1.0 PURPOSE:

Sympathomimetic Amines (Amphetamines, and its structural analogues; "SMA's") can cause profound behavioral changes and have high potential for abuse due to both chemical and psychological dependence. MDMA and MDA are metabolized to Methamphetamine and Amphetamine respectively. SMAs are water soluble and excreted in the urine. Blood and urine samples, screening positive for SMAs, are extracted using a solid phase extraction column, co-extracting materials are washed from the column and any SMAs that may be present are eluted using a methylene chloride/isopropanol/NH4OH mixture. After evaporation of the solvent, the extracted drug is derivatized with TFAA. Derivatized samples are analyzed by GC/MS.

The presence of SMAs is qualitatively evaluated by 3-ion SIM using retention time, and 2 qualitative ion ratios as the basis for identification, in comparison to co-extracted calibrator and control samples.

2.0 SPECIMEN

A. JusticeTrax can be used to generate a worklist for specimens requiring confirmation for BE/Cocaine.

B. All evidence transfers, either between individuals or between an individual and a storage location must be documented on the Chain of Custody for the case, either in the LIMS, or on hard-copy COC document maintained in the Case Jacket.

C. When not in the sampling or aliquot process samples will be maintained in locked storage within the Toxicology unit.

D. Samples must be maintained in such a manner so that they are protected from contamination or deleterious change. Depending on the nature of the sample, this may mean refrigeration or freezing when not in the analytical process.

E. When analysis of samples in the Toxicology unit is complete, they must be maintained "Under Proper Seal." Refer to SOP TX-19 for further guidance.

F. Samples are maintained in the Toxicology Section for a minimum of 8 weeks after case is completed, in the absence of notification of any legal action, or

other reason to maintain the samples. Samples from fatalities and sexual assaults are maintained indefinitely by the DSS. Cases with requests for retention are maintained by the laboratory based on the request. Upon request from the courts or submitting agency the evidence will be returned to the submitting agency.

3.0 EQUIPMENT:

A. GC/MS and associated data station/computer (HP6890/5973, Agilent Technologies 7890A, AT-7890B, AT-5975, 5977 or equivalent)
B. General laboratory glassware and equipment
C. Solid phase extraction manifold and associated vacuum equipment.
D. Analytical evaporator (Zymark Turbovap or equivalent)
E. UCT; ZSDAU020 “Clean Screen” extraction columns (or equivalent)

4.0 REAGENTS

A. **Reagents available as stock items** (Sigma or J.T. Baker Reagent Grade or equivalent unless otherwise specified):
   1. Methanol (Baker HPLC grade or equivalent)
   2. Ammonium Hydroxide (Baker reagent grade or equivalent)
   3. Ethyl acetate (Baker HPLC grade or equivalent)
   4. Methylene Chloride (Baker reagent grade or equivalent)
   5. Isopropanol (Baker reagent grade or equivalent)
   6. Triflouroacetic anhydride (TFAA) (Pierce or equivalent)
   7. Blank Blood (may be acquired from a hospital blood bank, or American Red Cross or other similar source)
   8. Drug free urine
   9. Deionized water (DIW; Millipore or equivalent In-House supply)
   10. Glacial Acetic acid (Baker reagent grade or equivalent)
   11. Sodium Phosphate Dibasic (Na_2HPO_4).
   12. Sodium Phosphate Monobasic (NaH_2PO_4).
   13. Hydrochloric acid (Baker reagent grade or equivalent)

B. **Reagents prepared in the Toxicology Laboratory**:
   1. <u>1 M Acetic acid:</u>
      a. Add 50 ml of DIW to a volumetric flask
      b. Add 28.6 ml of glacial acetic acid
      c. Mix, QS to 500 ml
   2. <u>0.1 M Acetic Acid</u>
      a. Add 50 ml 1 M acetic acid to a volumetric flask

b. Mix QS to 500 mL DIW (or equivalent ratio)

3. 0.1 M Phosphate Buffer pH 6
   a. Combine 48.6 g Na_2HPO_4 and 6.8 g NaH_2PO_4
   b. QS to 4000 ml using DWI

4. 1% Methanolic acid
   a. To 50 ml of DWI in a volumetric flask
   b. Add 1 ml HCl
   c. Mix, QS to 100 ml Methanol

5. Methylene chloride/Isopropanol/ammonium hydroxide (39/10/1)
   a. Prepare volume as appropriate
   b. Note: must be prepared fresh each day of use

C. Calibration Standards / In-house Control Standards/Internal Standard

Preparation of all calibrator and control solutions is documented in the 'Calibrator and Control Preparation' book, maintained in the Toxicology unit.

