# Benzodiazepines and Metabolites from Blood and Urine by LC/MS (MRM)

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## 1 INTRODUCTION

Benzodiazepines are one of the most commonly prescribed classes of drugs in the United States. They are also frequently abused. Therapeutic blood concentrations vary for benzodiazepines, depending on whether they are considered high dose benzodiazepines (e.g., diazepam) or low dose benzodiazepines (e.g., triazolam). Benzodiazepines are usually excreted into the urine as glucuronide metabolites and may persist in the urine for days after administration due to elimination half-lives that may exceed 24 hours.

## 2 SCOPE

| | |
|---|---|
| Analyses | ☒ Screening ☒ Confirmation ☒ Quantitation |
| Matrices | Blood, Urine |
| Analytes | 7-aminoclonazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxymidazolam, α-hydroxytriazolam, alprazolam, chlordiazepoxide (qualitative only), clonazepam, desalkylflurazepam, diazepam, flunitrazepam, flurazepam (qualitative only), lorazepam, midazolam, n-desmethylflunitrazepam, nordiazepam, oxazepam, temazepam, and triazolam |
| Personnel | This document applies to authorized personnel who perform the described tasks, singly or in combination. |

## 3 PRINCIPLE

Biological specimens are qualitatively assayed and/or quantified for benzodiazepines and their metabolites. Specimens are mixed with deuterated internal standards. Proteins are precipitated from blood before extraction. Both blood and urine samples are extracted using solid phase extraction (SPE). Analysis of extracts is by liquid chromatography/tandem mass spectrometry in the multiple reaction monitoring mode (LC/MS(MRM)).

## 4 SPECIMEN CRITERIA

This procedure uses 0.2 mL of blood (in duplicate for quantitation) or 0.4 mL of urine.

# 5 EQUIPMENT

## 5.1 Equipment

| | |
|---|---|
| A. | Centrifuge |
| B. | Evaporator/Concentrator with Nitrogen |
| C. | Graduated cylinders |
| D. | Incubator |
| E. | pH Meter |
| F. | Pipettes |
| G. | Vacuum or Positive Pressure Manifold |
| H. | Volumetric flasks |
| I. | Vortex/Mixer |

### *5.1.1 Column*

Phenomenex Kinetex XB-C18 (or equivalent) analytical column (150 mm x 2.1mm x 2.6 µ)

## 5.2 Consumables

| | |
|---|---|
| A. | Centrifuge tube filters (0.45 microns, Nylon) |
| B. | Oasis HLB 6 cc (500 mg) LP SPE cartridges |
| C. | 12 x 75 mm test tube |
| D. | 16 x 100 mm test tube |

## 5.3 Instruments

A. Sciex 6500+ QTRAP Mass Spectrometer

B. Shimadzu HPLC

## 5.4 Software

| Component | Software | Version |
|---|---|---|
| **Operating System** | Microsoft Windows | 10 Enterprise 2016 LTSB |
| **Mass Spectrometer** | Analyst | 1.7.1 |
| **Data Analysis** | MultiQuant | 3.0.3 |

## 5.5 Chemicals/Reagents

### *5.5.1 Purchased*

| | | |
|---|---|---|
| A. | Acetic acid, glacial | 17 M, ≥ ACS grade |
| B. | Ammonium acetate | 99.999% purity |
| C. | Ammonium formate | ≥ Reagent Grade |
| D. | Ammonium hydroxide, concentrated | 15 M, ≥ ACS grade |
| E. | Deionized water | 18 MΩ |
| F. | Disodium hydrogen phosphate (anhydrous) | ≥ Reagent Grade |
| G. | Formic acid | ≥ Reagent Grade |
| H. | Isopropanol (HPLC grade) | ≥ HPLC grade |
| I. | Methanol | ≥ Optima Grade |
| J. | Methylene chloride | ≥ Optima Grade |
| K. | Potassium dihydrogen phosphate | ≥ Reagent Grade |
| L. | Zinc sulfate heptahydrate | ≥ Reagent Grade |
| M. | β-Glucuronidase | >100,000 u/mL β-glucuronidase activity; from Red Abalone, *H. Rufescena*; available from Kura Biotec |

### *5.5.2 Prepared*

A. Zinc Sulfate (0.2 M)

Combine 50 mL deionized water and 5.75 g of zinc sulfate heptahydrate in a 100 mL volumetric flask. Mix until dissolved. Bring to the mark with deionized water. Store in glass at room temperature. Stable for at least 6 months.

B. Zinc Sulfate in Methanol

Combine 80 mL methanol and 20 mL zinc sulfate (0.2 M) in a volumetric flask and mix well. Store in glass at room temperature. Stable for at least 2 months.

C. Potassium Phosphate Buffer (KH2PO4)

Add 4.54 g potassium phosphate monobasic to a 0.5 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.

D. Sodium Phosphate Buffer (Na2HPO4)

Add 11.6 g sodium phosphate dibasic anhydrous to a 1 L volumetric flask and bring to the mark with deionized water. Store refrigerated in glass or plastic. Stable for at least three months.

E. Sorensen Buffer (pH 7.4)

Combine 500 mL of (D) Sodium Phosphate Buffer with 200 mL of (C) Potassium Phosphate Buffer. Add (C) to decrease pH or (D) to increase pH until pH reads 7.4 with a pH meter or tight range pH strip. Store refrigerated in glass or plastic. Stable for at least three months.

F. Ammonium Acetate Buffer (0.5 M; pH 5)

Add 3.854 g ammonium acetate to a 100-mL volumetric flask containing about 75 mL deionized water. Mix well to dissolve. Add glacial acetic acid until pH registers between 4.5 and 5.5. Bring to volume with deionized water and mix well. Store refrigerated in glass or plastic. Stable at least three months.

G. Methanol:Water:Ammonia (40:60:0.5)

Combine 40 mL methanol, 60 mL deionized water and 0.5 mL ammonium hydroxide and mix well. Store at room temperature in glass. Prepare fresh daily.

H. Methylene Chloride:Isopropanol (75:25)

Combine 188 mL methylene chloride and 62 mL isopropanol in a 250 mL graduated cylinder and mix well. Store at room temperature in glass. Stable for at least two months.

I. Water:Acetonitrile (90:10)

Combine 90 mL deionized water and 10 mL acetonitrile (Optima grade) and mix well. Store at room temperature in glass. Stable for at least three months.

J. Mobile Phase 1 (5 mM Ammonium Formate with formic acid; pH~3.5)

Add 0.3153 g ammonium formate to a 1 L volumetric flask. Add approximately 800 mL deionized water and mix well. Add 1 mL formic acid, and fill to the line with with deionized water. Store in glass at room temperature. Stable for at least one week.

