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1.0 Principle

1.1 Benzodiazepines are a class of Central Nervous System (CNS) depressant psychoactive compounds formed around the core structure of a diazepine ring linked to a benzene ring. These drugs generally enhance the activity of GABA at the $GABA_A$ receptor, resulting in anxiolytic, amnesic, hypnotic, and sedative effects.

1.2 Benzodiazepines can be extracted from whole blood using a process of cell lysis and solid phase extraction. Due to the nature of the analytes of interest, a Mixed-mode strong Cation eXchange (MCX) solid phase sorbent is used. Based on differential affinity for the organic solvents passed through the sorbent bed, other potentially interfering compounds such as phospholipids are removed from the resulting eluent.

1.3 The eluent is analyzed on the Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS) system. Chromatographic separation between the analytes of interest is achieved by using a 2.7 µm Raptor Biphenyl column. Each analyte will fragment in a unique and predictable pattern as its ions pass through the tandem mass spectrometer, allowing for confirmatory identification and quantitation.

2.0 Equipment

2.1 Pipettes

2.2 Class A volumetric glassware

2.3 Graduated cylinders

2.4 HPLC grade glass bottles

2.5 Glass culture tubes

2.6 Analytical balance

2.7 Vortex mixer

2.8 Centrifuge

2.9 Positive pressure manifold (PPM)

2.10 96-well Oasis PRiME MCX µElution plates

2.11 96-well collection plates and sealing mats

2.12 Waters Acquity H-Class UPLC / Xevo TQ-S micro MS system.

3.0 Solvents & Reagents

3.1 Materials

3.1.1 Zinc sulfate heptahydrate

3.1.2 Ammonium acetate

3.1.3 Ammonium formate

3.1.4 Ammonium hydroxide

3.1.5 4% phosphoric acid

3.1.6 Formic acid (HPLC grade)

3.1.7 Acetonitrile (HPLC grade)

3.1.8 Methanol (HPLC grade)

3.1.9 Isopropanol (HPLC grade)

3.1.10 Deionized water

3.2 Zinc sulfate/ammonium acetate 0.1 M solution

3.2.1 Add 28.76 g zinc sulfate heptahydrate and 7.71 g ammonium acetate to 500 mL diH_2O.

3.2.2 Fill bottle to 1L.

3.2.3 Assigned lot number is ZA-MMDDYYYY.

3.3 200 mM ammonium formate in 4% phosphoric acid (H_3PO_4)

3.3.1 Add 4 mL of 85% phosphoric acid to approximately 96 mL of diH_2O.

3.3.2 Add 1.26 g ammonium formate to solution.

3.3.3 Assigned lot number is AFP-MMDDYYYY.

3.4 Wash solvent – 25% methanol in water

3.4.1 Add 25 mL methanol to 75 mL diH_2O.

3.4.2 Assigned lot number is MH-MMDDYYYY.

3.5 Elution solvent – 50% acetonitrile/methanol containing 5% strong ammonia

3.5.1 Add 1 mL acetonitrile to 1 mL methanol.

3.5.2 Add 100 µL ammonium hydroxide solution.

3.5.3 Elution solvent expires 24 hours after preparation and is not assigned a lot number.

3.6 Dilution solvent – (97:2:1 diH20: acetonitrile: formic acid)

3.6.1 Add 97 mL diH_2O with 2 mL acetonitrile and 1 mL formic acid.

3.6.2 Assigned lot number is DIL-MMDDYYYY.

3.6.3 Solution expires six months from date of preparation.

3.6.4 Dilution solvent will be run on the LC-MS/MS system prior to use in casework to demonstrate that it is free from analytes of interest and other interfering compounds.

3.6.5 Chromatograms generated from the analysis will be reviewed and documentation of passing QC recorded in the Reagent Preparation Log. Analytical results will be kept on file with the Toxicology Section.

3.7 Solvents and reagents do not require a performance check prior to use unless otherwise noted.

3.8 All solvents and reagents are stored at room temperature and expire one year from date of preparation unless otherwise noted.

3.9 Solvent and reagent preparation will be recorded in the Reagent Preparation Log and containers labeled with lot number, preparation date, expiration date, and preparer initials, unless otherwise noted.

3.10 Volumes specified in each preparation can be changed, provided the final concentration or ratio of components remains consistent.