1. Stock Calibrator and Control Solutions:
   a. Comprised of Amphetamine, Methamphetamine, MDA, and MDMA. Reference material standards target analyte drug stocks are obtained from Sigma/Aldrich, Cerrilient, Lipomed, Grace or other equivalent manufacturers. In the form of 1 mL ampules of 1.0 mg/ml or 100 ug/mL.
   b. Composition of the calibration/control (analyte/concentration) will be dependent on the need of the casework.
2. Working Standard Solution 10 ug/mL
   a. Into a 10 mL volumetric flask partially filled with methanol
   b. Pipette 100 ul of each reference standard (1 mg/ml)
   c. QS with methanol, mix
   d. Store in freezer/refrigerator
   e. Stable for approximately 1 year, then it must be revalidated after that year

3. Diluted Working Standard Solution 1.0 ug / mL
   a. Into a 10 ml volumetric flask partially filled with methanol
   b. Pipette 1 mL of working stock solution 10 ug/mL
   c. QS with methanol, mix

d. Store in freezer/refrigerator
e. Stable for approximately 1 year, then it must be revalidated after that year

4. Internal Stock Standards:
Comprised of Amphetamine D6 or related deuterated SMA; (Cerrilient, Lipomed, Grace or other equivalent)
a. Working Internal Standard SMA Deuterated SMA: 100 ng/mL
b. Into a 10 mL volumetric flask partially filled with methanol
c. Pipette 1 ml of each reference standard (1 mg/mL)
d. QS with methanol, mix
e. Store in freezer/refrigerator
f. Stable for approximately 1 year, then must be revalidated after that year

D. Controls

1. Urine Qualitative
a. The target concentration for the positive urine control is 500 ng/mL
b. Pipette 50 ul of the working standard solution (10 ug/mL) into 1.0 mL blank matrix
c. Continue to follow sample preparation procedure.

2. Blood Quantitative
a. Refer to table in procedure section
b. Continue to follow sample preparation procedure

3. Quality Controls
Calibrators and controls must be independently prepared from a separate initial dilution or obtained from other sources. When available commercial references, controls will be purchased from an outside vendor. If commercial controls are not available, in-house controls should be prepared from a different provider. When only one supplier is available, a lot different from the calibrator should be used. At the least, when there is only one source, a separate preparation, different from the calibration standard may be used.

4. Urine Control DAU LC2, product #50703, UTAK Laboratories or equivalent
a. Remove cap from vial

b. Reconstitute control material by adding 10 mL of DWI, using a 10 mL volumetric pipette
c. Replace cap and let sit 10-15 minutes
d. Swirl gently 3-4 minutes to ensure homogenous mixture
e. Swirl gently each time an aliquoted is removed to ensure a homogenous mixture
f. Assay control material in the same manner as case specimens
g. For quantitative assays, record the results obtained on the Excel quality control chart in the DSS S drive.
h. Store reconstituted control material refrigerated at 2-8°C, stable for 25 days after reconstitution.

E. Validation of Reagents: Acceptable performance of all batch control materials and overall batch acceptability (although individual samples may fail) is considered as validation of reagents. Validated reagents are marked with a green dot, detailing the specific procedure for which the reagent was validated, and the batch on which that process was documented. Newly prepared reagents may be evaluated for validity on an analytical batch, prior to any consideration of sample results. Reagents so validated are marked with a green sticker as noted above. Preparation of reagents, and their validation is documented in the Toxicology Section Reagent Preparation Validation Logbook, maintained in the Toxicology unit.