K. Mobile Phase 2 (Acetonitrile with 0.1% Formic Acid)

Combine 1 mL formic acid and 1000 mL acetonitrile and mix well. Store in glass at room temperature. Stable for at least two months.

L. Methanol:Water (1:1)

Combine equal amounts methanol and water. Store in glass at room temperature. Stable for at least 12 months.

## 5.6 Standards/Controls

### *5.6.1 Purchased*

#### *5.6.1.1 Standard/Control Stock Solutions*

Standard and control stock Solutions (1.0 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX), Lipomed or an equivalent supplier. Solutions may be in methanol or acetonitrile and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

| | | |
|---|---|---|
| 7-aminoclonazepam | clonazepam | n-desmethylflunitrazepam |
| 7-aminoflunitrazepam | desalkylflurazepam | nordiazepam |
| α-hydroxyalprazolam | diazepam | oxazepam |
| α-hydroxymidazolam | flunitrazepam | temazepam |
| α-hydroxytriazolam | flurazepam | triazolam |
| alprazolam | lorazepam | |
| chlordiazepoxide | midazolam | |

#### *5.6.1.2 Hydrolysis Control Stock Solution*

Oxazepam-glucuronide (0.1 mg/mL) may be purchased from Cerilliant (Round Rock, TX), or an equivalent supplier; the S-isomer of the oxazepam-glucuronide is known to be cleaved by β-glucuronidase approximately 400 times faster than the R-isomer, making the R-isomer unsuitable as a control in this procedure.

#### *5.6.1.3 Internal Standard Stock Solutions*

Internal Standard Stock Solutions (0.1 mg/mL) of the following may be purchased from Cerilliant (Round Rock, TX) or an equivalent supplier. Solutions may be in methanol or acetonitrile and will be stored according to the manufacturer's recommendations. Stability is determined by the manufacturer.

| | | |
|---|---|---|
| 7-aminoclonazepam-$d_4$ | chlordiazepoxide-$d_5$ | midazolam-$d_4$ |
| 7-aminoflunitrazepam-$d_7$ | clonazepam-$d_4$ | n-desmethylflunitrazepam-$d_4$ |
| *α-hydroxyalprazolam-$d_5$ | desalkylflurazepam-$d_4$ | nordiazepam-$d_5$ |
| α-hydroxymidazolam-$d_4$ | diazepam-$d_5$ | oxazepam-$d_5$ |
| α-hydroxytriazolam-$d_4$ | flunitrazepam-$d_7$ | temazepam-$d_5$ |
| alprazolam-$d_5$ | lorazepam-$d_4$ | triazolam-$d_4$ |
| *used for flurazepam | | |

### *5.6.2 Prepared*

#### *5.6.2.1 Internal Standard Prepared Solutions*

A. Internal Standard Intermediate Solution (5 µg/mL):

Add 0.25 mL of each Internal Standard Stock Solution to a 5-mL volumetric flask and bring to the mark with methanol. Store in the freezer. Stable for at least 2 years.

B. Internal Standard Working Solution (500 ng/mL):

Combine 0.1 mL of the Internal Standard Intermediate Solution (5 µg/mL) and 0.9 mL methanol. Prepare fresh daily.

#### *5.6.2.2 Calibration Solutions*

| **A. High Calibration Solution** | | | |
|---|---|---|---|
| Starting Solution | 1 | mg/mL | stock solution(s) |
| Starting Solution Aliquot | 0.025 | mL | |
| Diluent Volume | 25 | mL | methanol, volumetric flask |
| Resulting Concentration | 1 | µg/mL | storage: freezer; stability: ≥ 1 year |
| **B. Low Calibration Solution** | | | |
| Starting Solution | 1 | µg/mL | High Calibrator Solution |
| Starting Solution Aliquot | 0.5 | mL | |
| Diluent Volume | 10 | mL | methanol, volumetric flask |
| Resulting Concentration | 50 | ng/mL | storage: freezer; stability: ≥ 1 year |
| **C. Calibration Scheme** | | | |
| | Low Cal | High Cal | Resulting Concentration, ng/mL |
| Calibrator Level | Spike (µL) | Spike (µL) | (in 0.2mL of blood) |
| 1 | 10 | | 2.5 |
| 2 | 20 | | 5 |
| 3 | 100 | | 25 |
| 4 | 200 | | 50 |
| 5 | | 20 | 100 |
| 6 | | 30 | 150 |
| 7 | | 50 | 250 |
| 8 | | 75 | 375 |

*5.6.2.3 Blood Controls*

Negative Control Blood is purchased from Cliniqa or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

At least one Positive Control Blood Sample will be analyzed with each blood assay. For quantitative analyses, both levels of Positive Control Blood Samples will be analyzed in duplicate.

| **A. High Control Solution** | | | |
|---|---|---|---|
| Starting Solution | 1 | mg/mL | stock solution(s) |
| Starting Solution Aliquot | 0.025 | mL | |
| Diluent Volume | 25 | mL | methanol, volumetric flask |
| Resulting Concentration | 1 | µg/mL | storage: freezer; stability: ≥ 1 year |

| **B. Low Control Solution** | | | |
|---|---|---|---|
| Starting Solution | 1 | µg/mL | High Control Solution |
| Starting Solution Aliquot | 0.5 | mL | |
| Diluent Volume | 10 | mL | methanol, volumetric flask |
| Resulting Concentration | 50 | ng/mL | storage: freezer; stability: ≥ 1 year |

| **C. Blood Control Scheme** | | | |
|---|---|---|---|
| | Low Control | High Control | Resulting Concentration, ng/mL |
| Control Level | Spike (µL) | Spike (µL) | (in 0.2mL of blood) |
| Negative | 0 | 0 | 0 |
| Low | 20 | 0 | 5 |
| High | 0 | 50 | 250 |

*5.6.2.4 Urine Hydrolysis Solution*

| A. Hydrolysis High Control Solution (1 µg/mL Oxazepam equivalent) |
|---|
| To a 10-mL volumetric flask, add 0.160 mL of the Oxazepam Glucuronide Stock Standard. Bring to volume with methanol. Store frozen in glass. Stable for ≥ 1 year. |

