Title: Benzodiazepine Quantitative/Qualitative Analysis using Liquid Chromatography/Mass Spectrometry (LC/MS) (TX 31)

1. Introduction

Benzodiazepines and the functionally related "Z drugs" (e.g., Zolpidem and Zopiclone) are drugs whose properties include anti-anxiety, hypnotic, and sedation. Benzodiazepines are class of anti-anxiety agents which interfere with the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Their effects include anti-anxiety, sedation, sleep, muscle relaxation, and anticonvulsant activity. Long-term benzodiazepine therapy can be associated with addiction and withdrawal symptoms. While benzodiazepines are considered safer than drugs in the barbiturate class, there is still significant potential for abuse and dependence with continued use. Samples that require confirmation by liquid chromatography/mass spectrometry (LC/MS) are extracted from buffered, diluted sample aliquots by adsorption onto solid phase extraction (SPE) columns. Benzodiazepines/Z-drugs that may be present are then eluted from the SPE columns, dried, and reconstituted before being analyzed using an LC/MS system. The detection of these types of drugs is determined using single point calibration cut off values. Quantitations are performed using a multipoint calibration graph with deuterated internal standards. Matrix-specific (blood and/or urine as needed) positive and negative controls are extracted and analyzed in each analytical batch. The presence of Benzodiazepines/Z-drugs may be confirmed in urine, blood or other aqueous fluids.

2. Scope

This procedure can be used for the qualitative and/or quantitative determination of biological specimens for the presence of select drugs and/or metabolites (e.g., alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, lorazepam, diazepam, nordiazepam, oxazepam, temazepam, Zolpidem, midazolam, flunitrazepam, 7-aminoflunitrazepam, flurazepam, N-desalkylflurazepam, triazolam, and zolpiclone). The addition of analytes to this group may not necessitate a complete validation to be performed of this procedure, but rather a verification that the analytes can be detected reproducibly and at a certain detection limit(s).

3. Principle

Biological specimens are analyzed for the presence of drugs of abuse (DoA) and/or their metabolites by extraction using solid phase extraction (SPE) columns. Final extracts are analyzed by LC/MS using ESI and can involve a combination of selected reaction monitoring (SRM) and full scan analysis modes.

4. Specimens

This procedure uses biological fluid(s) such as blood, urine, serum, and/or plasma. Qualitative analyses are usually limited to urine, blood, serum/plasma, and and/or vitreous samples while quantitative analyses are usually limited to blood (or serum/plasma). Blood sample collection tubes containing proper anticoagulant and preservative (i.e., 'gray-tops' containing potassium oxalate and sodium fluoride) should be used. Containers for urine and other sample types should be within sealed plastic (e.g., polypropylene) containers, however other containers may be acceptable. All samples should be received with proper documentation, have been properly sealed (i.e., prevent sample loss, contamination, or deleterious change), and have been properly stored. Once samples are received within the Toxicology Unit they will be properly stored within either a refrigerator or freezer. Typically 0.5 mL of sample is consumed during the analysis but varying volumes may be used, as necessary. Dilution of samples due to limited specimen or due to suspicion of high drug or metabolite concentration is acceptable.

5. Equipment/Materials/Reagents

5.1 General laboratory glassware

5.2 Disposable borosilicate test tubes (e.g., 16 x 100 mm, round bottom, borosilicate glass with Teflon caps)

5.3 Vortex mixer

5.4 Sonicator

5.5 Automatic pipettes (with disposable tips)

5.6 Positive pressure solid phase extraction device – SPEWare Cerex, 48 sample (or equivalent)

5.7 Sample concentrator with nitrogen – SPEWare Cerex 48 heated (or equivalent)

5.8 Centrifuge

5.9 pH paper (or equivalent)

5.10 Trace B Extraction Columns – SPEWare (or equivalent)

5.11 Liquid Chromatograph/Mass Spectrometer (Shimadzu LCMS-8030, or equivalent)

5.12 HPLC column – Kinetex, phenyl hexyl, 2.6 µm, 100Å, 50 mm x 4.6 mm (Phenomenex), or equivalent)

5.13 Pre-Column – SecurityGuard ULTRA Cartridge UHPLC Kinetex for 4.6mm ID Columns (Phenomenex or equivalent)

5.14 Autosample vials (LC/MS grade 1.8mL or equivalent)

5.15 Acetic acid, glacial ($CH_3COOH_{(l)}$, Reagent grade or equivalent)

5.16 Ammonium formate (NH_4CHOO, Reagent grade or equivalent)

5.17 β-Glucuronidase (p. vulgata ; Sigma or equivalent)

5.18 Formic acid (HCOOH, Reagent grade or equivalent)

5.19 Methanol (MeOH, Reagent grade, LC/MS Grade, or equivalent)

5.20 Sodium acetate trihydrate ($NaCH_3COO \cdot 3H_2O$, Reagent grade or equivalent)

5.21 Sodium bicarbonate ($NaHCO_3$; Reagent grade or equivalent)

5.22 Sodium carbonate (Na_2CO_3 ; Reagent grade or equivalent)

5.23 Water (Mobile Phase) (LC/MS Grade ; Optima or equivalent)

5.24 Certified Reference Materials (alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, diazepam, desmethyldiazepam, lorazepam, oxazepam, temazepam, Zolpidem ; 1 mg/mL) (Cerilliant, Lipomed, or equivalent)

5.25 Benzodiazepine Control Mixtures (UTAK ; Blood (#12100) ; Urine (#12090)) or equivalent

5.26 Benzodiazepine Multi-Component CRM Mixtures (Cerilliant B-033-1ML) or equivalent

5.27 Ammonium $Formate_{(aq)}$: ($NH_4CHOO_{(aq)}$; 5M ; 31.5% (w/v)): Can be prepared by dissolving 3.15 g of ammonium formate in 10 mL of water. Stable for at least one (1) year in glass container when refrigerated.