5.0 Procedure

A. Sample Preparation Blood/Urine:

1. Pipette 1 mL into an appropriately labeled 16 x 100 borosilicate culture tube
2. Add 20 ul of SMA deuterated working internal standard.
3. Add 3 mL phosphate buffer (pH 6.0)
4. Mix
   a. Note: If blood sample proceed to steps 5.1.5 and 5.1.6
5. Sonicate for 15 minutes
6. Centrifuge 10 minutes at ~5000 rpm
7. The chart below may be used as a guide, pipette 1 mL of sample, blank, calibrator and control to each appropriately labeled 16 x 100 screw top culture tube.

Calibrator Concentration ng/mL	uL Working standard (10 ug/mL)	uL diluted Working Standard (1 ug/mL)	Blank Blood uL

o-blank	0	0	1000
20	-	20	980
50	-	50	950
100	-	100	900
200	20		980
500	50		950
1000	100		900
QC Concentration	uL Stock Control (10 ug/mL)		
250	25		975
	Utak Control		
UTAK Low	500		500

8. Label SPE tubes to correspond with each culture tube. Load in the manifold.
   a. Note: Cases requiring reanalysis due to high concentrations (i.e. initial analysis is greater than the high control), should be diluted with 0.1 M pH 6.0 Phosphate buffer as appropriate. The initial quantitative values may be used as a guide for the dilution process The dilution process shall be documented in the case jacket.
   b. Note: This procedure utilizes controls prepared (spiked) in blank blood and/or urine (as appropriate to batch makeup) as follows: Each quantitative assay must incorporate a high and low control for each analyte. Blood matrix controls may be used to validate urine results, but not the reverse. Controls are prepared by addition of SMA drugs from validated stock solutions to blank sample matrix aliquots, prior to extraction, (details in Procedure below). Acceptable quantitative control performance is target value +/- 20%.

B. Extraction:
1. Condition the columns:
   a. 1 x 3 mL methanol; drain (vacuum assist)
   b. 1 x 3 mL DIW; drain (vacuum assist)
   c. 1 x 1 mL 100 mM Phosphate buffer; drain (vacuum assist)
   d. DO NOT LET COLUMN GO DRY!

2. Transfer contents of each tube to the appropriately labeled SPE tube, and allow gentle drop wise flow until the level reaches the top if the column bed.
3. Wash column
   a. 1 x 2 mL DIW; (vacuum assist)
   b. 1 x 2 mL 100mM Acetic acid; (vacuum assist)
   c. 1 x 3 mL Methanol; (vacuum assist)
   d. Dry column (15 minutes at > 10 inches Hg)
4. Position appropriately labeled 13x100 test tubes under each SPE column, with clean thru tip inside collection test tube.
5. Elute analytes:
   a. 1 x 3 mL CH2Cl2/Isopropanol/NH4OH (78:20:2).
   b. Collect Eluate at 1 to 2 mL/minute.
   c. Add 1 drop of 1% methanolic HCl to each calibrator, control, sample and blank tubes.
6. Remove receiver tubes and evaporate to dryness at < 40° C using a gentle flow of Nitrogen (Turbovap).
7. Reconstitute residue with 150 ul of ethyl acetate and transfer to GC/MS vial with limited volume insert. Add 10 micro liters TFAA (Trifluoroacetic anhydride).
   a. Note: Do not evaporate TFAA solution
   b. Note: Departure from procedures as specified in this SOP is not anticipated. Should an issue arise that may require such a departure, the issue must be reviewed and approved by with the Unit Lead and Deputy Director. Depending on the nature of the deviation DSS customers may need to be consulted, see GL-20 for guidance.

