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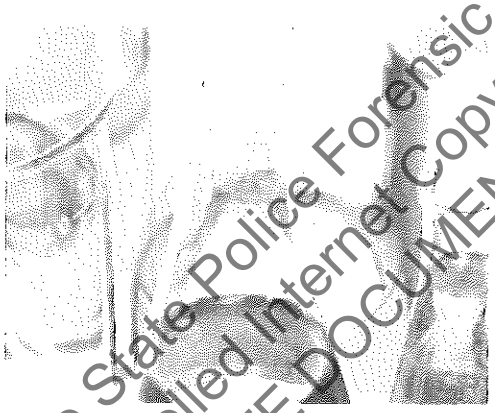

Discipline/Name of Document: Toxicology
3.10.2 – Extraction and Quantitation of Methamphetamine and Amphetamine from Blood Employing the Bond Elut Certify™ Extraction Column (**FOR QUALITATIVE USE ONLY)**

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Quality Manager

7/28/08
Date Signed

Section Three
Blood Toxicology

3.10 SPE Methods for Quantitative GC/MSD Confirmation

3.10.2 Extraction and Quantitation of Methamphetamine and Amphetamine from Blood Employing the Bond Elut Certify™ Extraction Column

3.10.2.1 BACKGROUND

Amphetamine dates back to 1887. It was used freely as a nasal decongestant, appetite suppressant, and to treat disorders such as narcolepsy in the early part of the 20th century until its potential for abuse was fully realized.[4,5,6] The use of amphetamine and methamphetamine to treat narcolepsy, attention deficit disorder and obesity continues in a more regulated environment. Amphetamine (figure 1) and Methamphetamine (figure 2)) are phenethylamines structurally related to norepinephrine and epinephrine, respectively.

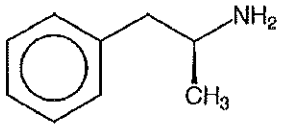

figure 1

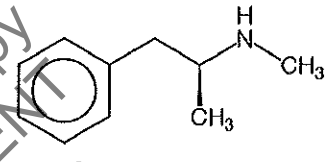

figure 2

The blood concentrations of methamphetamine and amphetamine should be considered in conjunction with all available information to determine the degree and nature of an individual's impairment.[2,3] Therapeutic levels for legitimate methamphetamine and amphetamine use are one to two orders of magnitude less than abuse and toxic levels.[6]

Consult provided references for additional information regarding the pharmacology of these compounds.

3.10.2.2 PRINCIPLE

Methamphetamine and amphetamine are recovered through the application of the Varian Bond Elut Certify® solid phase extraction (SPE) cartridge. The Bond Elut Certify® SPE cartridge contains a sorbent which utilizes cation exchange and non-polar mechanisms to recover methamphetamine and amphetamine from blood. Following the addition of deuterated internal standard mixture, the blood proteins are precipitated with cold acetonitrile. Following centrifugation, the supernatant is decanted and made basic with a 100mM phosphate buffer (pH 6). The sample is loaded onto the SPE cartridge that has been conditioned with methanol and a 100mM phosphate buffer (pH 6). The methanol conditioning opens up the coiled hydrophobic portion of the sorbent so that it interacts with the polar, buffered blood

matrix. The addition of the buffer removes excess methanol and creates an environment similar to the matrix thus allowing for optimal interaction between the sorbent and the analytes of interest. The analyte is retained by ionic interaction of the cationic functional groups present on the drug and the anionic sulfonic acid exchanger on the sorbent.

The cartridge is subsequently washed with 100mM acetic acid followed by methanol, to selectively remove matrix components and interfering substances from the cartridge. The wash also disrupts the hydrophobic and adsorption interactions leaving behind the ionically bound material. Next, the sorbent is thoroughly dried to remove traces of aqueous and organic solvents which could adversely affect the analyte recovery. When the sorbent is dry, the analytes of interest are recovered from the cartridge with alkaline ethyl acetate. The alkaline environment serves to disrupt the ionic interactions of the analyte with the sorbent and the methanol disrupts the hydrophobic interactions. Following the elution from the SPE cartridge the evaporated extract is acylated for confirmation on the GC/MSD. The quantitation is accomplished through the use of a deuterated internal standard and a five-point calibration curve. This method is based on the method utilized by the Bioaeronautical Sciences Research laboratory.[1]

