1. PROTOCOL FOR THE ANALYSIS OF BENZODIAZEPINES BY LC/MS-QQQ

PURPOSE

The following method describes the quantitation of benzodiazepines in blood, serum, plasma and the qualitative identification of benzodiazepines in urine by LC/MS-QQQ. 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) LC/MS-QQQ with a C18 column

(2) Positive Pressure Manifold

(3) SPE Column – Polymeric bead- Dual mode (hydrophobic and a strong cation exchanger) CEREX Polycrom Clin II

(4) Sample concentrator with UHP nitrogen

(5) Centrifuge

(6) Water bath

(7) Vortex mixer / Multi-tube vortex mixer

B. Reagents:

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

(2) **Sodium Acetate buffer 0.1M, pH 4.5**. Prepared by adding 13.6 g 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) **Ammonium formate buffer 0.01%.** Prepared by adding 0.34 g of ammonium formate and 100 µl of formic acid to 1.0 L deionized water. Stable until consumed.

(5) **Formic Acid (0.01%) in methanol**. Prepared by adding 100 µl of formic acid to 1.0 L methanol. Stable until consumed.

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

(7) **Methanol.** Stable until consumed.

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

(9) **Abalonase 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 or per manufacturer's recommendations.

C. Standards: (Store frozen or refrigerated. i.e. 7-aminoclonazepam. See individual ampuoles for manufacturer recommendations.) Stable 2 years if prepared in house or as per manufacturer's expiration date.

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

Group A

Clonazepam	7-aminoclonazepam	7-aminoflunitrazepam
N-desmethylflunitrazepam	Clonazolam	8-aminoclonazolam
Etizolam	α-hydroxyetizolam	α-hydroxytriazolam
Fluaplrazolam	α-hydroxyflualprazolam	Flubromazolam
Deschloroetizolam		

Group B

Alprazolam	α-hydroxyalprazolam	Lorazepam
Diazepam	Nordiazepam	Oxazepam
Temazepam	Midazolam	α-hydroxymidazolam
Chlordiazepoxide	desalkylflurazepam	2-hydroxyethylflurazepam
Bromazepam	Phenazepam	Estazolam
Flunitrazolam	Delorazepam	Diclazepam
Pyrazolam	Adinazolam	Bromazolam
Zolpidem		

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

D4-desmethylflunitrazepam	D4-clonazepam	D5-diazepam
D5-oxazepam	D5-α-hydroxyalprazolam	D7-zolpidem
D4-7-aminoclonazepam	D5-alprazolam	D4-lorazepam
D5-temazepam	D5-nordiazepam	

Oxazepam-glucuronide Temazepam-glucuronide Lorazepam-glucuronide

D. Calibrators and internal standards: (Store refrigerated. Calibrators stable for 1 year; internal standard stable for 2 years)

(1) **1.0 ng/µl mix Internal Standard solution.** In a 25 ml volumetric flask, add 250 µl each of D4-desmethylflunitrazepam, D4-clonazepam, D5-diazepam, D5-oxazepam, D5-α-hydroxyalprazolam, D7-zolpidem, D4-7-aminoclonazepam, D5-alprazolam, D4-lorazepam, D5-temazepam and D5-nordiazepam. Dilute to volume with methanol.

(2) **10 ng/µl mix Benzodiazepine calibrator stock solution**: In a 10 ml volumetric flask, add 100 µl of each 1 mg/ml stock standard and 1.0 ml of each 100 µg/ml stock standard. Dilute to volume with methanol.

Prepare Levels 1-6 mix Benzodiazepine calibrator stocks at the following concentrations:

Level 1 0.05 ng/µl	*Level 2* 0.10 ng/µl	*Level 3* 0.20 ng/µl
Level 4 0.50 ng/µl	*Level 5* 1.0 ng/µl	*Level 6* 2.5 ng/µl

E. Quality Controls: (Store refrigerated. Stable as per manufacturer's recommendation or 1 year if made in house)

(1) **Positive controls: (BLOOD) 15, 100, and 200 ng/ml / (URINE) 15 ng/ml mixed benzodiazepines control**. 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:** Urine produced in house will be used as negative control. Blank blood prepared in house consisting of 50% saline, 50% packed red blood cells, and 5g sodium fluoride/1g potassium oxalate (per 500ml prepared blood) will be used as negative control.

(3) **Hydrolysis control (0.3 ng/µl) stock solution**: Prepare interim 5 ng/µl stock by adding 81 µl of oxazepam-glucuronide (100 µg/ml stock), 80 µl of temazepam-glucuronide (100 µg/ml stock), and 77 µl of lorazepam-glucuronide (100 µg/ml stock). Dilute to 1ml volume with methanol. Pipette 60µl of interim stock and dilute to 1ml with methanol to make the 0.3 ng/µl stock solution. Store refrigerated in glass. Stable for 2 years. Using 50 µl stock solution, prepare hydrolysis control with negative urine on day of use at a target concentration of 30 ng/mL.