6.0 Chromatography and Mass Spectroscopy

A. Instrument and Setup. (see appendix 1 below):
1. GC/MS/Auto sampler: (Hewlett-Packard 6890/5973, or equivalent)
2. Column: 30M RTX-5 or 30M RTX-1 (MS) (0.25 mm ID; 0.25 micron film) (or equivalent).
3. Inj. Temp. 250 °
4. Det. Temp. 300 °
5. Oven (initial): 80°, 25 ° /min to 300 °, (1.50 min hold).
6. 13.80 min total run time
7. 1 ul injection

B. Injection sequence; Samples are injected on the GC/MS generally in the following sequence:

1. Urine Qualitative
   a. Solvent blank
   b. Positive urine calibrator/standard 500 ng/mL (i.e. In-house control)
   c. Urine blank
   d. Utak control
   e. Case samples
   f. End batch with calibrator or control

C. Blood Quantitation Multipoint Calibrators: Number of calibrators and concentration can change based on analyte of interest and the expected therapeutic range.
1. Solvent blank
2. Priming calibrator
3. Blood blank
4. Calibrator 1 (20 ng/ml)
5. Calibrator 2 (50 ng/mL)
6. Calibrator 3 (100 ng/mL)
7. Calibrator 4 (200 ng/mL)
8. Calibrator 5 (500 ng/mL)
9. Calibrator 6 (1000 ng/mL)
10. Blood blank
11. In-house and or UTAK Low control (if available)
12. Case samples
13. Run set of low and high controls midway through the batch as appropriate for longer batches)
14. End batch with a set of controls.

D. File name – Include case number (including year) in each sample data file.
E. Verify the sequence, verify the vial positions in the auto sampler tray match the sequence.

7.0 Detection and Identification:

A. Determination of the presence of SMAs in the sample extract is by appearance and ratio of the 3 ions characteristic of each species at the appropriate retention time. Hence, both retention time (a GC characteristic) and fragmentation pattern and ratio (MS characteristics) are used as the basis of qualitative identification. Retention time must be within 5% of the

corresponding analyte in the calibrator injection, and the ion ratios for both qualitative ions must be within 20% of the corresponding ratio calibrator sample. Qualitative identification of each analyte is independent.

B. Calibration:

1. Calibration is not performed on urine samples
2. Calibration for each batch is done independently through the use of method specific controls.

C. Quantitation:

1. Quantitation is accomplished by the comparison of the response ratio of the analyte in a specific sample, to the response ratios of the calibrators as expressed as a "standard curve". The concentration of the analyte in the sample is then extrapolated, from the standard curve, and corrected for any dilution that may have been performed to facilitate the analysis of relatively concentrated blood samples.
2. For multi-point calibrations, the criteria for acceptability of the calibration and for individual calibrators is that when the values are read back against the final calibration curve they should generally be within +/- 20% of their value. A slightly wider acceptance value (e.g. +/-25% or 30%) is acceptable for calibrators that approach the LOQ of the assay.
3. Note: The available quantitative range of this procedure is (in terms of amount of drug injected on the instrument (thereby allowing for appropriate calculation of diluted samples) is defined for , and validated on each batch by the high and low control, and the acceptable performance of each.
4. The criteria for a valid calibration GC/MS linear regression "r_2" value for a 3 point curve is ≥ 0.98. A significant change in the slope of the calibration line, monitored between runs, may indicate that corrective action needs to be taken.
5. When more than 3 calibration points are used, one point may be removed if it failed to fall into the acceptable quantitative range. If two or more points need to be removed consult with the Unit Lead if any results can be accepted. On a case-by-case basis, results may be reported qualitatively or semi-quantitative.

8.0 QUALITY CONTROL AND RUN EVALUATION:

A. Verification of Vial Sequence; Vial sequence is checked both before and after the injection of samples. The check after the samples are injected is documented on the run summary sheet.

B. Chromatography - Evaluation and Acceptance Criteria; Chromatographic quality is evaluated for each peak. General guidelines are that peaks should be symmetrical, and resolved to baseline on at least one side, with 90% resolution on the other side. Significant departures from those guidelines may be experienced with forensic samples. In many cases, chromatographic quality will warrant rejection of the chromatographic run, or specific samples, by the operator. Any such action should be clearly documented on the batch summary sheet. Questionable peak shape, resolution, or other problems with chromatography can be discussed with the Unit Lead or Deputy Director.