*5.6.2.5 Urine Controls*

| **A. Urine Negative Control** | | |
|---|---|---|
| Prepared in-house or purchased from an appropriate vendor. Stable for 6 months when refrigerated. A Negative Control Urine sample will be extracted and analyzed with every urine assay. | | |

| **B. Working Standard Control Solution** | | | |
|---|---|---|---|
| Starting Solution | 1 | µg/mL | High Control Solution |
| Starting Solution Aliquot | 0.5 | mL | |
| Diluent Volume | 10 | mL | 1:1 methanol:water, volumetric flask |
| Resulting Concentration | 50 | ng/mL | storage: freezer; stability: ≥ 1 year |

| **C. Hydrolysis Working Standard Control Solution** | | | |
|---|---|---|---|
| Starting Solution | 1 | µg/mL | Urine Hydrolysis Solution |
| Starting Solution Aliquot | 0.5 | mL | |
| Diluent Volume | 10 | mL | 1:1 methanol:water, volumetric flask |
| Resulting Concentration | 50 | ng/mL | storage: freezer; stability: ≥ 1 year |

**D. Urine Control Scheme**

| | WS Control Solution | High Control | Urine (mL) | Resulting Concentration, ng/mL |
|---|---|---|---|---|
| Control Level | Spike (µL) | Spike (µL) | | |
| Negative | 0 | 0 | 0.4 | 0 |
| Low | 20 | 0 | 1 | 1 |
| High | 0 | 10 | 1 | 10 |

Mix Low and High Controls well before removing 0.4 mL for analysis.

**E. Hydrolysis Urine Control Scheme**

| | Hydrolysis WS Control Solution | Hydrolysis High | Urine (mL) | Resulting Concentration, ng/mL |
|---|---|---|---|---|

| | | Control Solution | | |
|---|---|---|---|---|
| Control Level | Spike (µL) | Spike (µL) | | |
| Low Hydrolysis | 20 | 0 | 1 | 1 |
| High Hydrolysis | 0 | 10 | 1 | 10 |

Mix Low and High Hydrolysis Controls well before removing 0.4 mL for analysis.

*5.6.2.6 LC/MS Performance Standard (5 ng/mL)*

Add 5 µL of the Benzodiazepine High Calibrator Solution (1 µg/mL) and 10 µL of the Internal Standard Working Solution to 1 mL of Water:Acetonitrile (90:10). Store in refrigerated autosampler tray for up to one week or prepare fresh daily.

## 6 PROCEDURE

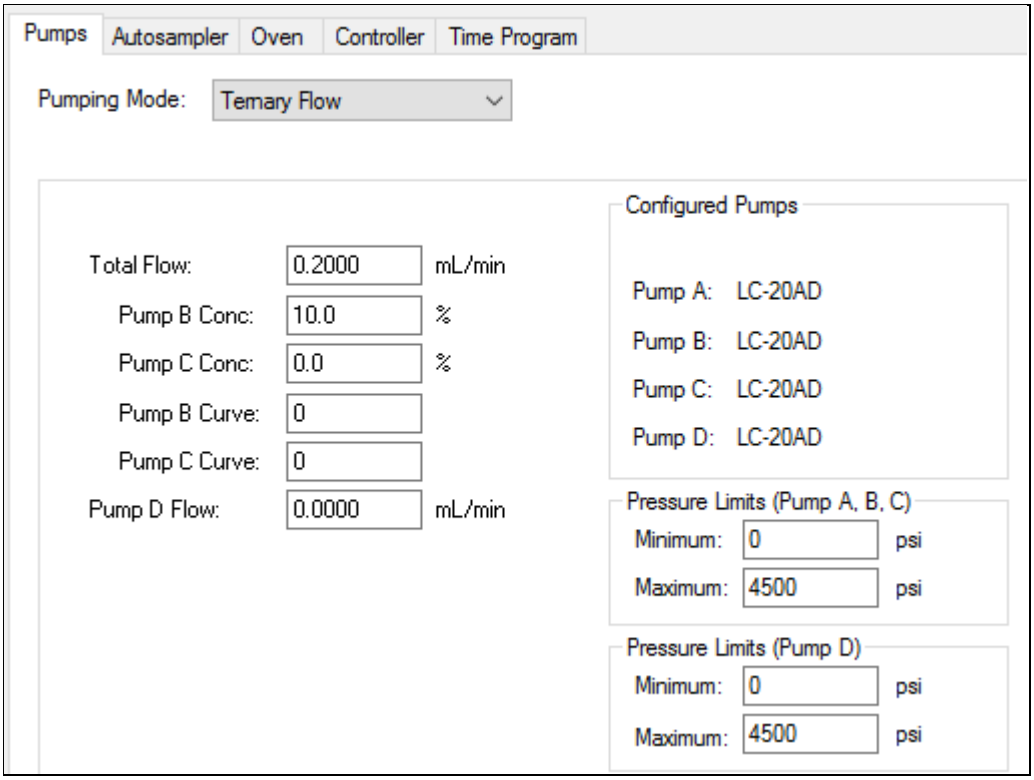
| | Step | Note | Reference/Lot |
|---|---|---|---|
| | **A. Sample Preparation for Blood Specimens** | | |
| | 1. Prepare blood calibrators as directed in Section 5.6.2.2 | | |
| ☐ | High Calibration Solution | | |
| ☐ | Low Calibration Solution | | |
| ☐ | Negative Control Matrix | | |
| ☐ | Pipettors Used | | |
| | 2. Prepare blood controls as directed in Section 5.6.2.3 | | |
| ☐ | High Control Solution | | |
| ☐ | Low Control Solution | | |
| ☐ | Negative Control Matrix (at least one negative control is required) | | |
| ☐ | Pipettors Used | | |
| | 3. Prepare case blood samples | | |
| ☐ | Add 0.2 mL of case blood sample to 12 x 75 mm test tube. (prepare in duplicate for quantitation and diluted as required for on-scale analysis) | | |
| | **B. Internal Standard(s) for Blood Specimens** | | |
| ☐ | 1. Add 0.020 mL of the Internal Standard Working Solution (500 ng/mL) to each sample | | |
| ☐ | 2. Vortex well | | |
| | **C. Protein Precipitation and Buffering for Blood Specimens** | | |
| ☐ | 1. Add 2 mL of zinc sulfate in methanol to each blood sample | | |
| ☐ | 2. Allow to sit for 1 minute | | |
| ☐ | 3. Vortex | | |
| ☐ | 4. Centrifuge samples for 5 minutes at 3000 rpm | | |
| ☐ | 5. Transfer supernatant to a new, properly labeled 16 x 100 mm test tube | | |
| ☐ | 6. Concentrate samples to ~ 0.4 mL under nitrogen at 60°C | | |
| ☐ | 7. Add 5.5 mL of Sorenson buffer to each tube | | |
| ☐ | 8. Vortex | | |
| ☐ | 9. Centrifuge samples for 1 minute at 3000 rpm | | |

