I. INTRODUCTION

A. Principle

Benzodiazepines and the functionally related "Z drugs" (zolpidem and zopiclone) are drugs whose properties include anti-anxiety, hypnotic, and sedation. These drugs are GABA-agonists, acting via specific allosteric-activating receptors on the GABA-A receptor, beta subunit. While benzodiazepines are considered safer than the barbiturate class they have mostly replaced, there is still significant potential for abuse and dependence with continued use.
Samples that require confirmation by LC/MS/MS are extracted from a buffered, diluted sample aliquot by adsorption onto a solid phase extraction column. Benzodiazepines/Z drugs that may be present are then eluted from the SPE column, dried, and reconstituted before injection onto the LC/MS system.
The detection of each specific benzodiazepine/ Z drug is determined by single point calibration cut off for urines. Bloods are quantitated with a multipoint calibration using a deuterated internal standard.
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.

B. Method Targets

1. Alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, lorazepam, diazepam, nordiazepam, oxazepam, temazepam, zolpidem are quantitative targets within this method.

2. Midazolam, flunitrazepam, 7-aminoflunitrazepam, flurazepam, N-desalkylflurazepam, triazolam, and zolpiclone are qualitative targets that upon toxicologist review can be quantitated (additional compounds can be added to this list).

C. Safety

This procedure is carried out in a laboratory environment and standard safety procedures should be utilized, including (minimally) safety glasses and lab coat when deemed necessary. Biological specimens subject to the analytical procedure should be handled using universal precautions. Potentially contaminated items and surfaces should be disinfected prior to and after use.

D. Specimen Requirements

1. 0.5 mL blood or urine

II. MATERIALS

A. Equipment

1. General laboratory glassware
2. Vortex, Thermolyne Maxi Mix 1 or equivalent
3. Sonicator, Fisher-Scientific or equivalent (for blood samples)
4. Centrifuge, Beckman TJ-6 or equivalent
5. Trace B Extraction Columns - SPEWARE (Baldwin Park, CA)
6. SPEWARE CEREX System-48 Solid phase extraction manifold
7. SPEWARE CEREX System-48 Sample Concentrator
8. pH paper
9. Shimadzu LC/MS/MS System consisting of:
   a. Degasser: Shimadzu DGU-20A
   b. Pumps: 2 Shimadzu LC-20AD Prominence
   c. Autosampler: Shimadzu SIL 20AC Prominence
   d. Column Oven: CTO-20A
   e. Pre-Column: SecurityGuard ULTRA Cartridge UHPLC Phenyl for 4.6mmID Columns (Phenomenex)
   f. Column: Kinetex Phenyl Hexyl (Phenomenex)
   g. Detector: Shimadzu LCMS-8030 Mass Spectrometer
   h. Controller: Shimadzu CBM-20A
   i. Data Station: Shimadzu LabSolutions software

B. Reagents available as stock items: Sigma or J.T. Baker reagent grade or equivalent unless specified

1. Methanol (CH_3OH): Fisher Optimum LCMS Grade or Burdick Jackson pesticide grade
2. Deionized water (DIW): Milli-Q or LCMS grade
3. Drug-free urine or blood (blood may be purchased or obtained from the Red Cross or other source)
4. Formic Acid (HCOOH)

5. Ammonium formate (NH_4COOH)

6. Glacial acetic acid (CH_3COOH)

7. Sodium acetate trihydrate ($NaCH_3COO{\cdot}H2O$)

8. Sodium carbonate (Na_2CO_3)

9. Sodium bicarbonate ($NaHCO_3$)

10. UTAK LC-2 Control – UTAK Laboratories (Valencia, CA)

C. Drug Standard Solutions – Cerilliant Corportation (Austin, TX)

Target Analytes	**Concentration**
Alprazolam	1.0 mg/mL
α-hydroxyalprazolam	1.0 mg/mL
Clonazepam	1.0 mg/mL
7-aminoclonazepam	1.0 mg/mL
Lorazepam	1.0 mg/mL
Diazepam	1.0 mg/mL
Nordiazepam	1.0 mg/mL
Oxazepam	1.0 mg/mL
Temazepam	1.0 mg/mL
Zolpidem tartrate	1.0 mg/mL

