1. PROTOCOL FOR THE ANALYSIS OF BARBITURATES IN BLOOD

PURPOSE

This protocol outlines the procedure to follow when analyzing blood, serum, plasma or other biological samples for barbiturates. Samples which have been screened positive by a preliminary test, as well as special requests or retest requests will follow the following protocol. Additionally, this protocol can also be used as a screening method.

PLAN

A. Equipment:

(1) GC/MS with a 5% diphenylpolysiloxane, 95% dimethylpolysiloxane, 15/30 meter, 0.25 micron film thickness column.

(2) Positive Pressure Manifold

(3) SPE Column – Silica gel - Co polymeric bonded phase with a hydrophobic cation exchange (CSDAU203 or XRDAH203).

(4) Sample concentrator with UHP Nitrogen

(5) Centrifuge

(6) Vortex mixer / Multi-tube vortex mixer

B. Reagents:

(1) **100 mM Phosphate buffer solution.** Dissolve 1.70 grams of Na_2HPO_4 and 12.14 g $NaH_2PO_4 \cdot H_2O$ in 800 ml of deionized water. Dilute to 1000 ml with deionized water. Mix well. pH should be 5.5-6.0. If necessary, adjust with 100 mM monobasic sodium phosphate (lowers pH) or 100 mM dibasic sodium phosphate (raises pH). Store refrigerated. Stable for six months.

(2) **Deionized Water** *(DI Water)* Label. Stable until consumed.

(3) **Methanol**. Prepare a transfer bottle of ACS/HPLC grade methanol. Label accordingly. Store in glass at room temperature. Stable until consumed.

(4) **100 mM Hydrochloric Acid (HCL).** To 400 ml of deionized water, add 8.4 ml concentrated HCl. Dilute to 1 L with deionized water. Mix well. Stable for 2 years.

(5) **Ethyl Acetate**. Prepare a transfer bottle of ACS/HPLC grade ethyl acetate. Label accordingly. Store in glass at room temperature. Stable until consumed.

(6) **Hexane**. Prepare a transfer bottle of ACS/HPLC grade hexane. Label accordingly. Store in glass at room temperature. Stable until consumed.

(7) **Hexane/Ethyl acetate (50/50).** Prepare fresh daily.

C. Standards: (Store refrigerated. Stable per manufacturer's recommendation or 2 years if prepared in-house):

(1) **1 mg/ml Amobarbital stock standard**. Prepare by weighing out 5.5 mg sodium amobarbital and dissolving in 5 ml of methanol or use purchased 1.0 mg/ml amobarbital stock from Cerilliant (A-020).

(2) **1 mg/ml Butalbital stock standard**. Prepare by weighing out 5.0 mg butalbital free acid and dissolve in 5 ml of methanol or use purchased 1.0 mg/ml butalbital stock from Cerilliant (B-006).

(3) **1 mg/ml Pentobarbital stock standard**. Prepare by weighing out 5.5 mg sodium pentobarbital and dissolve in 5 ml of methanol or use purchased 1.0 mg/ml ampoule purchased from Cerilliant (P-010).

(4) **1 mg/ml Phenobarbital stock standard**. Prepare by weighing out 5.0 mg phenobarbital free acid and dissolve in 5 ml of methanol or use purchased 1.0 mg/ml ampoule purchased from Cerilliant (P-008).

(5) **1 mg/ml Secobarbital stock standard**. Prepare by weighing out 5.0 mg secobarbital free acid and dissolve in 5 ml of methanol or use purchased 1.0 mg/ml ampoule purchased from Cerilliant (S-002).

(6) **Hexobarbital stock internal standard (1 mg/ml).** Prepare by weighing out 5.0 mg hexobarbital and dissolve in 5 ml of methanol or use purchased 1.0 mg/ml ampoule from Cerilliant (H013).

D. Calibrators and Internal Standard. (Store refrigerated. Stable for 2 years):

(1) **100 ng/µl mixed barbiturate calibrator stock solution**. In a 10 ml volumetric flask add 1 ml of each 1mg/ml amobarbital, butalbital, secobarbital, phenobarbital and pentobarbital stock standards. Dilute to volume with methanol.

