8.6 BENZODIAZEPINE PROCEDURE (INCLUDING ZOLPIDEM AND ZOPICLONE)

8.6.1 Purpose

To qualitatively and/or quantitatively identify benzodiazepines and related compounds in submitted evidence using protein precipitation followed by instrumental analysis with liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

8.6.2 Specimen Requirements

Samples for this analysis shall be presumptively screened positive by either ELISA or GC/MS and shall be confirmed via LC/MS/MS. There are instances of some benzodiazepine compounds that do not fall within either of these current screen capabilities. For these compounds it is acceptable to proceed with the analysis and two separate tests by Multiple Reaction Monitoring (MRM) and/or Enhanced Product Ion (EPI) would be required for confirmation and reporting purposes. Acceptable samples for analysis include blood and/or urine. For additional samples see Alternative Matrices (section 6.6).

8.6.3 Apparatus and Equipment

Disposable 10 mL culture tubes (2)
Volumetric pipettes and disposable tips
Assorted volumetric glassware
Disposable transfer pipettes
Sample mixer
Centrifuge
Evaporation station
11 mm autosampler vials, inserts, and caps
11 mm crimper
LC/MS/MS, Analyst and/or Cliquid software, compatible computer, and printer

8.6.4 Reagents and Standards

Negative blood, urine, or other matrix as needed
Reference standards
Alpha-hydroxyalprazolam-D_5 certified reference standard (internal standard)
Diazepam-D_5 certified reference standard (internal standard)
Water (H_2O)
Acetone ($(CH_3)_2CO$)
Methanol/water 1:1 (CH_3OH)
1 M Ammonium formate (NH_4HCO_2)
Formic acid (HCO_2H)
Mobile phase A (99.6% water, 0.2% 1 M ammonium formate, 0.2% formic acid)
Mobile phase B (97.6% HPLC suitable methanol, 2% water, 0.2% 1 M ammonium formate, 0.2% formic acid)

8.6.5 Standard and Reagent Preparation
The following are examples of how to prepare the standards and reagents used in this procedure.

8.6.5.1 Standards

Stock Reference Standard Solution (1) [500 ng/mL]
Pipette 50 µL of each [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Stock Reference Standard Solution (2) [5,000 ng/mL]
Pipette 50 µL of each [1 mg/mL] certified reference standard and dilute to 10 mL with acetonitrile.

Stock Reference Standard Control Solution [5,000 ng/mL]
Pipette 50 µL of each [1 mg/mL] certified reference standard and dilute to 10 mL with acetonitrile.

Intermediate Reference Standard Solution (1) [50 ng/mL]
Pipette 1 mL of the 500 ng/mL stock reference standard solution (1) and dilute to 10 mL with H_2O.

Intermediate Reference Standard Solution (2) [500 ng/mL]
Pipette 1 mL of the 5,000 ng/mL stock reference standard solution (2) and dilute to 10 mL with H_2O.

Intermediate Reference Standard Control Solution [500 ng/mL]
Pipette 1 mL of the 5000 ng/mL stock reference standard control solution and dilute to 10 mL with H_2O.

Qualitative only (Q_o) Stock Reference Standard Solution [500 ng/mL]
Pipette 50 µL of each [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Alpha-hydroxyalprazolam-D_5 /Diazepam-D_5 Reference Standard Solution [100 ng/mL] (Internal Standard)
Pipette 50 µL of alpha-hydroxyalprazolam-D_5 [100 µg/mL] certified reference standard and 50 µL diazepam-D_5 [100 µg/mL] certified reference standard and dilute to 50 mL with H_2O.

Working Reference Calibrator/Control Standard Solutions
To make the working reference calibrator/control standard solutions, add the following amounts to a final volume of 1 mL with blood or urine, or other matrix. Working reference standard solution should be made in the same sample matrix being analyzed.

Quantitative Standard Solution Prep

CONCENTRATION	AMOUNT USED	STD SOLUTION
*5 ng/mL	100 µL	Intermediate Reference Soln. 1
15 ng/mL	300 µL	Intermediate Reference Soln. 1
25 ng/mL	500 µL	Intermediate Reference Soln. 1
50 ng/mL	100 µL	Intermediate Reference Soln. 2
100 ng/mL	200 µL	Intermediate Reference Soln. 2 **or** Intermediate Reference Control Soln.
250 ng/mL	500 µL	Intermediate Reference Soln. 2

* Lower levels for sexual assault cases may be used.

Qualitative only (Q_o) Standard Solution Prep

CONCENTRATION	AMOUNT USED	STD SOLUTION
*10 ng/mL	20 µL	Q_o Stock Reference Standard Soln.
50 ng/mL	100 µL	Q_o Stock Reference Standard Soln.
100 ng/mL	200 µL	Q_o Stock Reference Standard Soln.

* Lowest level used to establish reporting limit.

8.6.5.2 Prepared Reagents

Methanol/Water 1:1 (Needle Rinse and Reconstitution Solution)
Add 500 mL of methanol to a volumetric flask and dilute to 1000 mL with H_2O.

1 M Ammonium Formate
Dissolve 63 g of ammonium formate and dilute to 1000 mL with H_2O.

Mobile Phase A
Add 2 mL 1 M ammonium formate and 2 mL formic acid to H_2O and dilute to 1000 mL with H_2O.