D. Deuterated Drug Standard Solution – Cerilliant Corporation (Austin, TX)

Diazepam-D5	1.0 mg/mL

E. Drug Standard Solutions – Lipomed Inc. (Cambridge, MA)

Target Analytes	**Concentration**
Alprazolam	1.0 mg/mL
α-hydroxyalprazolam	1.0 mg/mL
Clonazepam	1.0 mg/mL
7-aminoclonazepam	1.0 mg/mL
Lorazepam	1.0 mg/mL

Diazepam	1.0 mg/mL
Desmethyldiazepam (Nordiazepam)	1.0 mg/mL
Oxazepam	1.0 mg/mL
Temazepam	1.0 mg/mL
Zolpidem hemitartrate	1.0 mg/mL

F. Reagents prepared in the Toxicology Laboratory:

Reagent volumes can be adjusted using proportionate ratios depending on the needed volume.

- **5M Ammonium formate**:
  1. Dissolve 3.15 g of ammonium formate in 10 mL volumetric flask.
  2. Q.S. to 10 mL with high purity water. Stable for one year.
- **Mobile Phase A (H_20 with 0.01% formic acid and 5mM ammonium formate)**:
  1. Add 0.5 mL 5M Ammonium formate and 0.05 mL formic acid to a 500 mL volumetric flask.
  2. Q.S. to 500 mL with high purity water.
  3. Transfer solution to a glass bottle reserved for LC/MS use only. Stable for one week.

- **0.1M Sodium Acetate buffer (pH 4.5)**:
  1. Combine 5.86 g of sodium acetate trihydrate and 3.24 mL glacial acetic acid in a 1000 mL stoppered graduated cylinder.
  2. Q.S. to 1000 mL with deionized water.
  3. Check pH; should be approximately 4.5
  4. Store in glass container at room temperature (25^0C)
  5. Stable for one year
  6. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

0.1M Sodium Bicarbonate (pH 8.0):

1. Add 4.2 g of sodium bicarbonate to a **separate** 500 volumetric flask.
2. Q.S. to 500 mL with deionized water.
3. Check pH; should be approximately 8

4. Store in glass container at room temperature (25^{0}C)
5. Stable for one year
6. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

0.1M Sodium Carbonate (pH 11.0):

1. Add 5.3 g of sodium carbonate to a 500 mL volumetric flask.
2. Q.S. to 500 mL with deionized water.
3. Check pH; should be approximately 11
4. Store in glass container at room temperature (25^{0}C)
5. Stable for one year
6. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

Bicarbonate buffer pH 9.0: Mixture of 0.1 M Sodium Carbonate and 0.1 M Bicarbonate Solutions

1. Pour into a beaker the 0.1M Sodium Bicarbonate solution (lower pH solution)
2. Check pH, adjust with the 0.1M Sodium Carbonate solution (higher pH solution) until a pH of approximately 9 is reached.
3. Store in glass container at room temperature (25^{0}C)
4. Stable for one year
5. Inspect for contamination before use. If bacterial contamination is visible, prepare fresh before use.

- **Reconstitution Mixture: 20% Methanol in Deionized Water**
  1. Into a 100 mL graduated cylinder with a cap, add 80 mL of deionized water.
  2. Add 20 mL methanol.
  3. Cap and shake. Store at room temperature; stable for 6 months.

4. Before use check for clarity; if cloudy, discard and prepare fresh.

III. PREPARATION OF STANDARDS

Note: Equivalent reference standards may be substituted if needed. Calibrators and controls should be from different suppliers when possible.