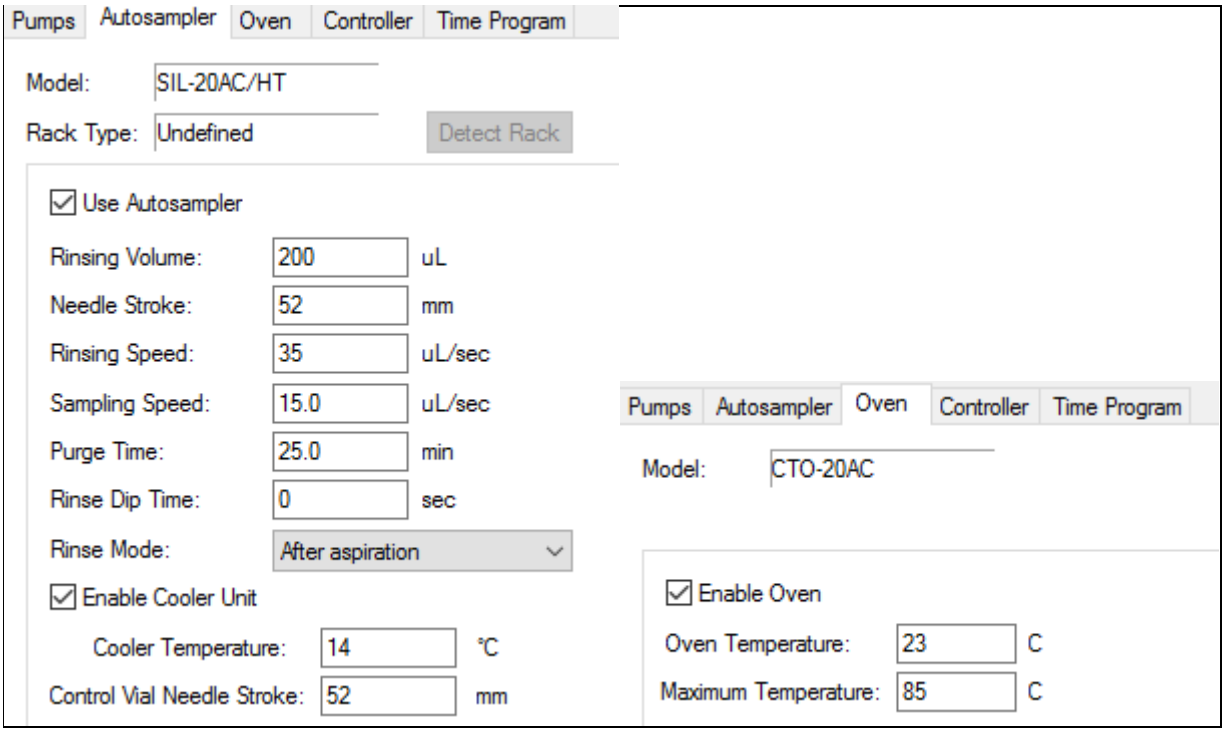
| | | | |
|---|---|---|---|
| | **D. Sample Preparation for Urine Specimens** | | |
| | 1. Prepare urine controls as directed in Section 5.6.2.5 | | |
| ☐ | **Low Positive Control Urine (1 ng/mL)** | | |
| | Benzodiazepine Working Standard Control Solution (50 ng/mL) | | |
| ☐ | **High Positive Control Urine (10 ng/mL)** | | |
| | High Control Solution (1 µg/mL) | | |
| ☐ | **Hydrolysis Low Positive Control Urine (1 ng/mL)** | | |
| | Urine Hydrolysis Working Standard Control Solution (50 ng/mL) | | |
| ☐ | **Hydrolysis High Positive Control Urine (10 ng/mL)** | | |
| | Hydrolysis High Control Solution (1 µg/mL) | | |
| ☐ | **Negative Control Matrix** | | |
| | 2. Prepare case urine samples | | |
| ☐ | i. Add 0.4 mL of each case urine sample into a properly labeled 16 x 100 mm test tube | | |
| | **E. Internal Standard(s) for Urine Specimens** | | |
| ☐ | 1. Add 0.010 mL of the Internal Standard Working Solution (500 ng/mL) to each sample | | |
| ☐ | 2. Vortex | | |
| | **F. Hydrolysis for Urine Specimens** | | |
| ☐ | 1. Add 0.6 mL ammonium acetate buffer (0.5 M, pH 5) | | |
| ☐ | 2. Add 0.1 mL β-glucuronidase | | |
| ☐ | 3. Cap and vortex | | |
| ☐ | 4. Incubate for 30 minutes at 68°C | | |
| ☐ | 5. Cool to room temperature | | |
| | **G. Protein Precipitation and Buffering for Urine Specimens** | | |
| ☐ | 1. Add 2mL zinc sulfate in methanol, allot to sit for 1 minute | | |
| ☐ | 2. Vortex. Centrifuge samples for 5 minutes at 3000 rpm | | |
| ☐ | 3. Transfer supernatant to a new 16 x 100 mm test tube | | |
| ☐ | 4. Concentrate samples to ~ 1.0 mL under nitrogen at 60°C | | |
| ☐ | 5. Add 5 mL of Sorenson buffer to each tube | | |
| ☐ | 6. Vortex and centrifuge samples for 1 minute at 3000 rpm | | |

| | | | |
|---|---|---|---|
| | **H. Solid Phase Extraction (Blood and Urine Samples)** | | |
| | 1. Condition cartridge (Oasis HLB 6 cc (500 mg) LP) | | |
| ☐ | a. Add 2 mL methanol | | |
| ☐ | b. Add 3 mL deionized water | | |
| ☐ | 2. Load samples on SPE cartridges | | |
| ☐ | 3. Wash cartridge with 2 mL of methanol:water:ammonia (40:60:0.5) | | |
| ☐ | 4. Dry cartridges at full vacuum for 15 minutes (use vacuum manifold; positive pressure manifold does not dry sufficiently) | | |
| ☐ | 5. Elute with 5 mL dichloromethane:isopropanol (75:25) under gravity | | |
| ☐ | 6. Evaporate eluent to dryness under nitrogen at 60°C | | |
| | **I. Reconstitution and Filtration** | | |
| | 1. Blood Samples | | |
| ☐ | i. Add 0.25 mL water:acetonitrile (90:10) | | |
| | 2. Urine Samples | | |
| ☐ | i. Add 0.1 mL water:acetonitrile (90:10) | | |
| | 3. Filter extracts through 0.45 micron filters (nylon) | | |
| ☐ | **J. Instrumental Analysis**<br>1. LC/MS:<br>i. Analyze LC/MS Performance Standard prior to batch analysis - 5 µL | | |
| ☐ | ii. Blood Extracts - 5 µL | | |
| ☐ | iii. Urine Extracts - 20 µL | | |
| | 2. Mobile Phase 1 (Aqueous) | | |
| | 3. Mobile Phase 2 (Organic) | | |
| | 4. LC Column | | |