(4) **Dilution control (10 ng/µl) stock solution**: Prepared from the control stock solution, this is a process control for qualitative confirmation of urine samples. Prepare on day of use by adding 50 µl of the dilution control to 450 µl negative urine to make a 1000 ng/ml sample. From this sample, prepare dilution controls consistent with case sample urine dilutions (for example 100 µl for x5, 50 µl for x10, 10 µl for x50, etc.) The target concentration should be 1000 ng/ml after the dilution factor is processed by LC-MS/MS software.

F. Solid Phase Extraction (SPE)

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

(a) Prepare a set of calibrators at 5, 10, 20, 50, 100, and 250 ng/ml using 50 µl of the Level 1-6 calibrator stocks respectively in 0.5 mL of blank blood along with 0.5 mL* negative control, positive controls, hydrolysis control (if applicable), case samples and additional controls at appropriate volumes and/or dilutions if applicable. Add 50µl* of working internal standard to each tube.

(optional for BLOOD/required for URINE) Perform enzymatic hydrolysis on samples, negative control and hydrolysis control by adding 50 µl of Abalone β-glucuronidase/ 200 µl of Hydrolysis Buffer solution to 0.5 ml of urine/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 sample is received use the same fraction of internal standard as the sample, as an example 0.25 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 0.25 mL sample with 50 µl of internal standard.

(b) Add 1.0 ml H_2O and vortex.

(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. Apply pressure to achieve a flow rate of about 1.0 ml/minute.

(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 approximately 1.5 ml of ethyl acetate:ammonium hydroxide (98:2) into labeled microvials *NOTE*: for 10 ml of ethyl acetate add 200 µl of ammonium hydroxide, mix thoroughly

(e) 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.

(f) To the microvials add 200 µl methanol and 200 µl water, cap and vortex.

G. Data Acquisition and Analysis:

(1) Perform Autotune or checktune if not previously done (weekly).

(2) Purge LC pump lines of any bubbles. At equilibrium the pump pressure for the method should reside near 100-120psi. Binary ripple should not exceed 0.3 %. Let LC stabilize before analysis.

(3) The trays for samples are P1 in front and P2 in back. They have rows a-f, columns 1-9 (Ultivo columns 1-11). Prepare a sequence beginning with two blanks (to stabilize LC column flow) followed by the calibrators, negative control, positive control; (positive controls should be included throughout the batch, i.e. beginning, mid-run and end of run) and LC solvent blanks prior to case samples. For samples requiring dilution add the appropriate sample multiplier in the sequence table. Load samples onto autosampler according to sequence and have it verified by another analyst before or after analysis but prior to unloading.

(4) Retention times and/or transition ratios can be updated with an unextrated standard or calibrator. Retention times should be set prior to running multiple samples.

(5) Analyze using the appropriate method on LC/MS-QQQ. (Benzo02DMRM)

H. Results and Acceptability

(BLOOD/quantitative):

(1) Calibration $R^2 \geq 0.99$ 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 < 25% of area count of cutoff calibrator

(5) Retention time within 5% as stored or set from an unextracted standard or calibrator

(6) Transition ratios within 20% as stored or set from an unextracted standard or calibrator

(7) Chromatographically acceptable i.e. peak purity ≥90%

(8) Blank prior to sample < 25% area count of cutoff calibrator

(9) Report analytes as positive from *Group A* ≥5 ng/ml and *Group B* ≥10 ng/ml [see 1.C.(1)]

(10) Quantitative if above criteria met except for Pyrazolam, 8-aminoclonazolam, Adinazolam, Bromazolam, Deschloroetizolam; 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.

(11) Results will be truncated and documented in case notes to two significant figures if applicable

(URINE/qualitative):

(1) Positive control is positive (≥ cutoff calibrator)

(2) Hydrolysis control is positive (≥ cutoff calibrator)

(3) Dilution control result verifies the appropriate order of magnitude from the dilution performed

(4) Negative control < 25% of area count of cutoff calibrator

(5) Retention time within 5% as stored or set from an unextracted standard or calibrator

(6) Transition ratios within 20% as stored or set from an unextracted standard or calibrator

(7) Chromatographically acceptable i.e. peak purity ≥90%, for primary transition

(8) Blank prior to sample < 25% area count of cutoff calibrator

(9) Report analytes as positive from *Group A* ≥5 ng/ml and *Group B* ≥10 ng/ml [see 1.C.(1)]