A. 100 µg/mL Mixed Cerilliant Benzodiazepine Stock Standard

1. Into a borosilicate glass screw-top culture tube, add 100 µL of the following Cerilliant 1 mg/mL standards:

Alprazolam	α-hydroxyalprazolam	Diazepam	Nordiazepam	Oxazepam
Temazepam	Clonazepam	7-aminoclonazepam	Lorazepam	Zolpidem tartrate

2. Store in freezer ($\leq -10^0$C)
3. Stable for 6 months when tightly capped.

B. 2000 ng/mL Cerilliant Benzodiazepine Working Standard (WS-1)

1. Into a 5 mL volumetric flask, add 100 µL of the 100 µg/mL Cerilliant Benzodiazepine Stock Standard.
2. Q.S. to 5 mL with DI water. Protect from light, make fresh with each run.

C. 200 ng/mL Cerilliant Benzodiazepine Diluted Working Standard (WS-2)

1. Into a 5 mL volumetric flask, add 500 µL of the 2000 ng/mL WS-1
2. QS to 5 mL with DI water. Protect from light; make fresh with each batch.

D. Working Internal Standard: 250 ng/mL

1. To a 100 mL volumetric flask add 25 µL of 1 mg/mL each reference standard (1 or all listed below may be used)

a. Diazepam D5
b. Amphetamine D6
c. Oxycodone D3
d. Benzoylegonine D3
    i. If other concentrations are used adjust volume accordingly.
e. 2. Q.S. to 1000 mL with methanol.

3. Store in freezer (≤ -10^{0}C)

4. Stable for 6 months when tightly capped.

IV. PREPARATION OF CONTROLS

A. In-House Control (100 µg/mL) Mixed Lipomed Stock Control

1. Into a screw-top glass culture tube, add 100 µL of the following Lipomed 1 mg/mL stock standards:

Alprazolam	α-hydroxyalprazolam	Diazepam	Desmethyldiazepam	Oxazepam
Temazepam	Clonazepam	7-aminoclonazepam	Lorazepam	Zolpidem hemitartrate

2. Store in freezer (≤ -10^{0}C)

3. Stable for 6 months when tightly capped.

B. In-House Control (2000 ng/mL) Lipomed Working Control (WC-1)

1. Into a 5 mL volumetric flask, add 100 µL of the 100 µg/mL Lipomed Mixed Stock Control.

2. Q.S. to 5 mL with DI water. Protect from light; make fresh with each run.

C. In-House Control (200 ng/mL) Lipomed Diluted Working Control (WC-2)

1. Into a 5 mL volumetric flask, add 500 µL of the 2000 ng/mL Lipomed WC-1.

2. QS to 5 mL with DI water. Protect from light; make fresh with each run.

D. External Controls

1. External controls UTAK preparation.

   a. Urine Control DAU LC 2, Product # 50703, Utak Laboratories Valencia, CA 91355
      1. Remove cap from vial
      2. Reconstitute control material by adding exactly 10.0 mL of DIW, using a 10 mL volumetric pipette.
      3. Replace cap and let sit ~10 – 15 minutes.
      4. Swirl gently ~3 – 4 minutes to ensure homogeneous mixture.
      5. Swirl gently each time an aliquot is removed to ensure a homogeneous mixture.
      6. Assay control material in the same manner as case specimens.
      7. For quantitative assays, record the results obtained on a quality control chart in Excel.
      8. Store reconstituted control material refrigerated at 2-8°C, stable for 25 days after reconstitution.