## 7 ANALYTICAL PARAMETERS

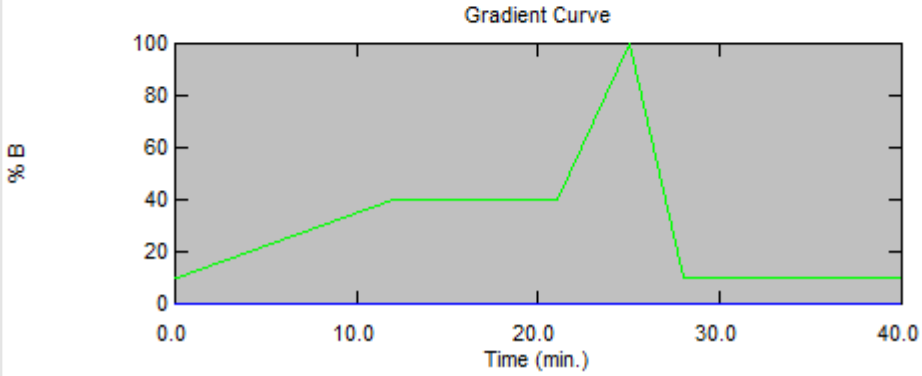
### 7.1 Liquid Chromatography



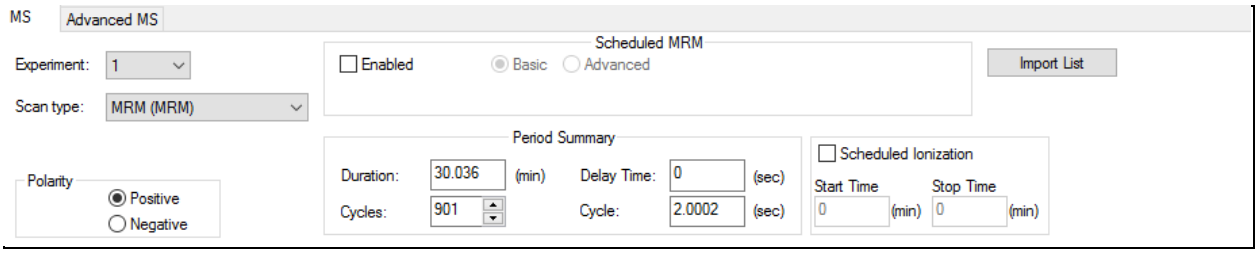



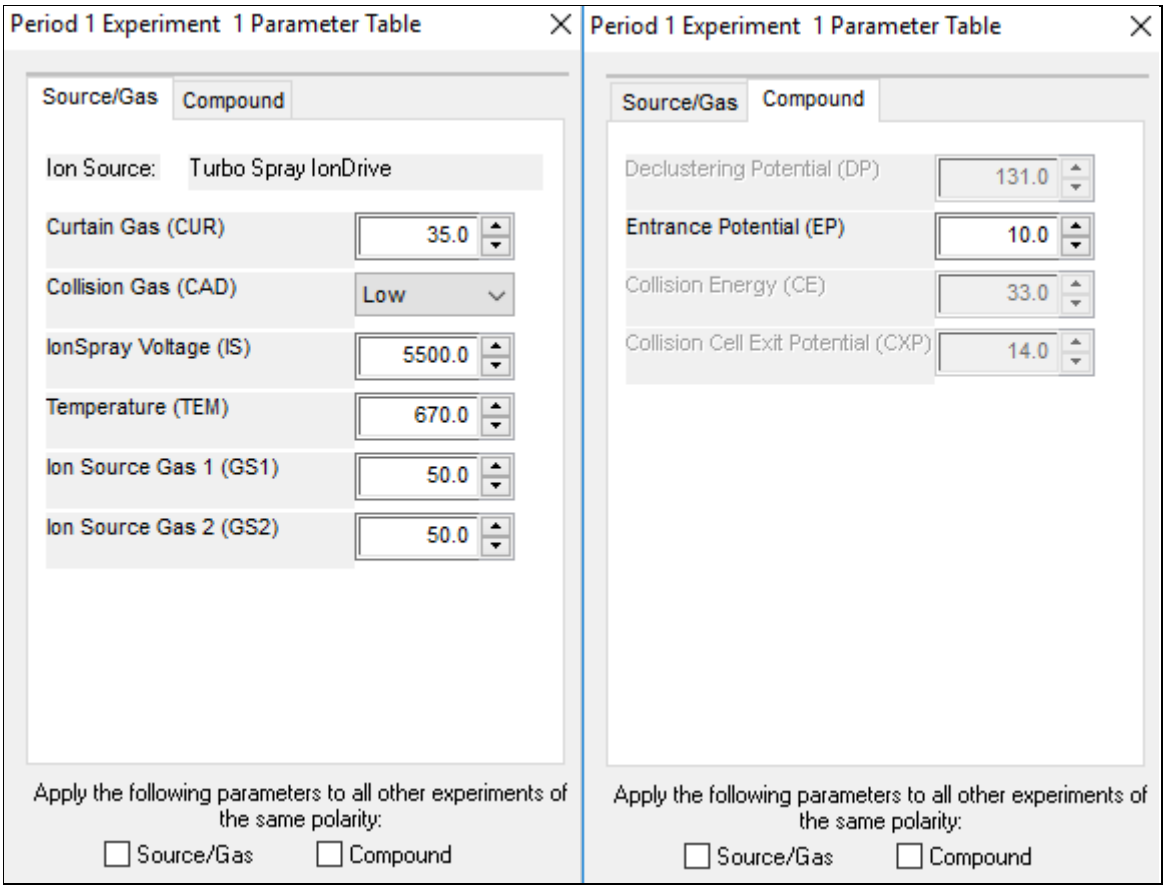
Pumps | Autosampler | Oven | Controller | Time Program

