3.3.7 Laboratory centrifuge capable of 3400 - 3500rpm
3.10.3.3.8 Vacuum Manifold/pump
3.10.3.3.9 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.
3.10.3.3.10 pH indicator strips
3.10.3.3.11 16 x 100mm silanized glass tubes
3.10.3.3.12 Screw Cap for 16mm O.D. tube
3.10.3.3.13 GC/MS Automated Liquid Sample (ALS) vials
3.10.3.3.14 Silanized GC/MS Vial Microinsert
3.10.3.3.15 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 <u>Elution Solvent</u>
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 as recommended by manufacturer.

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 as recommended by manufacturer.

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 **Optional: 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 (x2), SPE columns (x1), and GC/MSD vials with microinserts (x1) for calibrators, controls and case samples.

3.10.3.6.2 Calibrator Preparation
To prepare calibrators, use the same lot of negative blood used to prepare the negative control.

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

3.10.3.6.2.2 Add the volume of 1ng/µL Codeine-Morphine working 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 10ng/µL Codeine and Morphine working 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 are met.

3.10.3.6.3 Positive Control Sample Preparation

To prepare positive controls, use the same lot of negative blood used to prepare the negative control.

3.10.3.6.3.1 Add 1mL of negative whole blood to 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 to 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 **4**mL **DI water**, vortex.

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

3.10.3.6.7.3 Check pH. 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.

3.10.3.6.8 SPE Column Preparation

3.10.3.6.8.1 Insert valve liners and labeled SPE columns into appropriate location on vacuum manifold. For each following SPE step, allow to gravity flow or aspirate at ≤ 3 in. Hg to prevent sorbent drying.

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

Decant buffered blood extract onto the SPE column. *Care should be taken that very little solid matter (from centrifugation of whole blood) is applied to the SPE column.*

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.

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 centrifuge tubes.

3.10.3.6.11.2 Add 3mL **elution solvent** (3.10.3.4.11) 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 Add 50µL ethyl acetate. Vortex for ≅15 seconds.

3.10.3.6.13.2 Add 50µL 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 Into Sequence log table, enter the case sample, calibrators, blanks and control information..

3.10.3.6.14.2 Load samples, calibrators, blank and controls into the sample rack(s) as noted 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 Criteria

Acceptable retention time window established by calibrators is ± 0.2 minutes.

3.10.3.8.2 Qualitative Mass Spectral SIM Criteria

Ion ratios for the analyte and its corresponding internal standard, established by calibrators for target and qualifier ions, must not differ by more than ±20% (relative). Refer to section 3.10.3.8.4.5 for cutoff criteria.

3.10.3.8.3 Qualitative Mass Spectral Full Scan Criteria

Analytes may be confirmed from full scan data if the retention time for the sample versus applicable reference material does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

3.10.3.8.4 Quantitative Mass Spectral Criteria

3.10.3.8.4.1 Refer to Section 3.10.3.9.2 for determination of when this method may be used for quantitative purposes.

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

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

3.10.3.8.4.4 Quantitative values for case samples, calibrators and controls will be truncated for

reporting purposes.

3.10.3.8.4.5 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.

3.10.3.8.4.6 If the concentration exceeds the calibration range, the sample must be appropriately diluted with negative whole blood for reanalysis. Alternatively, the analyte(s) may be reported using full scan data; refer to section 3.10.3.8.3 for criteria.

3.10.3.9 REPORTING OF RESULTS

3.10.3.9.1 Qualitative Confirmation

If Codeine and Morphine meet confirmation criteria, they may be reported. The administrative cut-off of 25ng/mL, or the lowest calibrator meeting quality assurance requirements, will be used to determine if the analyte is detected.

3.10.3.9.2 Quantitative Value

Currently, this method is 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, 5.2, 5.8, and 5.10 for quality assurance and reference material authentication requirements.

3.10.3.10.2 Per Analysis Run Quality Requirements

3.10.3.10.2.1 Minimally, a solvent blank must follow the

highest calibrator, as well as precede 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. Controls should not be grouped at the beginning of the acquisition sequence. Rather, controls should be interspersed throughout the sequence.

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

3.10.3.10.2.4 Analysts may combine their samples into a single run to conserve supplies. However, each analyst with samples in the run must independently comply with the control requirements in section 3.10.3.10.2. A third-party reviewer must independently review the central file packet for compliance to method requirements.

3.10.3.10.2.5 If a drug other than Codeine or Morphine is to be identified in full scan acquisition mode, one additional in-run control verifying the extraction of that compound is required. Multiple compounds may be extracted simultaneously.

3.10.3.10.3 Monitoring of Control Values

Once the method has been approved for quantitative purposes, the following is required: upon the completion of analysis, input blood control values on a spreadsheet used to assess uncertainty for this method.

3.10.3.11 ANALYSIS DOCUMENTATION

3.10.3.11.1 Case results are to be recorded in the LIMS system.

3.10.3.11.2 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 or destruction.

3.10.3.11.3 A copy of data for 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.

3.10.3.12 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 Confirmation 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.
4	1-16-2014	Removed reference to quantitation in titles, added option for confirmation by full scan. Amendment to 3.10.3.11 in accordance with new LIMS system. Minor formatting changes.
5	02/13/2014	Clarified that method is not currently approved for quantitation. Clarified that all requirements pertaining to quantitation be suspended until method is approved for quantitation.
6	03/13/2015	Clarified aspiration in the SPE method section. Formatting for continuity. Clarified quality assurance and acceptance criteria; consolidated quality assurance paragraphs. Clarified control requirements; allowed for shared runs. Added control requirement for full scan identification of additional compounds. Added qualitative section in reporting of results. Removed Tune specifications from 3.10.3.6.14, and updated wording to be consistent with other methods.