C. Evaluation of Potential Carryover; Carryover in the chromatogram quality is evaluated by injection of a blank sample immediately following the calibrator. Carryover of greater than 2% (10 ng/mL) requires batch rejection, and remedial action for the instrument (e.g. replacement of injector insert, new septum and perhaps column trim or even replacement). Demonstrated carryover If less than 2% will require operator consideration with regard to the potential for effects on specific samples, and may require re-extraction of specific samples. In practice, when a question of potential carryover exists, coming from the previous case sample containing a high concentration of an analyte, the potentially affected sample replicates may be repeated at the end of the batch.

D. Evaluation of Controls: Positive and negative controls are evaluated to allow for procedural batch acceptability.

E. Qualitative: Control results are documented and evaluated on the batch summary sheet.

F. Qualitative Results: Controls must demonstrate the target analyte with acceptable chromatography and spectral characteristics.

G. Quantitative: Assays must have controls to verify the calibration and to monitor its stability. Each batch must have at least 10% controls including a positive and negative. The controls can be re-injected in the middle and end

to demonstrate the stability of the calibration. Acceptable results are the mean target +/-20% or +/-2 standard deviations.

H. Linearity: Linearity of the calibration curve is demonstrable in each batch, for each analyte as a function of quantitative results of control materials.

I. Sensitivity- LIMITS OF DETECTION (LOD) and QUANTITATION (LOQ): For the purposes of this procedure, the LOD and LOQ are defined as equal to the lowest concentration of the lowest control. Qualitative Identification and/or Quantitative analysis below the concentration of the low control may be accepted on a case by case basis with the concurrence of the analyst, technical reviewer, and Unit Lead.

J. Accuracy and Precision: Precision of the procedure is evaluated on a batch by batch basis or by repeat analyses of control or PT materials. Accuracy is expressed as a mean (absolute value) percentage difference between mean quantitative value of 10 reps of the specific control, and the target value. Precision is expressed as the CV of that value

K. Specificity: Specificity is a function of both the resolution of target analyte during the analytical process, and the mass spectral fragmentation that analyte molecules undergo during the instrumental analysis. There has been no report of any material either than SMAs that elute within 5% if the retention time of known standard materials, and produce the same fragmentation ions and ratios.

9.0 Reporting of Results: SMA runs are performed as part of GC/MS batches, containing controls and calibrators.

A. Once the batch is completed and the data is complied, the batch undergoes a batch review (refer to SOP TX-5 for guidance). Once the batch is accepted the results are entered into JusticeTrax. Wherever possible, analytical results must be reviewed with reference to whatever case history or other information is available.

B. Procedural Uncertainty is reported with all quantitative results, and is calculated and tabulated annually for each analytical method, (See SOP TX-19 section 6.3).

10.0 SOURCES OF ERROR

A. The utilization of 3-ion SIM methodology, with reference to procedural, controls and calibrators yields qualitative drug identification with essentially no uncertainty. Urine drug analyses are reported only as qualitative results.

11.0 REFERENCES:

A. Clarke's Isolation and Identification of Drugs. 2nd Edition
B. UCT United Chemical Technologies "Solid phase extraction methods

Appendix I:

GC/MS temperature program specifications. These are general parameters that may be adjusted based on instrument used or maintenance performed on any given instrument. Proper response of calibrators and controls work to demonstrate the appropriateness of the method.

Parameter			
Initial temp	80° C		
Initial Time	0.00min		
Ramps Rate/final temp/final time	rate	temp	time
	25.0	300	5.00
Post temp	80°c		
Post time	0.00		
Run Time	13.8 min		
Front inlet			
Equilibration time	0.5 min		
Mode	Pulsed split less		
Initial temp	250°c		
Constant Flow	1.0 mL/ min		
Pressure	9.34 psi		
Pulse pressure	35 psi		
Pulse time	0.50 min		
Purge flow	40.0 mL/ min		
Total flow	44.1 mL/min		
Gas type	Helium		
Injection volume	1microliter		
Post injection washes Solvent A / Solvent B	Solvent A -2 Solvent B - 2		
Tune file	ATUNE.U		
Acquisition mode	SIM		
Solvent delay	3.3 min		
MS Quad	150°c max 200		

MS Source	230°c max 250

Rev. #	History
3	Corrected typographical errors Loosened Instrument acquisition parameters and column choice restrictions. Updated choice of using In-house or commercial controls.