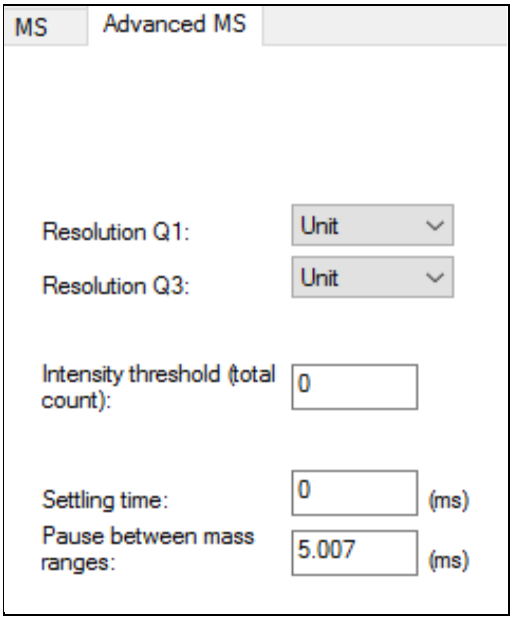
| | Time | Module | Event | Parameter |
|---|---|---|---|---|
| 1 | 0.10 | Pumps | Total Flow | 0.2 |
| 2 | 0.10 | Pumps | Pump B Conc. | 10 |
| 3 | 12.00 | Pumps | Pump B Conc. | 40 |
| 4 | 21.00 | Pumps | Pump B Conc. | 40 |
| 5 | 25.00 | Pumps | Pump B Conc. | 100 |
| 6 | 28.00 | Pumps | Pump B Conc. | 10 |
| 7 | 40.00 | Pumps | Pump B Conc. | 10 |
| 8 | 40.01 | Controller | Stop | |
| 9 | | | | |




## 7.2 Mass Spectrometry




Period 1 Experiment 1 Parameter Table
Source/Gas
Compound
Ion Source: Turbo Spray IonDrive
Curtain Gas (CUR) 35.0
Collision Gas (CAD) Low
IonSpray Voltage (IS) 5500.0
Temperature (TEM) 670.0
Ion Source Gas 1 (GS1) 50.0
Ion Source Gas 2 (GS2) 50.0
Apply the following parameters to all other experiments of the same polarity:
Source/Gas
Compound
Period 1 Experiment 1 Parameter Table
Source/Gas
Compound
Declustering Potential (DP) 131.0
Entrance Potential (EP) 10.0
Collision Energy (CE) 33.0
Collision Cell Exit Potential (CXP) 14.0
Apply the following parameters to all other experiments of the same polarity:
Source/Gas
Compound


MS
Advanced MS
Resolution Q1: Unit
Resolution Q3: Unit
Intensity threshold (total count): 0
Settling time: 0 (ms)
Pause between mass ranges: 5.007 (ms)

### 7.3 Analyte Specific Parameters

| Q1 Mass (Da) | Q3 Mass (Da) | Dwell Time (msec) | Analyte | DP(v) | CE(v) | CXP(v) |
|---|---|---|---|---|---|---|
| 286.233 | 222.200 | 12.4 | 7_aminoclonazepam_1 | 131 | 33 | 14 |
| 286.233 | 250.200 | 12.4 | 7_aminoclonazepam_2 | 131 | 29 | 14 |
| 286.233 | 195.200 | 12.4 | 7_aminoclonazepam_3 | 131 | 43 | 12 |
| 288.219 | 222.200 | 12.4 | 7_aminoclonazepam_4 | 100 | 33 | 12 |
| 290.218 | 226.200 | 12.3 | 7_aminoclonazepam_d4 | 126 | 35 | 14 |
| 284.285 | 227.300 | 14.1 | 7_aminoflunitrazepam_1 | 151 | 35 | 14 |
| 284.285 | 240.300 | 14.1 | 7_aminoflunitrazepam_2 | 151 | 45 | 14 |
| 284.285 | 135.000 | 14.1 | 7_aminoflunitrazepam_3 | 151 | 35 | 14 |
| 291.285 | 138.000 | 13.9 | 7_aminoflunitrazepam_d7 | 121 | 39 | 16 |
| 325.250 | 297.200 | 19.4 | alpha_hydroxyalprazolam_1 | 106 | 35 | 16 |
| 325.250 | 216.100 | 19.4 | alpha_hydroxyalprazolam_2 | 106 | 53 | 24 |
| 327.247 | 299.200 | 19.4 | alpha_hydroxyalprazolam_3 | 181 | 35 | 16 |
| 330.273 | 302.300 | 19.2 | alpha_hydroxyalprazolam_d5 | 136 | 37 | 16 |
| 342.211 | 203.000 | 16.6 | alpha_hydroxymidazolam_1 | 71 | 35 | 12 |
| 342.211 | 168.100 | 16.6 | alpha_hydroxymidazolam_2 | 71 | 49 | 18 |
| 344.216 | 205.000 | 16.6 | alpha_hydroxymidazolam_3 | 111 | 35 | 12 |
| 346.232 | 203.000 | 16.6 | alpha_hydroxymidazolam_d4 | 151 | 37 | 12 |
| 359.211 | 176.000 | 19.3 | alpha_hydroxytriazolam_1 | 151 | 37 | 20 |
| 359.211 | 331.300 | 19.3 | alpha_hydroxytriazolam_2 | 151 | 37 | 18 |
| 359.211 | 239.200 | 19.3 | alpha_hydroxytriazolam_3 | 151 | 61 | 14 |
| 361.195 | 333.200 | 19.3 | alpha_hydroxytriazolam_4 | 100 | 37 | 18 |
| 363.229 | 176.000 | 19.2 | alpha_hydroxytriazolam_d4 | 176 | 37 | 12 |
| 309.266 | 205.100 | 23.2 | Alprazolam_1 | 130 | 45 | 12 |
| 309.266 | 281.200 | 23.2 | Alprazolam_2 | 130 | 35 | 16 |
| 311.237 | 283.200 | 23.2 | Alprazolam_3 | 126 | 35 | 16 |
| 314.253 | 279.300 | 23.0 | Alprazolam_d5 | 131 | 35 | 16 |
| 300.217 | 227.100 | 14.9 | chlordiazepoxide_1 | 91 | 33 | 18 |
| 300.217 | 241.051 | 14.9 | chlordiazepoxide_2 | 91 | 31 | 16 |
| 300.217 | 165.100 | 14.9 | chlordiazepoxide_3 | 91 | 63 | 18 |
| 305.133 | 232.100 | 14.8 | chlordiazepoxide_d4 | 81 | 33 | 14 |
| 316.194 | 270.200 | 22.5 | clonazepam_1 | 146 | 35 | 16 |
| 316.194 | 214.100 | 22.5 | clonazepam_2 | 146 | 49 | 12 |
| 318.184 | 272.200 | 22.5 | clonazepam_3 | 141 | 35 | 14 |
| 320.238 | 274.200 | 22.3 | clonazepam_d4 | 191 | 35 | 16 |
| 289.173 | 226.200 | 24.3 | Desalkylflurazepam_1 | 36 | 39 | 12 |
| 289.173 | 179.100 | 24.3 | Desalkylflurazepam_2 | 36 | 59 | 20 |
| 289.173 | 140.004 | 24.3 | Desalkylflurazepam_3 | 36 | 39 | 16 |
| 291.132 | 226.200 | 24.3 | Desalkylflurazepam_4 | 111 | 39 | 14 |
| 293.205 | 230.200 | 24.1 | Desalkylflurazepam_d4 | 56 | 39 | 14 |