4.0 Standards & Controls

4.1 Materials

4.1.1 Negative Control Stock (NEG) see TOX_P700

4.1.2 Methanol (HPLC grade)

4.1.3 NIST traceable standards:

Analyte	Internal Standard
Alprazolam	Alprazolam D5
Alpha-Hydroxyalprazolam	Alpha-hydroxyalprazolam D5
Clonazepam	Clonazepam D4
7-Aminoclonazepam	7-Aminoclonazepam D4
Lorazepam	Lorazepam D4
Midazolam	Midazolam D4 maleate
Chlordiazepoxide	Chlordiazepoxide D5
Diazepam	Diazepam D5
Nordiazepam	Nordiazepam D5
Oxazepam	Oxazepam D5

Temazepam	Temazepam D5

4.2 Internal standard preparation

4.2.1 Add 5 mL methanol to a 10 mL volumetric flask.

4.2.2 Pipette the following volumes of each standard:

Standard	Concentration	Volume Added	Final Concentration
Alprazolam D5	1 mg/mL	50 µL	5 µg/mL
Alpha-hydroxyalprazolam D5	1 mg/mL	50 µL	5 µg/mL
Clonazepam D4	1 mg/mL	50 µL	5 µg/mL
7-Aminoclonazepam D4	1 mg/mL	50 µL	5 µg/mL
Lorazepam D4	1 mg/mL	50 µL	5 µg/mL
Midazolam D4 maleate	100 µg/mL	500 µL	5 µg/mL
Chlordiazepoxide D5	100 µg/mL	500 µL	5 µg/mL
Diazepam D5	1 mg/mL	200 µL	20 µg/mL
Nordiazepam D5	1 mg/mL	200 µL	20 µg/mL
Oxazepam D5	1 mg/mL	200 µL	20 µg/mL
Temazepam D5	1 mg/mL	200 µL	20 µg/mL

4.2.3 Fill flask to volume with methanol.

4.2.4 Assigned lot number is BNZ-IS-MMDDYYYY.

4.2.5 Prior to being placed in service, solutions must be performance checked.

4.2.5.1 Prepare one vial by adding 25 µL of currently in-service internal standard solution to 475 µL diH_2O and vortex to mix.

4.2.5.2 Prepare a second vial as above, using the newly prepared lot number of internal standard solution.

4.2.5.3 Analyze these vials on the UPLC system using the currently validated analytical method.

4.2.5.4 Retention times should be consistent between the currently in-service lot number and the newly prepared lot number.

4.2.5.5 The monitored transitions for each analyte of interest should exhibit no measurable interference from the deuterated internal standard compounds.

4.2.5.6 Peak area counts for each compound should match between the currently in-service lot number and the newly prepared lot number to within ± 30%.

4.3 Calibrator working stock preparation

4.3.1 Add 5 mL methanol to a 10 mL class A volumetric flask.

4.3.2 Pipette the following volumes of each standard:

Standard	Concentration	Volume Added	Final Concentration
Alprazolam	1 mg/mL	100 µL	10 µg/mL
Alpha-Hydroxyalprazolam	1 mg/mL	100 µL	10 µg/mL
Clonazepam	1 mg/mL	100 µL	10 µg/mL
7-Aminoclonazepam	1 mg/mL	100 µL	10 µg/mL
Lorazepam	1 mg/mL	100 µL	10 µg/mL
Midazolam	1 mg/mL	100 µL	10 µg/mL
Chlordiazepoxide	1 mg/mL	400 µL	40 µg/mL
Diazepam	1 mg/mL	400 µL	40 µg/mL
Nordiazepam	1 mg/mL	400 µL	40 µg/mL
Oxazepam	1 mg/mL	400 µL	40 µg/mL
Temazepam	1 mg/mL	400 µL	40 µg/mL

4.3.3 Fill flask to volume with methanol.

4.3.4 Assigned lot number is BNZ-CAL-MMDDYYYY.

4.3.5 Prior to being placed in service, solutions must be performance checked.

4.3.5.1 In triplicate, prepare samples at the highest calibrator concentration, as specified in section 5.1, using the newly prepared calibrator working stock.

4.3.5.2 Extract these samples and analyze them against a valid calibration curve prepared using the currently in-service calibrator working stock.

