1. PROTOCOL FOR THE ANALYSIS OF BENZODIAZEPINES IN URINE

PURPOSE

The following method describes the confirmation of benzodiazepines and their metabolites in urine by GC/MS. Samples which have been 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 Polychrom Clin II

(4) Heating block

(5) Sample concentrator with UHP Nitrogen

(6) Water bath

(7) Centrifuge

B. Reagents:

(1) **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

(2) **Deionized Water** (DI water). Stable until consumed.

(3) **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.

(4) **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 2 years.

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

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

(7) **Ethyl Acetate**. Prepare a 100ml transfer bottle of ACS/HPLC grade ethyl acetate. Label accordingly. Store in glass at room temperature. Stable until consumed.

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

(1) **Prepare (5ml) or purchase individual 1.0 mg/ml stock standards in methanol of the following:**

Nordiazepam	Oxazepam	7-aminoflunitrazepam
Temazepam	Diazepam	7-aminoclonazepam
Lorazepam	desalkylflurazepam	2-hydroxyethylflurazepam
Alprazolam	Estazolam	α-hydroxyalprazolam
α-hydroxytriazolam	N-desmethylflunitrazepam	

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

α-hydroxymidazolam	D4-7-aminoclonazepam	D4-desmethyflunitrazepam
D5-oxazepam	D5-α-hydroxyalprazolam	Temazepam-*glucuronide*
Lorazepam-*glucuronide*	Oxazepam-*glucuronide*	

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

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

(2) **50 ng/mL benzodiazepines calibrator**. To two ml of negative urine add 10 μl of mix benzodiazepine calibrator stock solution above.

(3) **100 ng/mL benzodiazepines calibrator**. To two ml of negative urine add 20 μl of mix benzodiazepine calibrator stock solution above.

(4) **200 ng/mL benzodiazepines calibrator**. To two ml of negative urine add 40 μl of mix benzodiazepine calibrator stock solution above.

(5) **8.0 ng/μl D5-oxazepam (Cerilliant 0-904), 8.0 ng/μl D5-α-hydroxyalprazolam (Cerilliant A-908), 4.0 ng/μl D4-7-aminoclonazepam (Cerilliant A-917) and 4.0 ng/μl D4-desmethylflunitrazepam (Cerilliant D-925) internal standard**: In a 10 ml volumetric flask add 800 μl each of the 100 μg/ml D5-oxazepam and 100 μg/ml D5-α-hydroxyalprazolam. Add 400 μl each of the 100 μg/ml D4-desmethyflunitrazepam and 100 μg/ml D4-7-aminoclonazepam stock standards. Dilute to volume with methanol.

E. Quality Controls. (Store refrigerated.)

(1) **Positive Control**. **100 ng/ml mixed benzodiazepines**. Prepared in house from a different lot of stock solution than that used to prepare calibrators or purchased from an external vendor.

(2) **10 ng/μL mixed benzodiazepines glucuronide control stock solution**. Dilute 774μl of Lorazepam-glucuronide, 807μl of oxazepam-glucuronide, and 797μl of temazepam-glucuronide stock standard to 5ml with methanol.

(3) **Hydrolysis Control**.**100 ng/ml lorazepam, oxazepam and temazepam glucuronide**. To two ml of negative urine add 20 μl of mixed benzodiazepine glucuronide stock solution above.

(4) **Negative Control**. Urine produced in house will be used as negative control.

F. Solid Phase Extraction (SPE)

(1) ***Sample Preparation***
Prepare in appropriately labeled culture tubes as follows:

(a) Pipette 2 ml negative control, 100 ng/ml glucuronide control, as well as samples. High samples may be diluted, as an example x2 by adding 1 ml sample/1 ml H_2O.

(b) Samples must be hydrolyzed in order to remove glucuronide-bound metabolites, and thus improve recovery. To each of the tubes, including the glucuronide and negative controls, add 100 µl of Abalone β-glucuronidase/ 400 µl of Hydrolysis Buffer solution and hydrolyze in a water bath at 50°C for minimum of one hour, or alternatively overnight (minimum of 12 hours) at room temperature. Remove from water bath and allow to cool.

(c) Prepare a set of calibrators at 50, 100, and 200 ng/ml in 2mL of negative urine. Prepare a 100 ng/ml positive control. Add 50 µl of working internal standard to each tube.

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

(e) 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 carbonate/bicarbonate buffer (pH 9.0)

(b) 2 ml of deionized water

(c) Dry column under maximum pressure (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 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 70 µl ethyl acetate, vortex, and then add 30 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 calibrator(s) 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 and negative controls, and solvent blanks between case samples. For samples requiring dilutions add the appropriate sample multiplier in the sequence table.

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

H. Results and Acceptability (Qualitative):

(1) Calibration $R^2 \geq 0.97$ and calibrators within 20% of set value

(2) Positive control is positive (≥ 50 ng/ml)

(3) Negative control <LOD

(4) Retention time within 2% as set or stored from calibrator

(5) Qualifier ion ratios within 20% as set or stored from calibrator

(6) Chromatographically acceptable i.e. peak purity ≥90% for target ion

(7) Report analytes as positive ≥ 50 ng/ml