| Q1 Mass (Da) | Q3 Mass (Da) | Dwell Time (msec) | Analyte | DP(v) | CE(v) | CXP(v) |
|---|---|---|---|---|---|---|
| 285.086 | 193.200 | 26.9 | diazepam_1 | 126 | 47 | 22 |
| 285.086 | 154.100 | 26.9 | diazepam_2 | 126 | 35 | 18 |
| 287.214 | 193.100 | 26.9 | diazepam_3 | 141 | 43 | 22 |
| 290.203 | 198.200 | 26.8 | diazepam_d5 | 46 | 45 | 24 |
| 314.220 | 268.200 | 25.3 | flunitrazepam_1 | 106 | 35 | 14 |
| 314.220 | 239.200 | 25.3 | flunitrazepam_2 | 106 | 45 | 14 |
| 314.220 | 183.100 | 25.3 | flunitrazepam_3 | 106 | 67 | 20 |
| 321.295 | 275.300 | 24.9 | flunitrazepam_d7 | 101 | 37 | 16 |
| 388.577 | 315.200 | 17.1 | flurazepam_1 | 56 | 31 | 18 |
| 388.577 | 288.200 | 17.1 | flurazepam_2 | 56 | 35 | 16 |
| 390.313 | 317.200 | 17.1 | flurazepam_3 | 121 | 31 | 18 |
| 321.203 | 275.100 | 21.5 | Lorazepam_1 | 91 | 33 | 14 |
| 321.203 | 229.200 | 21.5 | Lorazepam_2 | 91 | 43 | 12 |
| 323.186 | 277.200 | 21.5 | Lorazepam_3 | 96 | 29 | 16 |
| 327.203 | 281.100 | 21.4 | Lorazepam_Cl_d4 | 146 | 27 | 6 |
| 326.205 | 249.100 | 16.8 | Midazolam_1 | 176 | 51 | 14 |
| 326.205 | 291.300 | 16.8 | Midazolam_2 | 176 | 37 | 16 |
| 326.205 | 222.300 | 16.8 | Midazolam_3 | 176 | 63 | 28 |
| 328.190 | 291.200 | 16.8 | Midazolam_4 | 150 | 37 | 16 |
| 330.237 | 253.100 | 16.7 | midazolam_d4 | 126 | 53 | 14 |
| 300.173 | 198.100 | 20.4 | N_desmethylflunitrazepam_1 | 91 | 53 | 22 |
| 300.173 | 254.200 | 20.4 | N_desmethylflunitrazepam_2 | 91 | 37 | 14 |
| 300.173 | 225.100 | 20.4 | N_desmethylflunitrazepam_3 | 91 | 47 | 12 |
| 304.211 | 258.200 | 20.3 | N_desmethylflunitrazepam_d4 | 116 | 33 | 16 |
| 271.258 | 208.300 | 23.5 | Nordiazepam_1 | 141 | 39 | 12 |
| 271.258 | 165.300 | 23.5 | Nordiazepam_2 | 141 | 37 | 20 |
| 271.258 | 140.025 | 23.5 | Nordiazepam_3 | 141 | 37 | 16 |
| 276.382 | 213.200 | 23.1 | Nordiazepam_d5 | 26 | 39 | 12 |
| 287.231 | 241.300 | 20.2 | Oxazepam_1 | 161 | 31 | 14 |
| 287.231 | 231.200 | 20.2 | Oxazepam_2 | 161 | 31 | 12 |
| 289.213 | 243.200 | 20.2 | Oxazepam_3 | 106 | 29 | 14 |
| 292.196 | 246.200 | 20.1 | Oxazepam_d5 | 101 | 33 | 14 |
| 301.266 | 255.100 | 25.3 | Temazepam_1 | 101 | 29 | 22 |
| 301.266 | 177.000 | 25.3 | Temazepam_2 | 101 | 51 | 20 |
| 303.251 | 257.200 | 25.3 | Temazepam_3 | 71 | 29 | 16 |
| 306.215 | 260.200 | 25 | Temazepam_d5 | 81 | 33 | 16 |
| 343.213 | 239.200 | 24.3 | Triazolam_1 | 36 | 55 | 12 |
| 343.213 | 308.300 | 24.3 | Triazolam_2 | 36 | 35 | 18 |
| 345.212 | 241.100 | 24.3 | Triazolam_3 | 36 | 55 | 14 |
| 347.216 | 243.300 | 24.1 | Triazolam_d4 | 166 | 57 | 14 |

# 8 DATA ANALYSIS

## 8.1 Decision Criteria

### *8.1.1 LC/MS Performance Standard Decision Criteria*

Peaks should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. The authorized individual should ensure that the peaks entirely elute within their MRM windows, and adjust the MRM window times, if necessary.

### *8.1.2 Unknown Sample Decision Criteria*

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay.

#### *8.1.2.1 Batch Acceptance*

No analytes of interest should be detected in the Negative Control. For this purpose, analytes of interest are defined as any analytes that are being reported for this batch.

Each of the analytes in the Positive Control should be detected in the LC/MS data. High and Low Positive Controls should fall within ±20% of the target value. See TOX-101 for further guidance.

Oxazepam should be detected in the Urine Hydrolysis Positive Controls. The peak area of the first transition for oxazepam should be within ±50% of the area of oxazepam in the Low and High Positive Control Urine samples.

#### *8.1.2.2 Unknown Sample Criteria*

Each of the Internal Standards should be detectable in the LC/MS data.

##### *8.1.2.2.1 Chromatography*

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs.

##### *8.1.2.2.2 Retention Time*

The retention time of the peak should be within ± 2% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, an extracted Positive Control, or an appropriate deuterated analog.

##### *8.1.2.2.3* Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 10 when using the Analyst software. Further, the baseline signal for the peak of interest should be at least 10-fold greater than that for any observed peak at similar retention time in a Negative Control or solvent blank injected just prior to the sample.