5.28 Sodium $Acetate\ Buffer_{(aq)}$ (0.07M ; $NaCH_3COO$; pH~4.5): Can be prepared by combining 5.86 g of sodium acetate with 3.24 mL of glacial acetic acid in a 1 L volumetric cylinder and diluting to volume with water. Stable for at least one (1) year in glass container while at room temperature.

5.29 Sodium $Bicarbonate_{(aq)}$ (0.1M ; $NaHCO_3$; 0.84% (w/v) ; pH~8): Can be prepared by dissolving 4.2 g of sodium bicarbonate in water within a 500 mL volumetric flask and diluting to volume with water. Stable for at least one (1) year in glass container while at room temperature.

5.30 Sodium $Carbonate_{(aq)}$ (0.1M ; Na_2CO_3 ; 1.1% (w/v) ; pH~11): Can be prepared by dissolving 5.3 g of sodium carbonate in water within a 500 mL volumetric flask and diluting to volume with water. Stable for at least one (1) year in glass container while at room temperature.

5.31 $Bicarbonate/Carbonate\ Buffer_{(aq)}$ (pH~9): Can be prepared by transferring 0.1M sodium $bicarbonate_{(aq)}$ solution into a beaker and checking pH (should be pH~8). Adjust the pH to ~9 using the 0.1M sodium $carbonate_{(aq)}$ solution. Stable for at least one (1) year in glass container while at room temperature.

5.32 Solid Phase Extraction Elution Solution (SPEES) (e.g., 50 mL ; ~2 mL needed per SPE sample): {Dichloromethane (CH_2Cl_2) : IPA : NH_4OH (80 : 18 : 2)}.
Can be prepared by adding 9 mL of isopropanol to 1 mL of ammonium hydroxide within a 50 mL volumetric cylinder. To this mixture add 40 mL of methylene chloride and mix. This solution will be prepared when needed for use.

5.33 $MeOH_{(aq)}$ (20% (v/v)): Can be prepared by adding 80 mL of water and 20 mL of methanol to a 100 mL volumetric cylinder and mixing. Stable for at least one (1) month in glass container when refrigerated.

5.34 Methanol-HCl (1% (v/v)): Can be prepared by dissolving 10 µL of hydrochloric acid into 990 µL of methanol (or in different volume with an equivalent ratio). Stable for at least one (1) month in glass container when refrigerated. Verify pH is ≤ 3 prior to use.

5.35 Mobile Phase A – 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$:
Can be prepared by mixing 50 µL of formic acid with 0.5 mL of 5M ammonium formate in a 500 mL volumetric cylinder, diluting to volume with [Mobile Phase] water, and mixing well. Store in glass at room temperature. Stable for at least one (1) week while in a closed state.
{Note: 5mM $NH_4CHOO_{(aq)}$ is equivalent to 0.0032 % (w/v) $NH_4CHOO_{(aq)}$.}

5.36 Mobile Phase B – (MeOH or CH_3OH). Store in glass at room temperature – stable indefinitely in a closed state at room temperature.

Note-01: Volumes can be adjusted using appropriate ratios to account for the number of samples that are to be extracted (~2 mL are needed for each extraction sample).

Note-02: Unless otherwise noted the above solutions are stable for at least one (1) year when stored in a refrigerator/freezer from either their creation or from their last verification. Check clarity and check pH (when indicated as having a pH value) for all solutions prior to use.

6. Standards and Controls

Positive and Negative Controls and Calibrators:
Can be purchased (e.g., UTAK Labs) or generated in-house (e.g., spiked/un-spiked blank blood, spiked/un-spiked blank urine, spiked/un-spiked vitreous (or water)). Store frozen, refrigerated, or obtain fresh. If purchased, the stability should be determined by the manufacturer. Appropriate positive and negative controls will be extracted and analyzed with each assay or batch. Externally-prepared controls and calibrators are preferred, when feasible. Internally-prepared controls will be used only when externally-prepared controls are not available. When possible, control and calibrator solutions will be matrix-matched.

Internal Standard Analytes:
Purchased from suitable vendor(s) in solutions or neat solids (e.g., 0.1 mg/mL solution). Storage and stability is determined by manufacturer(s). Additional or alternative deuterated compounds may be used. If purchased solutions vary in concentration than what has typically been used, analysts may adjust the preparation volumes to account for differences. Any variations will be noted on solution preparation worksheets and, if such worksheets are not linked to case files, variations will be noted in case files (e.g., notes, summary sheets).

Preparation of Calibrators, Controls, Standards:

Internal Standard(s)

6.1 Internal Standard (I.S.) Working Solution (Diazepam–D_5; 250 ng/mL):
Can be prepared by adding 250 µL of the 100 µg/mL Diazepam-D_5 into a 100 mL volumetric flask and diluting with MeOH. Mix and store refrigerated in glass. Stable for at least 1 year.

Note-03: Additional internal standard analytes can be used but must be documented within batch notes:
Amphetamine-D_6
Oxycodone-D_3
Benzoylecgonine-D_3

6.2 [Optional] Instrument Performance Standard Mix Solution: ((Diazepam-D_5) ; 10 ng/mL)

This is optional and can be used to evaluate the instrument in addition to the performance check solution for the LC/MS instrument.