E. Negative Controls

1. Negative control: Drug-free human urine or drug-free blood.

V. PROCEDURE

1. Label clean screw cap tubes appropriately with blank, calibrator, control or case number designations.

2. Prepare calibrator and control samples, according to tables below. Note a new calibration curve is not required for each batch. Historical calibration curves can be used, the appropriateness of the curve will be demonstrated through the application of controls extracted with each batch of samples. New calibration curves should be extracted when there have been substantial instrument changes (i.e. a new column installed).

a) Blood Calibrators

Standard Concentration	Working Standard Volume to Add:	Working Standard Volume to Add:	Blank Blood Volume to Add:
	Cerilliant WS-1 (2000 ng/mL)	**Cerilliant Diluted WS-2 (200 ng/mL)**	
	Volume to Add uL:	**Volume to Add uL:**	
Blank	0 µL	0 µL	500 µL
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

b) In House Blood Controls

Control Concentration	Working Control Volume To Add:	Blank Blood Volume to Add:	Blank Blood Volume to Add:
	Lipomed WC-1 (2000 ng/mL)	**Lipomed Diluted WC-2 (200 ng/mL)**	
20 ng/mL		50 µL	500 µL

200 ng/mL	50 µL		500 µL
400 ng/mL	100 µL		500 µL

c) In House Urine Control

Control Concentration	Working Control Volume To Add:	Working Control Volume To Add:	Blank Urine Volume to Add:
	Lipomed WC-1 (2000 ng/mL)	**Lipomed Diluted WC-2 (200 ng/mL)**	
20 ng/mL		50 µL	500 µL

d) Commercial Controls

Control Concentration	Pipette Volume To Add:		Blank Urine Volume to Add:
UTAK LC 2 dilution x2	250 µL		250 µL
UTAK LC 2	500 µL		0 µL

Blood and Urine sample preparations

3. Add 0.5 mL case specimen, blood or urine, to appropriate labeled tubes.
4. Add 100 µL of internal standard to each tube.
5. Add 1 mL of 0.10 M sodium acetate buffer (pH 4.5) to each tube.
    a. **Blood sample preparations:** Add 500 µL DI water to each blood sample.
6. Cap tubes then vortex each tube for 10 seconds.
    a. **Blood sample preparations:** Sonicate blood samples for 15 minutes.
7. Centrifuge **all** tubes for ~10 min at ~5000 rpm.
8. Label SPE columns to correspond with each screw-top culture tube.

9. Place labeled Trace B extraction columns in the SPE column rack in the appropriate order. Position plastic waste tray labeled "Methanol" underneath SPE column rack.

10. Condition each column sequentially with:

    - 1 mL methanol; drain (≈3 psi) to solvent hazardous waste stream

11. Remove plastic waste tray labeled "Methanol" and replace with plastic tray labeled "Biohazardous/Buffers" (non-hazardous regulated waste stream)

    - 1 mL DI water; drain (≈ 3 psi) to non-hazardous regulated waste stream

12. Carefully transfer the sample to the center of the SPE column. Avoid any sediment found at the bottom of the tube and splashing. Allow gentle drop-wise flow to non-hazardous regulated waste stream. Use pressure if necessary to express sample through column.

13. Wash sequentially each SPE column with:

    - 1 mL bicarbonate buffer (pH 9.0); drain (≈ 3 psi) to non-hazardous regulated waste stream
    - 1 mL DI water; drain (≈ 3 psi) to non-hazardous regulated waste stream

14. Dry the columns for 10 minutes using maximum pressure, between 60-80 psi.

15. During this 10 minute window, label collection tubes LC vials and place in appropriate order in the SPE collection rack and prepare the elution solvent.

16. The elution solvent ratio is 80: 18: 2 Dichloromethane: IPA: NH_4OH. (Adjust volume prepared for the number of tubes, minimize excessive hazardous waste, 2mL needed for each tube)

    Add the IPA and NH_4OH together first before adding the dichloromethane portion (following this order prevents unsafe buildup of gases).

17. After 10 minutes, replace plastic waste tray with SPE collection rack with labeled auto sampler vials in order corresponding to SPE columns.

18. Elute column with two, 1.0mL aliquots of 80:18:2 Dichloromethane: IPA:NH4OH into the appropriate labeled tube. Flow at 2-4 mL/min to optimize recovery.