3.10.2.3 EQUIPMENT AND SUPPLIES

3.10.2.3.1 Varian Bond Elute Certify® SPE Cartridge
Product No: 1210-2051 (Laboratory Robot Compatible (LRC)) or 1211-3050 (Straight barrel) or equivalent
Sorbent type: Mixed mode octyl (C8) and benzenesulfonic acid (SCX), Sorbent mass: 130mg, Particle size: 40 µm

3.10.2.3.2 Drybath or laboratory oven

3.10.2.3.3 Evaporative concentrator equipped with nitrogen tank.

3.10.2.3.4 Vacuum manifold/pump

3.10.2.3.5 Tube rocker

3.10.2.3.6 Vortex mixer

3.10.2.3.7 Laboratory centrifuge capable of 3400rpm

3.10.2.3.8 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.

3.10.2.3.9 16 x 100mm round bottom glass tube

3.10.2.3.10 Screw Cap for 16mm O.D. tube

3.10.2.3.11 GC/MS Automated Liquid Sample (ALS) vials

3.10.2.3.12 GC/MS Vial Microinsert

3.10.2.3.13 GC equipped with a mass selective detector and a nonpolar capillary column with a phase composition comparable to 95%-dimethyl-polysiloxane with 5%-diphenyl.

3.10.2.4 REAGENTS

Refer to manual section 5.12 for solution preparation instructions.

3.10.2.4.1 Deionized/distilled (DI) water
3.10.2.4.2 Methanol (Certified ACS grade or better)
3.10.2.4.3 Hexane (Certified ACS grade or better)
3.10.2.4.4 Ethyl Acetate (Certified ACS grade or better)
3.10.2.4.5 Acetonitrile (Certified ACS grade or better)
3.10.2.4.6 Ammonium Hydroxide (Certified ACS grade or better)
3.10.2.4.7 Concentrated HCl (Certified ACS grade or better)
3.10.2.4.8 1% HCl in Methanol
3.10.2.4.9 100mM Phosphate Buffer (pH 6.0)
3.10.2.4.10 100mM Acetic Acid
3.10.2.4.11 Pentafluoropropionic acid anhydride (PFAA)

3.10.2.5 QUALITY ASSURANCE MATERIAL

3.10.2.5.1 Drug Stock Solutions

3.10.2.5.1.1 **1 mg/mL Calibrators and Controls**
(±)-Methamphetamine
(±)-Amphetamine

The source of a corresponding calibrator and control must be obtained from a different vendor.

3.10.2.5.2 Working Drug Solutions

3.10.2.5.2.1 10ng/µL
Add 100µL each 1mg/mL Amphetamine and Methamphetamine Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. Store remaining stock solution in ALS vial in freezer.

3.10.2.5.2.2 1ng/µL
Add 1mL 10ng/µL working drug solution to ≅5mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.10.2.5.2.3 Working solutions are stable for 6 months when stored at 4°C.

3.10.2.5.3 1mg/mL Internal Standard Stock Solutions
(±)-Methamphetamine-D_8
(±)-Amphetamine-D_8

3.10.2.5.4 10ng/µL Working Internal Standard Solution

Add 100µL each 1mg/mL Amphetamine-D_8 and Methamphetamine-D_8 Stock Solution to ≅9mL Methanol in a 10mL volumetric class A flask. QS to 10mL. Store remaining stock solution in ALS vial in freezer.

3.10.2.5.5 Vendor Obtained Whole Blood Controls

3.10.2.5.5.1 **Negative Whole Blood**

3.10.2.5.5.2 **Positive Whole Blood**

Control containing Amphetamine and Methamphetamine each at a specified target concentration. Refer to package insert for verified value and expected range.

3.10.2.6 PROCEDURE

3.10.2.6.1 Initial set-up

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

3.10.2.6.2 Calibrator Preparation

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

3.10.2.6.2.1 Add 2mL of negative whole blood to five screw-top extraction tubes. Use the same lot number of blood as negative control.

3.10.2.6.2.2 Add the volume of working 1ng/µL Amphetamine and Methamphetamine mixed reference material as indicated in the chart below.

Level	Desired ng/mL	µL Working Reference material
1	25	50
2	50	100

3.10.2.6.2.3 Add the volume of working 10ng/µL Amphetamine and Methamphetamine mixed

reference material as indicated in the chart below.

