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

The drug 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.

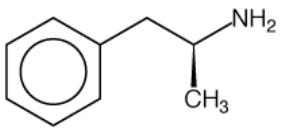

figure 1

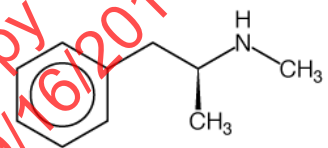

figure 2

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 & SCOPE

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 the pH adjusted 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 1M acetic acid followed by methanol, to selectively remove matrix components and interfering substances from the cartridge. The wash also disrupts 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 ethyl acetate disrupts the hydrophobic interactions. Following 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 Disposable inserts for SPE manifold ports

3.10.2.3.3 Drybath or laboratory oven capable of 70°C

3.10.2.3.4 Evaporative concentrator equipped with nitrogen tank.

3.10.2.3.5 Vacuum manifold/pump

3.10.2.3.6 Tube rocker

3.10.2.3.7 Vortex mixer

3.10.2.3.8 Laboratory centrifuge capable of 3400- 3500rpm

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

3.10.2.3.10 16 x 100mm round bottom glass tube

3.10.2.3.11 Screw Cap for 16mm O.D. tube

3.10.2.3.12 GC/MS Automated Liquid Sample (ALS) vials

3.10.2.3.13 GC/MS Vial Microinsert

3.10.2.3.14 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 1M Acetic Acid
3.10.2.4.11 Pentafluoropropionic acid anhydride (PFAA)

3.10.2.5 QUALITY ASSURANCE MATERIAL

3.10.2.5.1 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.

3.10.2.5.1.1 **Reference Material Stock Solutions**

Concentration: 1 mg/mL
(±)-Methamphetamine
(±)-Amphetamine

Store remaining stock solution as recommended by manufacturer.

3.10.2.5.1.2 **Reference Material Working Solutions**

Working solutions are stable for 6 months when stored under refrigeration.

Concentration: 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.

Concentration: 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 Internal Standard Stock Solutions

3.10.2.5.2.1 **Stock Solutions**
Concentration: 1mg/mL
(±)-Methamphetamine-D_8
(±)-Amphetamine-D_8

Store remaining stock solution as recommended by manufacturer..

3.10.2.5.2.2 **Working Internal Standard Solution**
Working internal standard solution is stable for 6 months when stored under refrigeration.

Concentration: 10ng/µL
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.

3.10.2.5.3 Commercial Whole Blood Controls

3.10.2.5.5.1 **Negative Whole Blood**

3.10.2.5.5.2 **Optional: Positive Whole Blood**
Control containing Amphetamine and Methamphetamine each at a target of 100ng/mL. Refer to package insert for verified value and expected range. Additional concentrations may also be utilized.

3.10.2.6 PROCEDURE

3.10.2.6.1 Initial set-up
Label extraction tubes (x3), SPE columns (x1), and GC/MSD vials with microinserts (x1) for calibrators, controls and case samples.

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

3.10.2.6.2.1 Add 2mL of negative whole blood to five screw-top extraction tubes.

3.10.2.6.2.2 Add the volume of 1ng/µL Amphetamine and Methamphetamine working solution 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 10ng/µL Amphetamine and Methamphetamine working solution 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

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

3.10.2.6.3.1 Add 2mL of negative whole blood to two screw top tubes.

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

Desired ng/mL	µL Working Control
75	15
300	60

3.10.2.6.3.3 Additional or alternative concentrations at the discretion of the analyst may be used as long as the requirements in 3.10.2.10.2 are met.

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 Place sample container on tube rocker for a minimum of five minutes. If sample is clotted, homogenize as necessary.

3.10.2.6.5.3 Transfer 2mL neat or diluted sample to labeled screw top tube.

3.10.2.6.5.4 If there is a low-volume sample, analyst may halve the volume of sample (1mL). Internal standard added should also be halved. One additional low and one high control must also be added to mirror the low-volume extraction.

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 (10ng/µL).

3.10.2.6.6.2 Vortex tube briefly and allow 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. Remove from rocker and place samples into centrifuge and let stand for 5 minutes.

3.10.2.6.7.3 Centrifuge at 3400 – 3500 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 Evaporative Concentrator. Evaporate sample to approximately 1mL

under nitrogen at approximately 40°C. *Do not allow extract to go to dryness.*

3.10.2.6.7.6 To concentrated extract, add **2**mL **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 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

3.10.2.6.8.2 Add **2**mL **methanol** to the column.

3.10.2.6.8.3 Add **2**mL **100mM Phosphate buffer (pH 6.00)** to the column.

3.10.2.6.9 Blood Extract Loading

Decant buffered blood extract onto the SPE column.