19. Remove top SPE column rack and transfer collection rack from SPE manifold to sample concentrator.

20. Evaporate all samples to dryness at $<40^0$ C.

21. Reconstitute each vial with slotted screw caps with 150 µL or less of the of starting mobile phase of 20% methanol in DIW. All calibrators, controls and samples must be reconstituted with the same volume of starting mobile phase. If the volume is different from 150 ul note the volume.

22. Individually transfer solutions to properly labeled auto sampler vials containing sample inserts.

23. Analyze by LC/MS/MS.

VI. INSTRUMENT PARAMETERS

A. LC Parameters

1. Shimadzu Prominence LC-20 System

a. Flow	0.5 mL/min
b. Auto sampler Temperature	15°C
c. Injection Volume	10 µL
d. Needle Wash	500 µL; before and after aspiration
e. Column	Kinetex 2.6um Phenyl-Hexyl 100A 50 x 4.6 cm Manufactured by Phenomenex (Torrance, CA)
f. Oven Temperature	50°C
g. Gradient	

Time (min)	Mobile Phase B (Methanol)	Mobile Phase A (0.01% Formic Acid and 5mM ammonium format in Water)
Initial	20%	80%
1.00	20%	80%
2.00	45%	55%
3.00	45%	55%
3.50	60%	40%
9.00	60%	40%
11.00	75%	25%
11.50	100%	0%
12.50	100%	0%
12.51	20%	80%
15.00	STOP	STOP

B. M/S Acquisition Parameters: The following conditions can be adjusted if needed based on availability of gases.

Interface	DUIS (APCI and ESI)
DL Temperature	250°C
Nebulizing Gas	3 L/min
Drying Gas	15 L/min

C. Transition Ions Monitored

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

Drug/Metabolite	**Precursor Ion**	**Quantification Ion**	**Reference Ion(s)**
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
7-aminoclonazepam	285.60	121.10	222.10, 250.05
Lorazepam	321.80	275.95	230.00, 195.00
Diazepam	284.90	154.10	91.10
Diazepam-D5	289.85	154.00	198.10, 227.00
Nordiazepam	270.70	140.00	165.00, 208.10
Oxazepam	286.80	241.05	104.05, 77.05
Temazepam	300.60	255.00	177.00, 193.10
Zolpidem	307.60	235.20	236.15, 263.15
Midazolam	325.60	291.10	223.05, 249.05
Flunitrazepam	313.60	268.05	239.10, 183.00

7-aminoflunitrazepam	283.90	135.15	227.05, 121.20
Triazolam	342.80	308.05	315.00, 239.00
Flurazepam	387.50	315.00	317.00, 288.00
Desalkylflurazepam	288.60	139.90	226.15, 165.00
Zopiclone	388.95	245.05	217.00, 112.05
Oxazepam glucuronide	462.90	286.85	241.10, 269.10
Temazepam glucuronide	476.70	301.00	254.95, 283.10

D. Detection and Identification: The analyst will review all chromatography, peak integrations, and transition ion ratios used for identifications.

Determination of the presence of target analytes in the sample extract are identified by appearance and ratio of product ions that are characteristic of each drug at the appropriate retention time. In this manner, both retention time (a LC characteristic) and fragmentation pattern and ratio (a MS characteristic) are used as the basis for qualitative identification. For the identification of an analyte to be made, the retention time of the chromatographic peak must be within 0.1 minute of the corresponding analyte in the calibrator sample as well as have ion ratios that are within the following limits. The 100 ng/mL Cerilliant Standard sample will be utilized to set the expected ion ratios. If ion ratios can be set from another standard, this should be disconnected.

Expected (Set) Ion Ratio	**Allowance**
> 50%	20%
20-50%	25%
< 20%	30%

E. Calibration: Calibration is accomplished by the addition of a known amount of analytes (in addition to the internal standard) into a blank sample of the matrix that is tested. The response of the system to this calibration defines the standard curve that is used as the basis for the quantitative calculations for controls and samples. The system for blood samples is "multi-point calibration, multi-point control."