Level	Desired ng/mL	µL Working Reference material
3	100	20
4	250	50
5	500	100

3.10.2.6.3 Positive Control Sample Preparation

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

3.10.2.6.3.1 Add 2mL of negative whole blood to two screw top tubes. Use the same lot number of blood as negative control.

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

Desired ng/mL	µL Working Control
75	15
300	60

3.10.2.6.4 Negative Control Sample Preparation

Add 2mL of negative whole blood to screw top tube.

3.10.2.6.5 Case Sample Preparation

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

3.10.2.6.5.2 Add 2mL neat or diluted sample to labeled screw top tube.

3.10.2.6.6 Internal Standard Addition

3.10.2.6.6.1 To calibrators, controls and case samples, add 20µL of internal standard mix (100ng/mL).

3.10.2.6.6.2 Cap tube and vortex tube briefly.

3.10.2.6.6.3 Allow tubes to stand 15 to 30 minutes for sample equilibration.

3.10.2.6.7 Protein Precipitation

3.10.2.6.7.1 While vortexing, add 5mL cold acetonitrile to case, calibrator and control samples.

3.10.2.6.7.2 Cap tubes and rock samples for approximately 15 minutes. Tubes should be at room temperature. Remove from rocker and place samples into centrifuge and let stand for 5 minutes.

3.10.2.6.7.3 Centrifuge at 3400 rpm for 10 minutes.

3.10.2.6.7.4 Transfer organic supernatant into second labeled tapered bottom centrifuge tube.

3.10.2.6.7.5 Transfer tube to TurboVap and evaporate under nitrogen at approximately 37°C to approximately 1mL. *Do not allow extract to go to dryness.*

3.10.2.6.7.6 To evaporated extract add 2mL 100mM phosphate buffer (pH 6). Vortex to mix.

3.10.2.6.7.7 If needed, centrifuge an additional 5 minutes to remove blood fragments or foam.

3.10.2.6.8 SPE Column Preparation

3.10.2.6.8.1 Insert labeled 200mg CLEAN SCREEN® Extraction column in the vacuum manifold.

3.10.2.6.8.2 Add 2mL methanol to the column. Aspirate at ≤ 3 in. Hg to prevent sorbent drying.

3.10.2.6.8.3 Add 2mL 100mM Phosphate buffer (pH 6.00) to the column. Aspirate at ≤ 3 in. Hg.

3.10.2.6.9 Blood Extract Loading

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

3.10.2.6.10 Column Clean-up

3.10.2.6.10.1 Add 1mL 100mM Acetic Acid.

Aspirate at ≤ 3 in. Hg.

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

3.10.2.6.10.3 Add 6mL methanol.

3.10.2.6.11 Pre-Elution Dry Disc

Increase vacuum to ≥10 in. Hg (≥34 kPa) for ≅2 minutes.

3.10.2.6.12 Compound Elution

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

3.10.2.6.12.2 Add 4mL 2% NH_4OH in ethyl acetate elution solvent to the column.
Collect with gravity flow or apply minimal vacuum.

3.10.2.6.12.3 Add 50µL 1% HCl in Methanol into each tube to minimize analyte loss.

3.10.2.6.13 Eluate Evaporation

Transfer centrifuge tube to TurboVap. Take solvent to dryness, under a gentle stream of nitrogen at ≤ 40°C.

3.10.2.6.14 Derivatization

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

3.10.2.6.14.2 Add 50µL PFAA.

3.10.2.6.14.3 Cap tubes and vortex briefly.

3.10.2.6.14.4 Place tubes in 70°C dry bath or oven for 20 minutes.

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

3.10.2.6.14.6 Return tubes to TurboVap and evaporate to dryness under nitrogen at approximately 37°C.

3.10.2.6.14.7 Reconstitute extract with 50µL ethyl acetate.

3.10.2.6.14.8 Transfer reconstituted extract to labeled GC/MSD ALS vial with microinsert.

3.10.2.6.15 Preparation for GC-MS Run

3.10.2.6.15.1 Perform an AUTOTUNE and TUNE EVALUATION.

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

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

3.10.2.6.16 GC-MS Calibration Curve

3.10.2.6.16.1 The calibration curve should be established with a minimum of five data points.

3.10.2.6.16.2 All reported results must be bracketed by calibrators.

3.10.2.6.16.3 Calibrators should be analyzed in order of increasing concentration.

3.10.2.6.16.4 The least squares line resulting from the analysis of calibrators must have a coefficient of correlation of ≥0.99.