*8.1.2.2.4* Mass Spectrometry

At least three independent MS/MS experiments are conducted for each analyte. Two ion ratios are calculated for each analyte. The mass spectrum of the analyte of interest should match that of a reference standard, extracted calibrator, or an extracted Positive Control. See TOX-104 for further guidance.

### 8.2 Calculations

*8.2.1 Calibration*

| | |
|---|---|
| **Model** | Linear |
| **Weighting** | 1/x2 |

Refer to TOX-101 for further guidance.

*8.2.2 Software*

Calculations may be performed by one or more of the following software packages:

A. Sciex
   1. Analyst
   2. Multiquant
   3. Report Builder

B. Microsoft
   1. Excel

## 9 REPORTING

### 9.1 Measurement Uncertainty

Refer to CHEM-100 and TOX-101.

## 10 CORRECTIVE MEASURES

Refer to TOX-101 for guidance on action steps in the event of a quality control failure.

## 11 PERFORMANCE CHARACTERISTICS

### 11.1 LOD

Blood: 1.25 ng/mL (or lower)

Urine: 0.5 ng/mL (or lower)

### 11.2 LOQ

Blood: 2.5 ng/mL

### 11.3 Linearity

Blood: 2.5 to 375 ng/mL

### 11.4 Bias

n=15 for values in table

| | Bias (%; at 5 ng/mL) | Bias (%; at 100 ng/mL) | Bias (%; at 250 ng/mL) |
|---|---|---|---|
| 7-aminoclonazepam | -3.63 | 2.06 | 3.65 |
| 7-aminoflunitrazepam | 2.71 | 5.89 | 1.32 |
| α-OH midazolam | 3.93 | 3.76 | 0.44 |
| α-OH alprazolam | 3.83 | 3.88 | -1.22 |
| diazepam | 5.21 | 4.54 | -2.80 |
| clonazepam | 0.39 | 3.69 | 1.41 |
| alprazolam | 6.84 | 6.45 | -1.85 |
| flunitrazepam | 1.23 | 2.09 | -0.17 |
| desalkylflurazepam | 2.71 | 2.42 | 0.36 |
| lorazepam | 2.04 | 2.89 | 0.69 |
| n-desmethylflunitrazepam | 0.09 | 2.11 | -0.78 |
| midazolam | 0.03 | 2.17 | 1.01 |
| nordiazepam | 2.29 | 4.72 | -2.76 |
| oxazepam | 0.52 | 0.97 | -1.00 |
| temazepam | 5.29 | 5.26 | -2.21 |
| triazolam | 7.57 | 5.43 | -3.56 |
| α-OH triazolam | 2.31 | 4.94 | -0.14 |

### 11.5 Precision

As both repeatability and intermediate precision; n=15 for all values in tables

| | Repeatability (%; at 5 ng/mL) | Repeatability (%; at 100 ng/mL) | Repeatability (%; at 250 ng/mL) |
|---|---|---|---|
| 7-aminoclonazepam | 7.20 | 9.71 | 8.96 |
| 7-aminoflunitrazepam | 7.29 | 6.49 | 11.09 |
| α-OH midazolam | 5.45 | 3.22 | 4.26 |
| α-OH alprazolam | 4.95 | 2.78 | 4.22 |
| diazepam | 5.26 | 2.34 | 3.33 |
| clonazepam | 4.72 | 2.47 | 3.86 |
| alprazolam | 4.83 | 3.66 | 2.85 |
| flunitrazepam | 4.54 | 3.02 | 2.76 |
| desalkylflurazepam | 4.79 | 4.03 | 4.01 |
| lorazepam | 6.79 | 3.28 | 3.12 |
| n-desmethylflunitrazepam | 3.94 | 3.62 | 3.76 |
| midazolam | 4.31 | 3.17 | 3.75 |

| | Repeatability (%; at 5 ng/mL) | Repeatability (%; at 100 ng/mL) | Repeatability (%; at 250 ng/mL) |
|---|---|---|---|
| nordiazepam | 5.00 | 2.79 | 3.73 |
| oxazepam | 5.08 | 2.65 | 3.08 |
| temazepam | 4.32 | 2.70 | 2.96 |
| triazolam | 4.59 | 3.17 | 1.92 |
| α-OH triazolam | 4.67 | 3.37 | 4.00 |
| | Intermediate Precision (%; at 5 ng/mL) | Intermediate Precision (%; at 100 ng/mL) | Intermediate Precision (%; at 250 ng/mL) |
| 7-aminoclonazepam | 10.29 | 9.71 | 9.17 |
| 7-aminoflunitrazepam | 7.29 | 6.49 | 11.65 |
| α-OH midazolam | 6.45 | 5.25 | 5.74 |
| α-OH alprazolam | 5.72 | 5.04 | 5.37 |
| diazepam | 5.36 | 3.94 | 5.95 |
| clonazepam | 4.91 | 4.05 | 5.37 |
| alprazolam | 5.63 | 5.04 | 6.66 |
| flunitrazepam | 5.04 | 4.74 | 4.47 |
| desalkylflurazepam | 5.44 | 4.64 | 6.60 |
| lorazepam | 7.67 | 7.38 | 7.53 |
| flurazepam | 10.45 | 7.50 | 6.29 |
| n-desmethylflunitrazepam | 5.40 | 4.50 | 4.81 |
| midazolam | 5.29 | 5.09 | 6.33 |
| nordiazepam | 5.40 | 5.20 | 5.40 |
| oxazepam | 5.68 | 3.29 | 3.16 |
| temazepam | 5.52 | 5.84 | 5.45 |
| triazolam | 5.11 | 5.21 | 5.34 |
| α-OH triazolam | 4.91 | 5.83 | 6.22 |

### 11.6 Carryover

No significant carryover detected for the linear range established.

## 12 LIMITATIONS

Oxazepam may be unstable in methanol.

## 13 SAFETY

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

## 14 REVISION HISTORY

| Revision | Issued | Changes |
|---|---|---|
| 08 | 06/01/2023 | Complete document reformat.<br><br>2 – Clarified chlordiazepoxide suitable for qualitative analysis only<br><br>5.6.1.2, 5.6.2.5.c, 5.6.2.5.e – updated to use d0-oxazepam glucuronide versus d5 version (which is no longer available) as a hydrolysis process control<br><br>6-j – specified injection volumes for extracts<br><br>8.1.2.1 – updated guidance for evaluating hydrolysis control to account for change from d5 to d0 glucuronide |