(2) **Hexobarbital Internal Standard (10 ng/µl).** Prepare by diluting 100 µl of a 1 mg/ml Hexobarbital (Cerilliant H013) stock internal standard with methanol in a 10 ml volumetric flask.

E. Quality Controls: (Store refrigerated)

(1) **Positive Controls**. 1200, 3000, and 8000 ng/ml mixed barbiturate control. Prepared in house from a different lot of stock solution than that used to prepare calibrators or purchased from an external vendor. Additional controls shall be prepared when appropriate, to coincide with any limited sample volumes and/or dilution of case samples.

(2) **Negative Control**. Blank blood prepared in house consisting of 50% saline, 50% packed red blood cells, and 5g sodium fluoride/1g potassium oxalate (per 500 ml prepared blood) will be used as negative control.

F. Solid Phase Extraction (SPE)

(1) ***Sample Preparation***.
Prepare in appropriately labeled culture tubes as follows:

(a) Prepare a set of 500, 1,000, 2,000, 5,000, 10,000 ng/ml calibrators using the above calibrator stock solution in 1mL of blank blood along with 1mL* negative control, positive controls, case samples and additional controls at appropriate volumes and/or dilutions if applicable. Add 50 µl* of Hexobarbital internal standard

*In case samples where a limited amount of blood is received use the same fraction of internal standard as the sample, as an example ½ ml of blood and 25 µl of internal standard. Case samples which must be diluted to fall within the calibration range will receive full internal standard, as an example x2 by using ½ mL sample with 50 µl of internal standard.

(b) Add 1.5 ml DI water and vortex until thoroughly mixed.

(c) Add 1 ml of 100 mM phosphate buffer and vortex until thoroughly mixed.

(d) Centrifuge at 3,500 rpm for 5 minutes.

(2) ***Column Conditioning***
Pass through the column sequentially the following reagents at <1.0 ml/min, or gravity only:

(a) 2 ml of methanol

(b) 2 ml of deionized water

(c) 1 ml of 100 mM phosphate buffer (pH 6.0)

Take care to prevent sorbent from drying out.

(3) ***Sample Application***
Apply sample to column, being careful to not allow the sediment, if present, which will be in the base of the centrifuge tube to pass. Flow rate should be 1 - 2 ml/minute.

(4) ***Column Rinse***
Pass through the column sequentially the following reagents, at 1-2 ml/min:

(a) 3 ml of deionized water

(b) 1 ml of 100 mM HCl

(c) Dry the column for 15 minutes under full pressure.

(d) 1 ml of hexane

(5) Elute Acidic and Neutral drugs

(a) Elute into autosampler vial with 2 ml of 50:50 hexane/ethyl acetate under gravity or <1.0 ml/minute. Check no water is present.

(b) Evaporate to dryness under nitrogen.

(c) Reconstitute with 100 µl of ethyl acetate, vortex (transfer to vial insert if using screw cap autosampler vials), and cap.

G. Data Acquisition and Analysis:

(1) Perform Autotune, fill rinse vials, etc.

(2) Set up a sequence with the calibrators injected first in order to calibrate the instrument used. Subsequent injections to include positive controls, negative control and solvent blanks prior to case samples. For samples requiring dilutions add the appropriate sample multiplier in the sequence table. Load samples onto autosampler according to sequence and have it verified by another analyst before or after analysis but prior to unloading.

(3) The ion ratios and retention times shold be set by a mid-level calibrator.

(4) Analyze using the appropriate method on GC/MS.

H. Results and Acceptability:

(1) Calibration $R^2 \geq 0.99$ and calibrators within 20% of set value.

(2) Positive control within 20% of target concentration.

(3) If the above two criteria are not met the analyte may be reported qualitatively

(4) Negative control < 25% of area count of cutoff calibrator

(5) Retention time within 2% as set from calibrator.

(6) Qualifier ion ratios within 20% as set from calibrator.

(7) Chromatographically acceptable i.e. peak purity ≥90% for target/quantitative ion.

(8) Quantitation ≥ 500 ng/ml; results greater than highest calibrator will be reported qualitatively as such, and samples which included a dilution factor will be reported greater than the highest calibrator multiplied by the applicable dilution factor.

(9) Blank prior to sample < 25% area count of cutoff calibrator

(10)Results will be truncated and documented in case notes to two significant figures