While a calibration curve for each drug and metabolite may be analyzed with each batch, a previously established (or "historical") calibration curve may be used. The calibration correlation coefficient must be ≥ 0.990 when using deuterated internal standards. When using non-deuterated internal standards a correlation coefficient ≥ 0.98 is acceptable. A calibrator may be removed to attain a correlation coefficient of ≥ 0.990. 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). The lower limit of quantitation (LOQ) is the concentration of the lowest calibrator. The urine control will be quantitated based on the blood calibration curve.

F. Quantitation: Quantitation is accomplished by the comparison of the response ratio of the analyte and the internal standard in a specific sample relative to the response ratios of the calibration curve. The concentration of the analyte in the sample is then extrapolated from the standard curve.

VII. RESULTS

A. Retention Times of 10 Calibrator Analytes and Internal Standard. Times are approximate.

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-D5	8.75
α-hydroxyalprazolam	6.54	Diazepam	8.83
Oxazepam	6.58		

VIII. RESULTS INTERPRETATION

A. Positive results will be reported only when:

1. Analyte identification is based on at least two transitions with relative abundances within allowable expected, set, ion ratio range, relative to a known calibrator/control.
2. Retention times are within 0.1 min, or +/- 3% relative to a calibrator analyzed in same batch.
3. Qualitative results have at least a 3x signal to noise (S/N) ratio.
4. The integration of the analyte peak has acceptable symmetrical shape and chromatography.

5. All MRMs show peaks at the appropriate retention times.
6. Quality control sample results are acceptable.

IX. QUALITY CONTROL

A. Criteria for Quantitative Results

1. Statistics will be maintained on all controls.
2. Results of controls must fall within ±20% of the analytes' target concentration.
3. All results are recorded.

B. Verification of Vial Sequence: The vial sequence is checked prior to and following injection of samples. These checks will be documented.

C. Evaluation of Potential Carryover: will be determined by a blank sample after the highest calibrator. In addition, a solvent blank will be run after each sample to insure there is no carryover in the next sample coming from the sample before it with a high concentration of analyte.

D. Linearity: Linearity of the calibration curve is demonstrable for each analyte as a function of r^2 correlation coefficient and quantitative results of control materials.

E. Sensitivity (LOD, LOQ): For the purposes of this procedure the limit of quantitation (LOQ) is defined as equal to the lowest concentration of the lowest calibrator. The Limit of Detection (LOD) must have a response of at least 3 times the signal to noise ratio and have acceptable ion ratios.

A. Specificity: Specificity is a function of both the resolution of target analyte during the analytical process and the mass spectral fragmentation that analyte molecules undergo during the instrumental analysis.

X. QUALITY ASSURANCE

A. Quality Assurance is provided by the multi-layer program described below:

1. The LC/MS analysis is thoroughly checked by the instrument operator. This includes the review of all data, including the determination of run acceptability and a check of vial position (prior to and following the injection of samples).

2. The LC/MS data (batch) is reviewed and signed off by a reviewer (batch reviewer) distinct from the operator; this review includes an evaluation of qualitative and quantitative (where applicable) results containing:
    a. Control Results
    b. Chromatographic Characteristics
    c. Transcription Errors
3. An overall case review by a Final Technical Reviewer.

XI. SOURCES OF ERROR

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

XII. References

A. Screening and Confirmation of Benzodiazepines in Blood by Electrospray LCMSMS. West Chester County Department of Laboratories and Research: Division of Forensic Toxicology.
B. Benzodiazepines. Connecticut Division of Scientific Services: Toxicology Laboratory.
C. Solid-Phase Extraction Method for Trace B Columns. SPEWARE: Baldwin Park, CA.

Revision #	History
4	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.