Mobile Phase B
Add 2 mL 1 M ammonium formate and 2 mL formic acid to 20 mL H_2O and dilute to 1000 mL with methanol.

8.6.6 Procedure

1. Allow all reference standards and case samples to equilibrate to room temperature before beginning procedure.
2. Label, check, and load/unload all samples in accordance with the "Sample Pipetting Check List" (see Appendix section).
3. Prepare working reference calibrator and/or control standards from the intermediate reference standard solutions. See example above.
4. Pipette 100 µL of corresponding case sample, calibrator, positive control, or negative control into the appropriately labeled 10 mL culture tube.
   Note: Smaller sample volumes may be analyzed on a case-by-case basis.
5. Pipette 50 µL of internal standard into each sample to make a final concentration of 50 ng/mL.
6. Add 2.5 mL of acetone, mix approximately 10 seconds, and centrifuge until separated (approximately 10 minutes).
7. Decant the supernatant to another clean, labeled 10 mL culture tube. Discard the bottom layer.
8. Evaporate to dryness with heat (optional) and a dry gas (e.g. nitrogen) in an evaporation station (approximately 10 minutes).
9. Reconstitute the residue with 100 µL of methanol/water 1:1, mix, and centrifuge until separated (approximately 5 minutes).
   Note: Over centrifugation can lead to a reduced sample volume.
10. Transfer to an 11 mm autosampler vial with insert, attempting to avoid transfer any of the particulate matter in the bottom of the tube, and seal with cap.
11. Analyze and quantitate the samples by LC/MS/MS.

8.6.7. Reporting

8.6.7.1 Qualitative

8.6.7.1.1 Retention times of drugs identified and internal standards must fall within ± 2% of calibrator or control standards.
Note: Some drug retention times are concentration dependent and a comparison of ± 2% to a calibrator used in the calibration curve or control standard of similar concentration shall be acceptable.

8.6.7.1.2 Multiple reactions monitoring (MRM) ion ratios must fall within ± 20% of the calibrators or control standards. If a calibration point is removed then the ion ratio range shall be recalculated from the calibrators used to establish the curve. All 3 Q_o reference standard ion ratios are required to generate the ion ratio range for qualitative only benzodiazepines.
Note: Some drug MRM ion ratios are concentration dependent and a comparison of ± 20% to a calibrator used in the curve or control standard of similar concentration shall be acceptable.

8.6.7.1.3 If the control standard concentration is outside the expected value, the drug may be reported as "positive" if the retention time criteria and ion ratio criteria are met.

8.6.7.1.4 Drug concentrations in casework may be reported as "positive" if the drug response ratio (i.e., area of drug/area of internal standard) is equal to or greater than the drug response ratio of the lowest calibrator used to establish the calibration curve or the lowest reference standard used to establish the reporting limit.

8.6.7.1.5 EPI mass spectrums (when required) must be consistent with those of analyzed reference material.

8.6.7.2 Quantitative

8.6.7.2.1 All of the qualitative result criteria above must be met.

8.6.7.2.2 Sample drug concentrations greater than the highest calibrator level where results are not necessary for interpretation in the case may be reported as "greater than …." The highest calibrator level.
Note 1: Drug concentrations of 1000 ng/mL for Clobazam, Clonazolam, Delorazepam, Diclazepam, Flualprazolam, Flubromazepam, and Flubromazolam produced no carryover using this procedure.
Note 2: Drug concentrations of 3000 ng/mL for 7-aminoclonazepam, 7-aminoflunitrazepam, Alpha-hydroxyalprazolam, Alprazolam, Chlordiazepoxide, Clonazepam, Desalkylflurazepam, Diazepam, Estazolam, Etizolam, Flunitrazepam, Flurazepam, Lorazepam, Midazolam, Nordiazepam, Oxazepam, Temazepam, Triazolam, Zolpidem, and Zopiclone produced no carryover using this procedure.

8.6.7.2.3 Sample drug concentrations greater than the highest calibration level where the results are necessary for interpretation in the case shall be reanalyzed with a smaller sample amount (including dilution factor). When diluting a sample for reanalysis, the diluent should be made of the same matrix as the sample.

8.6.7.2.4 In cases of drug facilitated sexual assault or in other cases as circumstances dictate, the reporting limit/limit of quantification may be reduced by successfully analyzing calibration standards of lower concentration.

8.6.7.3 Results

8.6.7.3.1 Any qualitative or quantitative report data not used in a case shall either be lined through and initialed or all the data used shall be highlighted.

8.6.7.3.2 Sample drug concentrations below the lowest calibrator level or sample drug response ratios below the reporting limit shall be reported as "No benzodiazepines detected".

8.6.7.3.3 Qualitative results shall be expressed as "positive" and include any clarifying remarks, if applicable.

8.6.7.3.4 Quantitative results shall be reported in ng/mL and truncated to the whole number.

8.6.7.3.5 When a definitive conclusion cannot be made, the reason shall be documented on the report (e.g., "insufficient sample for analysis", "sample unsuitable for analysis", "results are inconclusive due to sample condition", etc.).

8.6.8 References

See method validation for extensive bibliography.