Dilute the I.S. Working Solution 1:25 with water to make a 10 ng/mL final concentration. This can be done by mixing 20 µL of the I.S. Working Solution, combining it with 500 µL of deionized water, placing it into an autosample vial, and capping. This solution will be prepared when needed for use.

Note-04: Other analytes within this [Optional] I.S. Solution (if present) are not evaluated – just the Diazepam-D_5 analyte is used for evaluation purposes.

Controls

6.3 Negative Control Blood:

Purchased (e.g., Diagnostics Products Corporation, UTAK Labs), donated (e.g., American Red Cross, hospital blood bank), or in-house demonstrated negative control blood (e.g., from a proven blank specimen). Store frozen or refrigerated. If purchased or donated, stability should, when possible, be determined by manufacturer.

6.4 Negative Control Urine:

Purchased, acquired (e.g., Office of the Chief Medical Examiner (OCME)), or in-house demonstrated to be negative. Store refrigerated or frozen.

6.5 Negative Control [Other Matrix]

Synthetic or in-house negative controls [other matrix] which are analyte-free. Store refrigerated or obtain fresh. If purchased, stability should be determined by manufacturer. A negative control [other matrix] should be extracted and analyzed with each appropriate assay or batch.

6.6 Positive Control Blood (Externally prepared ; 100 ng/mL)

Lyophilized whole blood containing select analytes can be used (e.g., UTAK Benzodiazepines Plus 100 ng/mL ; Product #12100 ; approximately 23 analytes). These are usually reconstituted with water to a certain volume (e.g., 5 mL). Follow manufacturer's recommendation for preparation. Prepare fresh and mix adequately. Store reconstituted control material refrigerated. Stability (e.g., 25 days) should be listed by manufacturer but can be longer if verified prior to use.

6.7 Positive Control Urine (Externally prepared ; 100 ng/mL)

Lyophilized urine containing select analytes can be used (e.g., UTAK Benzodiazepines Plus 100 ng/mL ; Product #12090 ; approximately 25 analytes). These are usually reconstituted with

water to a certain volume (e.g., 5 mL). Follow manufacturer's recommendation for preparation. Prepare fresh and mix adequately. Store reconstituted control material refrigerated. Stability (e.g., 25 days) should be listed by manufacturer but can be longer if verified prior to use.

Note-05: The externally-prepared Urine positive control can be used as a positive control for other matrices if they have a similar make-up (e.g., water in both vitreous and urine).

6.8 Positive Control Blood and Urine Solutions (In-House prepared ; 100 ng/mL ; e.g., from Lipomed) (Only used when External Positive Controls are not available)
{alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, diazepam, nordiazepam, lorazepam, oxazepam, temazepam, Zolpidem}

6.8.1 In-House Positive Control Stock Solution (100 µg/mL ; e.g., Lipomed ; 10 analytes above)

Combine 100 µL of the following ten (10) 1 mg/mL reference standards (e.g., from Lipomed) into properly labeled screw-capped test tubes and mix (total volume = 1 mL):

alprazolam	α-hydroxyalprazolam	clonazepam	7-aminoclonazepam	diazepam
desmethyldiazepam	lorazepam	oxazepam	temazepam	Zolpidem (tartrate)

6.8.2 In-House Positive Control Working Solution (2.0 µg/mL ; 10 analytes (above))

Can be prepared by adding 100 µL of the In-House Positive Control Stock Solution (100 µg/mL) into a 5 mL volumetric flask that contains some methanol and bringing to volume with methanol. Tightly cap, mix, protect from light, properly label, and store in freezer. Stable for at least six (6) months.

6.8.3 In-House Positive Control Blood Solution (100 ng/mL ; 10 analytes (above))

Can be prepared by adding 50 µL of the In-House Positive Control Working Solution (2.0 µg/mL) to 500 µL of blank blood. Tightly cap, mix, protect from light, properly label, and store in freezer. Stable for at least six (6) months.

6.8.4 In-House Positive Control Urine Solution (100 ng/mL ; 10 analytes (above))

Can be prepared by adding 50 µL of the In-House Positive Control Working Solution (2.0 µg/mL) to 500 µL of blank urine. Tightly cap, mix, protect from light, properly label, and store in freezer. Stable for at least six (6) months.

6.8.5 In-House Positive Control [Other] (100 ng/mL ; 10 analytes (above))

Can be prepared by adding 50 µL of the In-House Positive Control Working Solution (2.0 µg/mL) with 500 µL of appropriate matrix. Tightly cap, mix, protect from light, properly label, and store in freezer. Stable for at least six (6) months.

Calibrators

Solutions used for calibrations should be prepared using as few dilutions as possible and will be from certified reference materials (CRM). While this procedure lists specific analytes, equivalent reference standards may be substituted, if needed. Calibrators should be from different suppliers than those used for control solutions, when possible. If the same supplier is used for both, then calibrator and control solutions should be prepared from solutions containing differing lot numbers. When purchased standards are not 1 mg/mL concentrations, when different analytes are prepared as calibrators, or when the number of analytes differ, then appropriate adjustments will be made and a second Analyst should confirm calculations, dilutions, and other preparatory work so as to ensure quality. Such changes will be recorded within appropriate case notes, batch preparation documents, and/or reagent log books. Older calibration curves may be used and fresh calibrators do not need to be prepared for every new batch (see note below).