3.10.2.6.16.5 If calibration reference materials are run in duplicate, it is not required that duplicate calibration points are included as long as the linearity requirement is met.

3.10.2.7 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.

3.10.2.7.1 GC Temperature Parameter

Injection Port: 250°C

3.10.2.7.2 MSD Instrument Parameters
Detector/Transfer Line: 280°C

3.10.2.7.3 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.

3.10.2.7.4 MS SIM Parameters

Analyte	Target Ion	Qualifier Ion 1	Qualifier Ion 2
Amphetamine	190	118	91
Amphetamine-D8	193	126	96
Methamphetamine	204	160	118
Methamphetamine-D8	211	163	123

3.10.2.8 REPORTING CRITERIA

3.10.2.8.1 Qualitative Chromatographic Criteria
Acceptable retention time window established by calibrators is ± 0.2 minute.

3.10.2.8.2 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%.

3.10.2.8.3 Quantitative Mass Spectral and Control Criteria

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

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

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

3.10.2.8.3.4 Administrative limit of detection (LOD) for Amphetamine and Methamphetamine is 25ng/mL. Results < this LOD should be reported as negative unless there are extenuating circumstances. The Toxicology Discipline Leader must be consulted to evaluate exceptions.

3.10.2.8.3.5 If the concentration exceeds the calibration range, the sample needs to be appropriately diluted with negative whole blood for reanalysis.

3.10.2.9 REPORTING OF RESULTS

3.10.2.9.1 Quantitative Value

Analysis results should be truncated and reported out without decimal places.

3.10.2.9.2 Uncertainty Value

Based on the current uncertainty assessment, the +/– range should be included on the analysis report. Refer to method quality monitoring spreadsheet for current uncertainty figure.

3.10.2.10 QUALITY ASSURANCE REQUIREMENTS

3.10.2.10.1 General

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

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

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

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

3.10.2.10.1.5 Refer to toxicology manual section 5.10 for reference material authentication requirements.

3.10.2.10.2 Per Analysis Run Quality Requirements

3.10.2.10.2.1 Solvent blank should follow the highest calibrator as well as each case sample.

3.10.2.10.2.2 A minimum of two vendor obtained prepared blood controls and the spiked blood controls described in section 3.10.3.6.3 must be run per batch of samples.

3.10.2.10.2.3 In addition to the four blood controls indicated above, for each additional 10 case samples, one blood control must be run. The preparation of spiked controls is outlined in analytical method section 3.10.2.6.3. Additional concentrations may be used.

3.10.2.10.3 Monitoring of Control Values

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

3.10.2.11 ANALYSIS DOCUMENTATION

3.10.2.11.1 A packet containing original data for controls and calibrators will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed until archiving.

3.10.2.11.2 A copy of controls and calibrators need not be included in individual case files. When necessary, a copy of the control and calibrator printouts can be prepared from the centrally stored document.

3.10.2.12 REFERENCES AND RECOMMENDED READING

3.10.2.12.1 Chaturevidi, A.K., Cardona, P.S., Soper, J.W. and Canfield, D.V., *Distribution and Optical Purity of Methamphetamine Found in Toxic Concentration in a Civil Aviation Accident Pilot Fatality*, U.S. Department of Transportation Federal Aviation Administration Technical Report, December 2004.

3.10.2.12.2 Logan, B.K., *Methamphetamine - Effects on Human Performance and Behavior,* Forensic Science Rev. 14(1/2): 133-151, 2002.

3.10.2.12.3 Logan, B.K., *Methamphetamine and Driving Impairment.* J Forensic Sci, 1996, 41(3):457-464.

3.10.2.12.4 Drummer, O.H., *Stimulants*, pp. 49-96. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

3.10.2.12.5 Moore, K.A., *Amphetamine/Sympathomimetic amines.* pp. 245-264. *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, 2003.

3.10.2.12.6 Baselt, R.C., d-Methamphetamine, pp. 683-685. and Amphetamine, pp. 66-69. *in:* Disposition of Toxic Drugs and Chemicals in Man, Seventh ed., 2004.

Revision History

Section Three
Blood Toxicology

3.10 SPE Methods for Quantitative GC/MSD Confirmation
3.10.2 Extraction and Quantitation of Methamphetamine and Amphetamine from Blood Employing the Bond Elut Certify™ 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.