1. PROTOCOL FOR THE ANALYSIS OF AMPHETAMINES IN BLOOD

PURPOSE

The following method describes the quantitation of amphetamine, methamphetamine, methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) in blood, serum, plasma or other biological samples by GC/MS. Samples which have screened positive by a preliminary test, as well as special requests or retest requests will follow the following protocol. Additionally, this protocol may 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) **78:20:2 methylene chloride: isopropanol: ammonium hydroxide Elution Solvent**. Prepare fresh daily. Add ammonium hydroxide to isopropanol, followed by methylene chloride (i.e. per 10 mL of elution solvent add approximately 200 µL ammonium hydroxide). Mix thoroughly, elution solvent should have a turbid appearance when thoroughly mixed.

(6) **2% glacial acetic acid**. To 100 ml of methanol add 2.0 ml glacial acetic acid. Store in glass at room temperature. Stable for 2 years.

(7) **Trifluoroacetic Anhydride TFAA**. Stable until consumed. Crimp cap with breakaway seal after use.

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

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

(1) **1 mg/ml amphetamine (sulfate) stock standard**. Prepare by weighing out 13.5 mg amphetamine sulfate and dissolving in 10 ml of methanol or use purchased 1.0 mg/ml amphetamine stock from Cerilliant (A-007).

(2) **1 mg/ml methamphetamine (hydrochloride) stock standard**. Prepare by weighing out 12.4 mg methamphetamine hydrochloride and dissolve in 10 ml of methanol or use purchased 1.0 mg/ml methamphetamine stock from Cerilliant (M-009).

(3) **1 mg/ml MDA stock standard**. Purchase a 1 mg/ml ampoule from Cerilliant (M-012)

(4) **1 mg/ml MDMA stock standard**. Purchase a 1 mg/ml ampoule from Cerilliant (M-013)

(5) **1 mg/ml D6-Amphetamine stock internal standard**. Purchase a 1mg/ml ampoule from Cerilliant (A-045).

(6) **1 mg/ml D9-Methamphetamine stock internal standard**. Purchase a 1 mg/ml ampoule from Cerilliant (M-091)

(7) **1 mg/ml D5-MDA stock internal standard**. Purchase a 1 mg/ml ampoule from Cerilliant (M-027)

(8) **1 mg/ml D5-MDMA stock internal standard**. Purchase a 1 mg/ml ampoule from Cerilliant (M-029)

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

(1) **10 ng/µl amphetamine, methamphetamine, MDA, MDMA calibrator stock solution**. In a 10 ml volumetric flask add 100 µl of 1 mg/ml amphetamine, methamphetamine, MDA, and MDMA stock standards. Dilute to volume with methanol.

(2) **1 ng/µl amphetamine, methamphetamine, MDA, MDMA calibrator stock solution**. Prepare on day of use from the above 10 ng/µl mixed calibrator stock solution.

(3) **1 ng/µl D6-amphetamine/ D9-methamphetamine/ D5-MDA/ D5-MDMA internal standard**. Add 10 µl each of the 1 mg/ml D6-amphetamine, D9-methamphetamine, D5-MDA and D5-MDMA stock internal standards. Dilute to 10 ml with methanol. (May also prepare larger stock volume by adjusting volumes accordingly to account for equivalent concentrations of each analyte.)

E. Quality Controls: (Store refrigerated)

(1) **Positive Controls. 30, 60, and 400 ng/ml mixed amphetamines**. Prepared on day of use 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 calibrators at 10, 25, 50,100, and 500 ng/ml using the above calibrator stock solutions in 1 mL of blank blood along with 1 mL* negative control, positive controls, case samples and additional controls at appropriate volumes and/or dilutions if applicable. Add 50 µl* of working internal standard to each tube.

*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 of water and vortex each tube until thoroughly mixed.

(c) Add 1.0 ml of 100 mM phosphate buffer and vortex each tube 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 by gravity only:

(a) 2 ml of methanol

(b) 2 ml of deionized water

(c) 1 ml of 100 mM phosphate buffer

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 about 1.0 ml/minute or gravity only.

(4) ***Column Rinse and Elution***
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) 2 ml methanol

(d) Dry column under full pressure (20-25psi) for 15 minutes.

(e) Elute by gravity with 1.5 ml of freshly prepared 78:20:2 methylene chloride: isopropanol: ammonium hydroxide solution directly into appropriately labeled microvials

(5) ***Derivatization***

(a) Add 25 µl of 2% glacial acetic acid into each microvial.

(b) Evaporate samples to dryness under nitrogen.

(c) To the microvials add 50 µl of ethyl acetate, vortex and then add 50 µl of TFAA. Crimp using the red PTFE crimp caps, and vortex.

(d) Derivatize at room temperature for at least 20 min.

(e) When derivatization is complete, remove cap and dry down under nitrogen.

(f) Reconstitute with 60 µl of ethyl acetate. Crimp cap microvials and vortex.

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 negative control, positive controls, (positive controls should be included throughout the batch, i.e. beginning, mid-run and end of run) and solvent blanks prior to case samples. For samples requiring dilution, 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 should 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) Blank prior to sample < 25% area count of cutoff calibrator

(9) Quantitation ≥ 10 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.

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