1. PROTOCOL FOR THE ANALYSIS OF BENZODIAZEPINES IN BLOOD

PURPOSE

The following method describes the quantitation of benzodiazepines 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 – Polymeric bead- Dual mode (hydrophobic and a strong cation exchanger) CEREX Polycrom Clin II

(4) Heating block

(5) Sample concentrator with UHP nitrogen

(6) Centrifuge

(7) Water bath

B. Reagents:

(1) **Deionized Water** (DI water). Label. Stable until consumed.

(2) **Sodium Acetate buffer 0.1M, pH 4.5**. Prepared by adding 13.6g of sodium acetate crystals and 6.0 ml of acetic acid to 1.0 L deionized water. Stable until consumed.

(3) **Carbonate/bicarbonate buffer, pH 9.0**. Prepared by adding 17g of $NaHCO_3$ and 8g of Na_2CO_3 to 1.0 L deionized water. Stable until consumed.

(4) **Ethyl Acetate: Ammonium Hydroxide (98:2).** Prepare fresh daily.

(5) **BSTFA with 1%TMCS**. Stable until consumed. Crimp cap and label appropriately if transferred.

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

(7) **Abalone β-glucuronidase enzyme (>50,000 units/mL) and Hydrolysis Buffer solution.** Purchased from United Chemical Technologies (UCT) or equivalent. These solutions are kept separate and pipetted separately into case samples and quality controls on the day of use. Stable for one year.

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

(1) **Prepare (as free base) or purchase individual 1.0 mg/ml stock standards in methanol of the following:**

Nordiazepam	Oxazepam	Midazolam	desalkylflurazepam
Temazepam	Diazepam	Bromazepam	N-desmethylflunitrazepam
Clonazepam	Lorazepam	Phenazepam	7-aminoclonazepam
Alprazolam	Estazolam	Zolpidem	α-hydroxyalprazolam
2-hydroxyethylflurazepam	7-aminoflunitrazepam		α-hydroxytriazolam
Etizolam			

(2) **Purchase individual 100 μg/ml stock standards in methanol or acetonitrile (depending on the ampoule) of the following**:

D4-desmethylflunitrazepam	D4-clonazepam	α-hydroxymidazolam
D5-oxazepam	D5-α-hydroxyalprazolam	
D4-7-aminoclonazepam	D5-alprazolam	

D. Calibrators and internal standards: (Store refrigerated. Stable for 2 years)

(1) **4.0 ng/μl D5-oxazepam (Cerilliant 0-904), 2.0 ng/μl D4-clonazepam (Cerilliant C-905), 2.0 ng/μl D4-7-aminoclonazepam-D4 (Cerilliant A-917), 2.0 ng/μl desmethylflunitrazepam (Cerilliant D-925), 1.5 ng/μl D5-alprazolam (Cerilliant A-902), and 1.5 ng/μl D5-α-hydroxyalprazolam (Cerilliant A-908) internal standard** : In a 10 ml volumetric flask, add 400μl of 100 μg/ml D5-Oxazepam; 200 μl each of 100 μg/ml D4-clonazepam, D4-7-aminoclonazepam, D4-desmethylflunitrazepam; and 150 μl each of 100 μg/ml D5-alprazolam, & D5-α-hydroxyalprazolam. Dilute to volume with methanol. (May also prepare larger stock volume by adjusting volumes accordingly to account for equivalent concentrations of each analyte.)

(2) **10 ng/μl mix Benzodiazepine calibrator stock solution**: Dilute 100 μl of each 1mg/ml stock standard, and 1 ml of each 100μg/ml stock standard to 10 ml with methanol in a 10 ml volumetric flask.

(3) **1ng/μl mix Benzodiazepine calibrator**: Prepare on day of use with above 10 ng/ml mixed calibrator stock solution.

E. Quality Controls: (Store refrigerated. Stable as per manufacturer's recommendation

(1) **Positive controls: 30, 100, 400 ng/ml mixed benzodiazepines controls**. 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 calibrators at 10, 25, 50,100, and 500ng/ml using the above calibrator stocks in 1 mL of blank blood along with 1mL* negative control, positive controls, case samples and additional controls at appropriate volumes and/or dilutions if applicable.

(OPTIONAL) Perform enzymatic hydrolysis on preliminary positive samples, positive hydrolysis control, and negative control by adding 100 µl of Abalone β-glucuronidase/ 400 µl of Hydrolysis Buffer solution to 1.0 ml of blood in labeled culture tubes, mix and incubate samples in a water bath at 50°C for a minimum of one hour.

*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 50µl* of working internal standard to each tube.

(c) Add 2.0 ml of 0.1M sodium acetate buffer (pH 4.5) and vortex each tube until thoroughly mixed.

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

(2) ***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 1.0ml /minute or gravity only.

(3) ***Column rinse and elution***
Pass through the column sequentially the following reagents, at 1-2 ml/min:

(a) 2 ml of pH 9 carbonate/bicarbonate buffer

(b) 2 ml of deionized water

(c) Dry column under maximum flow (25 psi) for 10 minutes

(d) Elute by gravity or <1.0 ml/minute with 1.5 ml of ethyl acetate:ammonium hydroxide (98:2) into labeled silanized microvials. Check no water is present.

(4) ***Derivatization***

(a) Evaporate the extracts under nitrogen to dryness.

Note: it is important to dry down samples immediately, as some Benzodiazepines are unstable in the elution solvent.

(b) To the microvials add 50 µl ethyl acetate, vortex, and then add 20 µl BSTFA with 1% TMCS to each, crimp cap and vortex again.

(c) Heat microvials at 70°C for at least 20 minutes

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. The ion ratios and retention times should be set by a mid-level calibrator. Subsequent injections to include positive controls, negative control and solvent blanks between case samples. For samples requiring dilution add the appropriate sample multiplier in the sequence table.

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

H. Results and Acceptability:

(1) Calibration $R^2 \geq 0.97$ 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 < LOD

(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 ≥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.

(9) Results will be truncated and reported to two significant figures