6.9 Benzodiazepine Calibrator Stock Sol'n (10 component calibrator mix ; 100 µg/mL)
{e.g., alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, diazepam, desmethyldiazepam, lorazepam, oxazepam, temazepam, Zolpidem}

6.9.1 Combine 100 µL of the following ten (10) 1 mg/mL reference standards (e.g., from Cerilliant) into properly labeled screw-capped test tubes and mix (total volume = 1 mL):

alprazolam	α-hydroxyalprazolam	clonazepam	7-aminoclonazepam	diazepam
desmethyldiazepam	lorazepam	oxazepam	temazepam	Zolpidem (tartrate)

6.9.2 Tightly cap, protect from light, and store in freezer.

6.9.3 Stable for at least six (6) months when stored in freezer.

6.10 Benzodiazepine Calibrator Working Solution-01 (WS-01 ; 10 component mixture ; 2000 ng/mL)

6.10.1 Add 100 µL of the Benzodiazepine Calibrator Stock Solution (100 µg/mL) into a properly labeled 5 mL volumetric flask that contains some methanol.

6.10.2 Dilute to volume with methanol, tightly cap, mix, protect from light, and store in freezer.

6.10.3 Stable for at least six (6) months when stored in freezer.

6.11 Benzodiazepine Calibrator Working Solution-02 (WS-02 ; 10 component mixture ; 200 ng/mL)

6.11.1 Add 500 µL of the WS-01 solution (2000 ng/mL) into a properly labeled 5 mL volumetric flask that contains some methanol.

6.11.2 Dilute to volume with methanol, tightly cap, mix, protect from light, and store in freezer.

6.11.3 Stable for at least six (6) months when stored in freezer.

6.12 Blood Calibrator Solutions

Combine the following Calibrator Working Solutions (WS-01 and WS-02) to 500 µL of matrix (blood and/or urine) to appropriately labeled screw-capped test tubes according to the table, tightly cap, and mix:

Calibrator Concentration	Volume of Calibrator Working Sol'n-01 (WS-01 ; 2 µg/mL)	Volume of Calibrator Working Sol'n-02 (WS-02 ; 200 ng/mL)	Volume of Matrix
5 ng/mL	–	12.5 µL	500 µL
10 ng/mL	–	25 µL	500 µL
20 ng/mL	–	50 µL	500 µL
50 ng/mL	–	125 µL	500 µL
100 ng/mL	25 µL	–	500 µL
200 ng/mL	50 µL	–	500 µL
500 ng/mL	125 µL	–	500 µL

Note-06: Pre-made CRM mixtures containing benzodiazepines can be used and diluted appropriately to make alternative calibrator solutions (e.g., using 250 µg/mL ; Cerilliant ; B-033-1ML). Appropriate quality measures will occur (as stated above) in such situations.

Note-07: A new calibration curve is not required for each batch. Historical calibration curves can be used. The validation of the curve will be demonstrated through the application of positive controls extracted with each batch of samples. When there have been substantial instrument changes (e.g., a new column installed) then a new calibration curve should be prepared and used.

7. Procedure

7.1 Prepare all necessary solutions (e.g., calibrators, controls, reagents, mobile phases).

7.2 Individually add 0.5 mL of evidentiary samples into properly labeled screw-capped test tubes. Within each batch of samples ensure that the correct samples transfer into the correct tubes. Cap after each sample transfer or otherwise reduce the risk of cross-contamination between samples (e.g., by moving tubes after each addition).

7.3 Add 0.5 mL of control matrix solutions into properly labeled screw-capped test tubes.

7.4 Individually add 100 µL of internal standard (Diazepam–D_5 ; 250 ng/mL) into each sample test tube (25 ng of Diazepam–D_5), cap, and briefly vortex-mix (e.g., ~10 seconds).

7.5 Individually add 1 mL of 0.10 M sodium acetate buffer (pH 4.5) to each tube.

7.6 For blood samples only: Add 500 µL of DI water to each blood sample.

7.7 Cap, briefly vortex-mix, and then centrifuge **all** tubes for ~10 min at ~5000 rpm.

7.8 Appropriately label Trace B solid phase extraction (SPE) columns to correspond with each sample.

7.9 Place labeled Trace B extraction columns in the SPE column rack in the appropriate order and position the plastic waste tray (labeled "Methanol") underneath the SPE column rack.

7.10 Condition each SPE column sequentially with:

7.10.1 Methanol (1 mL)

7.10.2 Drain (≈ 3 psi) to solvent hazardous waste stream

7.11 Remove the plastic waste tray labeled "Methanol" and replace with the plastic tray labeled "Biohazardous/Buffers" (non-hazardous regulated waste stream) underneath the SPE column rack.

7.12 Rinse each SPE column sequentially with:

7.12.1 Water (1 mL)

7.12.2 Drain (≈ 3 psi) to non-hazardous regulated waste stream

7.13 Individually and carefully transfer samples to the centers of the appropriate SPE columns. Avoid any sediment found at the bottoms of the test tubes and avoid splashing.

7.14 Allow gentle drop-wise flow to the non-hazardous regulated waste stream via gravity. Use some pressure (e.g., ≤3 psi), if necessary, to express samples through the columns.

7.15 Sequentially perform the following wash/rinse on each SPE column:

7.15.1 Bicarbonate/Carbonate $Buffer_{(aq)}$ (1 mL ; pH~9)

7.15.2 Drain (~3 psi) to non-hazardous regulated waste stream

7.15.3 Water (1 mL)

7.15.4 Drain (~3 psi) to non-hazardous regulated waste stream

7.16 Dry the columns for ~10 minutes using maximum pressure (e.g., between 60-80 psi).

7.17 During this 10 minute window (or earlier) actions such as the labeling the collection tubes (or autosample vials) and placing them in the appropriate order into the SPE collection rack to prepare for elution collection can be performed.