3.10.2.6.10 Column Clean-up

3.10.2.6.10.1 Add **1**mL **1M Acetic Acid**.

3.10.2.6.10.2 Turn on/increase vacuum to ≥10 in. Hg (≥34 kPa) for ≥ 5 minutes.

3.10.2.6.10.3 Add **6**mL **methanol**.

3.10.2.6.11 Pre-Elution Dry Disc

Turn on/increase vacuum to ≥10 in. Hg (≥34 kPa) for ≥ 5 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 evaporative concentrator. Take solvent to dryness, under a gentle stream of nitrogen at approximately 40°C.

3.10.2.6.14 Derivatization

3.10.2.6.14.1 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 Heat tubes at 70°C 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 evaporative concentrator and evaporate to dryness under nitrogen at approximately 40°C. ***Never inject PFAA extract directly into GC/MSD.***

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 Into Sequence log table, enter the case sample, calibrators, blanks and control information.

3.10.2.6.15.2 Load samples, calibrators, blank and controls into the quadrant rack as noted 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 four data points.

3.10.2.6.16.2 Calibrators should be analyzed in order of increasing concentration.

3.10.2.6.16.3 The least squares line resulting from the analysis of calibrators must have a coefficient of correlation of ≥0.98.

3.10.2.6.16.4 If calibration reference materials are run in duplicate, it is not required that duplicate calibration points be 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 ± 0.1 minutes.

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% (relative). Refer to section 3.10.2.8.4.4 for administrative cutoff criteria.

3.10.2.8.3 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 ±0.1 minutes and there are no significant differences in the mass spectral data.

3.10.2.8.4 Quantitative Mass Spectral and Control Criteria

3.10.2.8.4.1 Refer to Section 3.10.2.9.1 for determination of when this method may be used for quantitative purposes.

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

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

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

3.10.2.8.4.4 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.

3.10.2.8.4.5 If the concentration exceeds the calibration range, the sample needs to be appropriately

diluted with negative whole blood for reanalysis. Alternatively, the analyte(s) may be reported using full scan data; refer to section 3.10.2.8.3 for criteria.

3.10.2.9 REPORTING OF RESULTS

3.10.2.9.1 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.

3.10.2.10 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, 5.2, 5.8, and 5.10 for quality assurance and reference material authentication requirements.

3.10.2.10.2 Per Analysis Run Quality Requirements

3.10.2.10.2.1 A solvent blank must follow the highest calibrator, as well as precede each case sample.

3.10.2.10.2.2 A minimum of the spiked blood controls described in section 3.10.2.6.3 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.

3.10.2.10.2.3 If the number of case samples exceeds 10, in addition to the two spiked controls described in 3.10.2.6.3, one spiked or commercially-obtained blood control must be run for each additional 10 case samples. Additional concentrations may be used.

3.10.2.10.2.4 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 3.10.2.10.2.2. A third-party reviewer must independently review the central file packet for compliance to method requirements.

3.10.2.10.2.5 If a drug other than Amphetamine or Methamphetamine 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.

3.10.2.10.3 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 spreadsheet used to assess uncertainty for this method.

3.10.2.11 ANALYSIS DOCUMENTATION

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

3.10.2.11.2 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 or destruction.

3.10.2.11.3 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.
2	03-07-2011	Storage condition specifications updated, reformatted reference material section for clarity, emphasized need for sample homogeneity, updated nomenclature.
3	01-07-2013	Clarified that method is currently limited to qualitative reporting only. Reduced acceptable retention time window from 0.2 minutes to 0.1. Clarified that samples should be concentrated not evaporated completely.
4	02-13-2014	Further clarified that method is not currently approved for quantitation. Clarified that all requirements pertaining to quantitation be suspended until method is approved for quantitation. Allowed for possibility for full scan confirmation of analytes.
5	03/13/2015	Deleted repetitions of aspiration from the SPE method section; made single instruction at beginning of SPE section to replace repetitions. Formatting for continuity. Added LIMS reporting requirement. Added low-volume sample contingency. 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. Removed Tune specifications from 3.10.2.6.15, and changed wording to be consistent with other methods.