4.3.5.3 Each analyte should fall within ± 20% of the target value at each concentration.

4.3.5.4 Retention times should be consistent between the currently in-service lot number and the newly prepared lot number.

4.3.5.5 Qualifier ion ratios for the newly prepared lot number should fall within ± 20% of the currently in-service lot number average ion ratio.

4.4 Quality control working stock preparation

4.4.1 Add 5 mL methanol to a 10 mL volumetric flask.

4.4.2 Pipette the following volumes of each standard:

Standard	Concentration	Volume Added	Final Concentration
Alprazolam	1 mg/mL	80 µL	8 µg/mL
Alpha-Hydroxyalprazolam	100 µg/mL	800 µL	8 µg/mL
Clonazepam	1 mg/mL	80 µL	8 µg/mL
7-Aminoclonazepam	1 mg/mL	80 µL	8 µg/mL
Lorazepam	1 mg/mL	80 µL	8 µg/mL
Midazolam	1 mg/mL	80 µL	8 µg/mL
Chlordiazepoxide	1 mg/mL	320 µL	32 µg/mL
Diazepam	1 mg/mL	320 µL	32 µg/mL
Nordiazepam	1 mg/mL	320 µL	32 µg/mL
Oxazepam	1 mg/mL	320 µL	32 µg/mL
Temazepam	1 mg/mL	320 µL	32 µg/mL

4.4.3 Fill flask to volume with methanol.

4.4.4 Assigned lot number is BNZ-QC-MMDDYYYY.

4.4.5 Prior to being placed in service, solutions must be performance checked.

4.4.5.1 In triplicate, prepare samples at the high-range quality control concentration, as specified in section 5.2, using the newly prepared quality control working stock.

4.4.5.2 Extract these samples and analyze them against a valid calibration curve prepared using the currently in-service calibrator working stock.

4.4.5.3 Each analyte should fall within ± 20% of the target value at each concentration.

4.4.5.4 Retention times for each analyte should be consistent with the calibration standards.

4.4.5.5 Qualifier ion ratios for the newly prepared lot number should fall within ± 20% of the calibration average ion ratio.

4.5 Reagent preparation will be recorded in the Reagent Preparation Log and containers labeled with lot number, preparation date, expiration date, and preparer initials, unless otherwise noted.

4.6 All working stocks are stored in the freezer and expire one year from date of preparation.

4.7 Chromatograms from the analysis will be reviewed and documentation of passing QC recorded in the Reagent Preparation Log. Analytical results will be kept on file with the Toxicology Section.

4.8 Volumes specified in each preparation can be changed, provided the final concentration or ratio of analytes and components remains consistent.

4.9 Concentrations of purchased reference materials may vary depending on availability from the manufacturer. Alternative ratios or volumes may be used in reagent preparation, provided the final concentration of analytes remains consistent.

4.10 If the currently in-service lot number is not adequate for comparison purposes (e.g. insufficient volume, expired, failing acceptability criteria), reagent performance can be based on the general acceptability criteria for the analytical method.

5.0 Procedure

5.1 Prepare calibration samples

5.1.1 Serially dilute calibration working stock material in methanol to each calibration concentration.

5.1.1.1 For alprazolam, alpha-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, lorazepam, and midazolam, calibrators will be prepared at 5.0, 10, 25, 50, 250, and 500 ng/mL.

5.1.1.2 For chlordiazepoxide, diazepam, nordiazepam, oxazepam, and

temazepam, calibrators will be prepared at 20, 40, 100, 200, 1000, and 2000 ng/mL.

5.1.1.3 Refer to BNZ sample preparation sheet for suggested volumes and concentrations to use in serial dilution.

5.1.2 Aliquot 450 µL NEG into clean glass culture tubes and label one for each concentration.

5.1.3 Add 25 µL of the appropriate calibrator concentration to each tube.

5.1.4 Add 25 µL of internal standard to each tube.

5.1.5 Vortex to mix.

5.2 Prepare quality control samples

5.2.1 Dilute quality control working stock material in methanol to each of the required quality control concentrations.

5.2.1.1 For alprazolam, alpha-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, lorazepam, and midazolam, QC samples will be prepared at low (15 ng/mL), mid (250 ng/mL), and high (400 ng/mL) concentrations.