7.18 Once the SPE columns are dry replace the plastic waste tray with the SPE collection rack containing the collection tubes (or autosample vials).

7.19 Important: Ensure proper placement of collection tubes and that each corresponds to correctly labeled SPE column.

7.20 Elute the SPE columns:

7.20.1 Elution #1:

7.20.1.1 Add 1.0 mL of the Solid Phase Extraction Elution Solution (80:18:2 CH_2Cl_2:IPA:NH_4OH) to each SPE column.

7.20.1.2 Set the flow at ~2-4 mL/min for optimal recovery.

7.20.1.3 Collect eluent into appropriately labeled tubes.

7.20.2 Elution #2:

7.20.2.1 Add 1.0 mL of the Solid Phase Extraction Elution Solution (80:18:2 CH_2Cl_2:IPA:NH_4OH) to each SPE column.

7.20.2.2 Set the flow at ~2-4 mL/min for optimal recovery.

7.20.2.3 Collect eluent into appropriately labeled tubes.

7.21 Remove the top SPE column rack and transfer the collection rack from the SPE manifold to the sample concentrator. Ensure concentrator parts are clean and free of contamination.

7.22 Add one (1) drop of MeOH-$HCl_{(aq)}$ and evaporate all samples to dryness at ~<40^0 C.

7.23 If samples are not to be immediately analyzed they should be tightly capped, protected from light, and placed in a refrigerator or freezer under proper evidentiary seal.

Note-08: The word 'immediately' refers to instrumental sequences being started on the same day.

7.24 Reconstitute each collection tube (or autosample vial) with the same volume of $MeOH_{(aq)}$ (20% (v/v)) – no more than 150 µL.
All calibrators, controls, and samples must be reconstituted with the same volume of MeOH.
If the volume is different from 150 µL then this must be recorded within the appropriate documentation (e.g., case notes, summary sheets).

7.25 If not collecting into autosample vials, individually transfer elution solutions into properly labeled auto sampler vials containing sample inserts.

Setting-up Instrument with Samples:

7.26 Ensure the appropriate instrumental quality assurance/quality control (QA/QC) procedures were performed. The instrument must have passing QA/QC results prior to preparing and loading of samples.

7.27 Prepare the sequence and enter the samples in the appropriate order. Negative controls will be analyzed prior to evidentiary samples. Calibrators can be analyzed either before or after evidentiary samples. Blank samples (i.e., those containing just $MeOH_{(aq)}$ (20% (v/v))) should be analyzed in-between evidentiary samples to avoid carry-over and will be analyzed after the highest calibrator is injected.

7.28 Place the labeled autosample vials in the appropriate order within the instrument.

7.29 Save the sequence to the day's date. Ensure lab number, operator/analyst's name(s), and instrument name are recorded within each sample.

7.30 Sequence Verification:

7.30.1 Print the sequence list.

7.30.2 Check that the physical placement of the autosample vials and the vial positions within the instrument's sequence list match.

7.30.3 Once the check has been completed place an indication of the sample check (e.g., 'sequence checked' or 'sequence verified') on the sequence page along with Analysts initials and date.

7.31 Print the instrument method and include both the method and the sequence printouts with the batch documents.

7.32 Begin the sequence and analyze the samples.

7.33 After the batch has completed:
Re-check that the physical placement of the autosample vials and the vial positions within the instrument's sequence list match. Once the re-check has been completed place an indication of the sample re-check (e.g., 'sequence re-checked post analysis' or 'sequence re-verified post analysis') on the sequence page along with Analyst's initials and date.

7.34 Analyze extract solutions using LC/MS.
Blank solutions (i.e., solvent, mobile phase) will be analyzed in-between samples and after highest calibrator solution.

8. Instrumental Parameters

The following are the typical operating parameters for the instrument used in this procedure. With approval from the Lead Examiner and Deputy Director (or higher), the instrument conditions may be modified to adjust or improve the procedure. Documentation of such changes must be included with batch data so that any instrumental parameter change can be associated with data and casework until this procedure has been updated.
The appendix contains an abbreviated version of the procedure. The checklist can be used by analysts. Any changes within the instrumental parameters, if listed on the checklist, will be reflected on the checklist by the analyst filling it out.

HPLC Parameters:

Mobile Phase A: 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$
Mobile Phase B: $MeOH_{(l)}$ (neat)
HPLC Column: Kinetex, 2.6 µm Phenyl-Hexyl, 100 Å, 50 mm x 4.6 mm (Phenomenex)
Column Temperature: 40 °C
Constant Flow: 0.6 mL/min.
Autosampler Temperature: 15 °C
Injection Volume: 10 µL
Needle Wash: 500 µL before and after aspiration

Gradient Program: Initial 20% B ; Flow 0.6 mL/min

Time (min.)	Mobile Phase A [0.01 %(v/v) $HCOOH_{(aq)}$ + 5mM $NH_4CHOO_{(aq)}$] (%)	Mobile Phase B $MeOH_{(neat)}$ (%)
Initial	80	20
1.00	80	20
2.00	55	45
3.00	55	45
3.50	40	60
9.00	40	60
11.00	25	75
11.50	0	100
12.50	0	100
12.51	80	20
15.00	STOP	STOP

Mass Spectrometer Parameters:

Ionization Source	Electrospray	**Polarity**	Positive Ion
Ion Spray Potential	+4.5 kV	**Heat Block**	400 °C
Nebulizer Gas	Nitrogen	**Nebulizer**	3 L/min
Drying Gas	Nitrogen	**Drying Gas**	15 L/min.
Scan Type	MRM (&/or Full Scan)	**Resolution**	Unit
Desolvation Line (DL)	250 °C		

Transition Ions Monitored

Using the LabSolutions® optimization program, the following transitions were identified:

Drug/Metabolite	Precursor Ion	Quantification Ion	Reference Ion(s)
7-Aminoclonazepam	285.60	121.10	222.10, 250.05
7-Aminoflunitrazepam	283.90	135.15	227.05, 121.20
Alprazolam	308.45	281.10	205.10, 165.15
α-Hydroxyalprazolam	324.85	297.10	216.05, 205.05
Clonazepam	315.60	270.0	214.00, 241.00
Desalkylflurazepam	288.60	139.90	226.15, 165.00
Diazepam	284.90	154.10	91.10
Flunitrazepam	313.60	268.05	239.10, 183.00
Flurazepam	387.50	315.00	317.00, 288.00
Lorazepam	321.80	275.95	230.00, 195.00
Midazolam	325.60	291.10	223.05, 249.05

Nordiazepam	270.70	140.00	165.00, 208.10
Oxazepam	286.80	241.05	104.05, 77.05
Oxazepam	462.90	286.85	241.10, 269.10
Temazepam	300.60	255.00	177.00, 193.10
Temazepam	476.70	301.00	254.95, 283.10
Triazolam	342.80	308.05	315.00, 239.00
Zolpidem	307.60	235.20	236.15, 263.15
Zopiclone	388.95	245.05	217.00, 112.05
Diazepam-D_5	289.85	154.00	198.10, 227.00

9. Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. Retention time (chromatographic characteristic), fragmentation pattern and qualified ion ratios (mass spectrometric characteristics), and other characteristics are used as the basis for detection and identification. In most cases all of the criteria below should be met in order to identify the appropriate drugs within biological specimens.

9.1 Chromatography

All chromatographic peaks for the analytes of interest should show good chromatographic characteristics, with reasonable peak shape, width, and resolution. For low concentrations of an analyte (e.g., ≤5 ng/mL), there may be transitions that are not optimal. In order to be determined as acceptable, a chromatographic peak in a sample should compare favorably to the same analyte's chromatographic peak in a known sample which has been analyzed on the same system and in the same, or subsequent, analytical timeframe. Additionally, the following two criteria should be met:

9.1.1 Retention Time (RT)

The retention time of a peak of interest should be within 0.1 minute of, or +/- 3% relative (relative or absolute) to, the retention time of a reference standard (i.e., calibrator or positive control).

Note-09: Instrumental evaluation of retention time using +/-0.1 minute as a criteria is acceptable. Using ±2 minute window is also acceptable for sample/control evaluation.

If analyzed, the retention times of the analytes within the Procedural Performance Solution should be within ±2 % of their previously analyzed times and/or to a set acceptable value. Minor changes in mobile phase percentage may account for slight retention time shifts.

The following table can be used for approximate retention times:

Analyte	Retention Time (min)	Analyte	Retention Time (min)
7-Aminoclonazepam	4.29	Alprazolam	7.07
Zolpidem	5.26	Temazepam	7.38
Clonazepam	6.35	Nordiazepam	7.63
Lorazepam	6.42	Diazepam-D_5	8.75
α-Hydroxyalprazolam	6.54	Diazepam	8.83
Oxazepam	6.58		

9.1.2 Signal-to-Noise Ratio (SNR)

To justify the existence of a peak, its baseline signal-to-noise ratio (SNR) will exceed 3. Furthermore, the baseline signal for the peak of interest will be at least 10-fold greater than that for any observed peak (corresponding to the analyte of interest) at a similar retention time in a negative control or blank sample that was injected just prior to that sample.

9.2 Mass Spectrometry

In order for the MS to be considered in good operating condition, the correct mass assignments for each of the analytes in the performance standard should be present. Independent MS/MS experiments are conducted for each analyte.

Analysts will review transition ion ratios and use them for identifications. Determination of the presence of target analytes in sample extracts are identified by appearance and ratio of product ions that are characteristic of analytes at appropriate retention times. In this manner, both retention times, mass spectral fragmentation patterns, and ion ratios are used as the basis for qualitative identification. For the identification of an analyte to be made retention times of chromatographic peaks should be within 0.1 minute of corresponding analytes from reference standards (i.e., calibrators or positive controls). However, since ion rations are not infallible, the meeting of this criteria is not mandatory.

Ion ratios should compare favorably to ion ratios from reference standards or an extracted positive control at a comparable concentration (e.g., positive control). Generally, ion ratios are within the following limits:

Expected [Set] Ion Ratio	Allowance
>50 %	20%
20% - 50%	25%
<20%	30%

9.3 Batch Acceptance:

In order for a batch to be acceptable:

9.3.1 No analytes of interest will be detected in the Negative Control.

9.3.2 Chromatography evaluation (above) should be met.

9.3.3 Mass spectral evaluation (above) should be met. The 100 ng/mL positive control will be utilized to set expected ion ratios for applicable analytes.

9.3.4 Significant carry-over will be brought to the attention of the lead Examiner to determine if evidentiary samples have been negatively impacted. If so, re-analyses will occur and sample re-extraction may be necessary. Appropriate case documentation will accompany these instances within affected case files to record events.

Note-10: An analyte is considered applicable if it is important to the batch.
Analytes of interest are considered those compounds that are being reported.