5.2.1.2 For chlordiazepoxide, diazepam, nordiazepam, oxazepam, and temazepam, QC samples will be prepared at low (60 ng/mL), mid (1000 ng/mL), and high (1600 ng/mL) concentrations.

5.2.1.3 Refer to BNZ sample preparation sheet for suggested volumes and concentrations to use in serial dilution.

5.2.2 Aliquot 450 µL NEG into clean, labeled glass culture tubes. The number of tubes at each concentration will depend upon the QC requirements of the analytical batch.

5.2.3 Add 25 µL of the appropriate QC dilution to each tube.

5.2.4 Add 25 µL of internal standard to each tube.

5.2.5 Vortex to mix.

5.3 Prepare negative control samples

5.3.1 Aliquot 450 µL NEG into clean, labeled glass culture tubes.

5.3.2 Add 25 µL of methanol to each tube.

5.3.3 Add 25 µL of internal standard to each tube.

5.3.4 Vortex to mix.

5.4 Prepare casework samples

5.4.1 Select one item from each evidential blood kit for analysis.

5.4.2 Thoroughly vortex each tube to ensure homogeneity of the sample.

5.4.3 Pipette 450 µL of blood into a clean, labeled glass culture tube.

5.4.4 Add 25 µL of methanol to each tube.

5.4.5 Add 25 µL of internal standard to each tube.

5.4.6 Vortex to mix.

5.5 Add 600 µL 0.1 M zinc sulfate/ammonium acetate solution to clean, labeled glass culture tubes.

5.6 Pipette 200 µL of prepared samples into each zinc sulfate/ammonium acetate tube; vortex to mix.

5.7 Centrifuge samples at 3030 rcf for 20 min.

5.8 Transfer supernatant into clean, labeled glass culture tubes containing 600 µL 200 mM ammonium formate in 4% phosphoric acid.

5.9 Transfer samples into µElution plate wells in two aliquots of ~600 µL; apply each at 1-2 mL/min using PPM.

5.9.1 Ensure each sample position is documented on BNZ sample preparation sheet.

5.10 Wash with 500 µL of wash solvent.

5.11 Add 100 µL dilution solvent to each sample well in 96-well collection plate.

5.12 Elute into collection plate wells using two aliquots of 50 µL elution solvent.

5.13 Cap collection plate with pre-slit sealing mat.

5.14 Gently vortex plate before loading onto instrument.

6.0 Instrumental Analysis

6.1 Complete all required maintenance procedures as outlined in the Toxicology Confirmation Manual (TOX_P700) and document in the Instrument Maintenance Log.

6.2 Ensure the “BNZ” inlet method and tune files are loaded and active.

6.3 Turn on gas flows, source electronics, and mobile phase pumps; allow all metrics to stabilize before running samples.

6.4 Place the collection plate in the autosampler.

6.5 In MassLynx, generate a sequence list for the analytical batch.

6.5.1 Inlet method, tune file, and MRM method columns should all be set to "BNZ".

6.5.2 All calibration and control standards should be set to "standard" and "QC" sample types, respectively, and include concentration levels in the appropriate columns.

6.5.3 All calibration standards should include a "1" in the quantitative reference column.

6.6 Save sequence list and begin sample acquisition.

6.7 After analysis, sample plates or vials may be kept at room temperature if reinjection is required.

6.7.1 Plates must be covered with a sealing mat or parafilm to prevent sample evaporation.

6.7.2 Calibrators, controls, and casework samples may be reinjected up to 48 hours after preparation.

7.0 Data

7.1 Upon completion of the run, process all acquired samples in TargetLynx using the "BNZ" method or appropriate submethod for reanalysis.

7.2 Ensure all samples meet the quality criteria outlined in section 7.0 of the Toxicology Confirmation Manual (TOX_P700).

7.3 Generate a data packet and perform analyst review as outlined in section 9.0 of the Toxicology Confirmation Manual (TOX_P700).

8.0 References

8.1 Toxicology Screening Manual (TOX_P600)

8.2 Toxicology Confirmation Manual (TOX_P700)

8.3 Reagent Preparation Log

8.4 Instrument Maintenance Log

8.5 BNZ Control Chart

DOCUMENT HISTORY			
DATE	**VERSION**	**APPROVED BY**	**ACTIVITY OR REVISION**
6/14/2021	1	Lab Director	First edition