9.3.5 All applicable analytes of interest within Positive Controls, as well as internal standards, will be identified.

9.3.6 Quantitative results of positive controls must fall within ±20% of the analyte's target concentration.

9.3.7 Statistical data of positive controls should be recorded and evaluated within appropriate charts. If controls fall out of acceptable range then applicable controls should be discarded and new controls utilized.

9.4 Unknown Sample Compound Detection:

This procedure is intended to be used for quantitative purposes. However, if enough data is present, confirmations of analytes not quantitated can be reported if a different chromatographic or mass spectrometric experiment is employed (or with approval of Lead Examiner and Deputy Director (or higher)).

10. Calibration

Calibration is accomplished by the addition of known amounts of analytes (in addition to an internal standard) into matrix-matched blank samples. A calibration graph is generated from the resulting data and used as the basis for the quantitation of unknown samples. Blood samples are typically the only matrix quantitated so only a blood calibration graph for each drug and/or metabolite is used with each batch. A correlation coefficient should be ≥ 0.990 when using deuterated internal standards. If the correlation coefficient is lower than 0.990 then approval from the appropriate lead analyst or higher must occur and, if deemed acceptable, justification must accompany the applicable data. If using non-deuterated internal standards a correlation coefficient ≥ 0.98 is acceptable. While a calibration curve for each drug and/or metabolite may be analyzed with each batch, a previously established (or "historical") calibration curve may be used. Following linear regression, reprocessed calibrators generally should be within 20% of their target value, but this is not mandatory. Calibrators will not be removed from calibration graphs without approval from the lead Examiner or higher. Such removal of calibration points will be appropriately documented within batches and within each case file (e.g., case notes, summary sheets). Calibration data are used for the analysis of each batch and are done independently.

Samples resulting in concentrations higher than the highest calibrator should be diluted (approval not to dilute must be obtained by the Unit Lead or higher). Such instances may involve reporting results as

'greater than the highest calibrator' may be acceptable and would avoid dilution and re-analysis. The lower limit of quantitation (LLOQ) is the concentration of the lowest calibrator. The upper limit of quantitation (ULOQ) is the concentration of the highest calibrator. Extrapolation of the calibration graph beyond the LLOQ and ULOQ values can be done with permission of the lead Examiner, but must be documented within each applicable case file. The urine control is used for qualitative purposes only but, if necessary, it can be quantitated based on the blood calibration curve.

Calibration graphs and quantitative results are obtained by comparing chromatographic peak responses [or peak areas] to corresponding peak responses [areas] from internal standards. Calibration graphs are created and equations are obtained by plotting peak area ratios against corresponding calibrant concentrations for each analyte of interest. Based on resulting calibration equations concentrations of select analytes within evidentiary samples are obtained based on corresponding ratios of analyte peak areas to internal standard areas.

11. Uncertainty

Some sources of measurement uncertainty in this procedure may include:

- Historical random uncertainty of repeated measurements
- Accuracy of the pipette(s) used to deliver sample volumes and/or calibrators
- Uncertainty in the concentration(s) of calibration standards and/or positive controls
- Instrument performance
- Human error

Uncertainty information for this procedure/instrument has been determined and can be included when quantitation values are reported.

It has been established that no known inferences are present within the calibrators or controls. Ion suppression or enhancement and potential interferences from other analytes has not been found for the common drugs and metabolites typically seen within casework.

12. Limitations

Limits of Detection (LOD), Lower Limits of Quantitation (LLOQ), Upper Limits of Quantitation (ULOQ)

Analyte	LOD – Urine (ng/mL)	LOD – Blood (ng/mL)	LLOQ – Blood (ng/mL)	ULOQ – Blood (ng/mL)
7-Aminoclonazepam	5*	5*	5*	500**
7-Aminoflunitrazepam	5*	5*	5*	500**
Alprazolam	5*	5*	5*	500**
α-Hydroxyalprazolam	5*	5*	5*	500**
Clonazepam	5*	5*	5*	500**
Desalkylflurazepam	5*	5*	5*	500**
Diazepam	5*	5*	5*	500**
Flunitrazepam	5*	5*	5*	500**
Flurazepam	5*	5*	5*	500**
Lorazepam	5*	5*	5*	500**
Midazolam	5*	5*	5*	500**
Nordiazepam	5*	5*	5*	500**
Oxazepam	5*	5*	5*	500**
Oxazepam	5*	5*	5*	500**
Temazepam	5*	5*	5*	500**
Temazepam	5*	5*	5*	500**
Triazolam	5*	5*	5*	500**
Zolpidem	5*	5*	5*	500**
Zopiclone	5*	5*	5*	500**

*Administratively set – values may actually be lower

**Administratively set – values may actually be higher

13. Safety

This procedure is carried out in a laboratory environment and standard safety procedures appropriate for such an environment should be utilized, including gloves, safety glasses, and protective clothing (e.g., lab coat). Biological specimens will be handled using universal precautions and will be treated as biohazardous. Potentially contaminated items and surfaces will be cleaned prior to use, especially. When casework samples are being processed/analyzed brown paper (or other similar barrior) should be placed in between the surface and the specimens.

14. References

14.1 Screening and Confirmation of Benzodiazepines in Blood by Electrospray LCMSMS. West Chester County Department of Laboratories and Research: Division of Forensic Toxicology.

14.2 Solid-Phase Extraction Method for Trace B Columns. SPEWARE: Baldwin Park, CA.

Appendix:

Bench Method: Benzodiazepine Quantitative/Qualitative Analysis using LC/MS

The following is an abbreviated version of this procedure and a pdf-copy can be used at the bench level by analysts.

Negative Control [drug-free] Blood	**Lot:**
Negative Control [drug-free] Urine (or Other)	**Lot:**
Positive Control Blood	**Lot:**
Positive Control Urine (or Other)	**Lot:**

Procedure:

	For Blood Specimens	
Prepare all necessary solutions (e.g., calibrators, controls, reagents, mobile phases).		
Add 0.5 mL sample/negative control/positive control into properly labeled screw-capped test tubes (e.g., 16 x 100 mm)		
	Add 100 µL of I.S. Working Sol'n to each sample (25 ng Diazepam–D_5):	**Lot:**
Add 1 mL of Sodium Acetate $Buffer_{(aq)}$ (0.1M ; pH 4.5) to each test tube.		**Lot:**
Add 500 µL of water to each blood sample, cap and vortex-mix samples, sonicate, Centrifuge.		
Label Trace-B extraction [SPE] columns appropriately.		**Lot:**
Place labeled Trace-B extraction columns in the SPE column rack in the appropriate order.		
Add 1 mL MeOH (to condition SPE), drain (~3 psi)		**Lot:**
Add 1 mL of water (to rinse each SPE column), then drain (~3 psi)		**Lot:**
Individually transfer samples/controls/calibrators to the center of their respective [properly labeled] SPE columns. Avoid sediment / splashing. Allow to migrate through SPE column via gravity. Apply small pressure, if needed (≤ 3psi).		
To each SPE column:		
	Wash with 1 mL of Bicarbonate/Carbonate buffer (pH ~9), then drain (~3 psi)	**Lot:**
	Rinse with 1 mL water, then drain (~3 psi)	**Lot:**
Dry the columns for 10 minutes using maximum pressure (e.g., between 60-80 psi)		
During 10 minute window (or earlier) actions such as proper labeling, placement in rack, preparation of SPEES can occur.		
The Solid Phase Extraction Elution Solution (SPEES) ratio is 80:18:2 CH_2Cl_2:IPA:NH_4OH.		
	Methylene Chloride (CH_2Cl_2)	**Lot:**
	Isopropyl Alcohol (2-Propanol ; IPA)	**Lot:**
	Ammonium Hydroxide (NH_4OH)	**Lot:**
After ~10 minutes replace plastic waste tray with SPE rack containing collection tubes. Ensure they are properly placed.		
Elute columns with 2x with 1 mL of Elution Solution. Flow using gravity (≤3 psi if needed)		**Lot:**
Transfer collection rack from SPE manifold to sample concentrator		
Add ~1 drop of $MeOH\text{-}HCl_{(aq)}$ and evaporate to dryness (~<40 ˚C) ; Store refrigerated, protected from light, and properly sealed if not immediately analyzed.		
Reconstitute each extract sample with 150 µL of 20% $MeOH_{(aq)}$; only if analyzing immediately		**Lot:**
Load samples, set up sequence, printout sequence/method, verify sequence w/ initials, and analyze using LC/MS		

Appendix (Cont'd):

Specimen Procedure (Cont'd)

Analyze by LC/MS:

Instrument: LC/MS	Instrument Identifier:
HPLC Column: Phenomenex ; Kinetex Phenyl-Hexyl ; 100 Å ; 2.6 µm ; 50 mm x 4.6 mm):	Manufacturer: Lot:
Mobile Phase A: [0.01 %(v/v) $HCOOH_{(aq)}$ + 5mM $NH_4CHOO_{(aq)}$]	Lot:
Mobile Phase B: MeOH (CH_3OH) – 100%	Lot:

Gradient Program:
Initial 20% B ; Flow 0.6 mL/min

Time (min.)	%B
Initial	20
1.00	20
2.00	45
3.00	45
3.50	60
9.00	60
11.00	75
11.50	100
12.50	100
12.51	20
15.00	STOP

Mass Spectrometer Parameters

Ion Source	Electrospray
Spray Voltage	+4.5
Polarity	Positive
Nebulizer Gas	Nitrogen (3 L/min.)
Drying Gas	Nitrogen (15 L/min.)
Scan Type	MRM (&/or Full Scan)
Heat Block	400 °C
Resolution	Unit

Revision #	Revision History
4	Section V.I E. Calibration removed requirement to compare the calibrators to the calibration curve generated. Section V. replaced autosampler vial with collection tube in multiple places. V.21 changed the reconstitution volume from 520 to 150 ul. Added line 22 Individually transfer solutions to properly labeled auto sampler vials containing sample inserts.
5	Use of pH meters removed. Added pH paper to equipment list and added check of pH for 0.1M sodium acetate buffer.
6	Updated information on the preparation of standards and controls allowing for them to be made up in methanol and stored frozen as opposed to making them each day of use. Added statement about use of primary purchased standards that are not 1mg/mL.
7	Title change. Reformatting throughout document. Minor wording changes throughout introduction/scope/principle/specimens-parts of procedure. Added statement about using externally-prepared controls before internally-prepared controls, when possible. Updated reagents, preparation of calibrators and controls, decreased number of controls, ensured use of external calibrator solutions and controls over internally-prepared solutions, edited procedure for clarity, added MeOH-HCl step prior to evaporation to dryness. Added verification of sequence step and printing out of documents. Added need to get approval and to document record any changes from SOP. Organized instrumental parameters, updated decision criteria, updated Calibration and Uncertainty, and Limitation sections. Updated Appendix for method summary.