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 that have been previously screened positive by ELISA or GC/MS for a relevant drug shall require confirmation by LC/MS/MS Multiple Reaction Monitoring (MRM).

Samples that have not previously screened positive, for example limited sample or low suspected drug levels such as in Drug Facilitated Sexual Assault cases, shall require LC/MS/MS testing by both Multiple Reaction Monitoring (MRM) and Enhanced Product Ion (EPI) to identify a relevant drug. These cases may be reported as negative with only LC/MS/MS Multiple Reaction Monitoring (MRM) testing.

Acceptable samples for analysis include blood and 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-D₅ certified reference standard (internal standard)
Diazepam-D₅ certified reference standard (internal standard)
Water (H₂O)
Acetone ((CH₃)₂CO)
Methanol/water 1:1 (CH₃OH)
1 M Ammonium formate (NH₄HCO₂)
Formic acid (HCO₂H)
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 H₂O.

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 H₂O.

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 H₂O.

Alpha-hydroxyalprazolam-D₅/Diazepam-D₅ Reference Standard Solution [100 ng/mL] (Internal Standard)
Pipette 50 µL of alpha-hydroxyalprazolam-D₅ [100 µg/mL] certified reference standard and 50 µL diazepam-D₅ [100 µg/mL] certified reference standard and dilute to 50 mL with H₂O.

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.
### 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 H₂O.

**1 M Ammonium Formate**

Dissolve 63 g of ammonium formate and dilute to 1000 mL with H₂O.

**Mobile Phase A**

Add 2 mL 1 M ammonium formate and 2 mL formic acid to H₂O and dilute to 1000 mL with H₂O.

**Mobile Phase B**

Add 2 mL 1 M ammonium formate and 2 mL formic acid to 20 mL H₂O 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 \( \mu \)L 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 of Results

Results can be reported if the following criteria are met:

8.6.7.1 MRM ion ratios for the drug and internal standard must fall within +/- 20% of those of a calibrator or control standard of similar concentration.

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

8.6.7.3 The retention times of the drug and internal standard are within +/- 2% of those of a calibrator or control standard of similar concentration.

8.6.7.4 Drug concentrations below 15 ng/ml or the lowest calibrator shall be reported as “not detected” in routine casework.

8.6.7.5 In cases of drug facilitated sexual assault or in other cases as circumstances dictate the limit of detection may be reduced by successfully analyzing calibration standards of lower concentration.

8.6.7.6 Drug concentrations may be reported as “positive” if the drug to internal standard ratio of the case sample is equal to or greater than that of the lowest calibrator used to establish the calibration curve. Any clarifying remarks must be included, if applicable.

8.6.7.7 Drug concentrations greater than the highest calibration point where the results are necessary for interpretation in the case shall be reanalyzed with a smaller sample size (dilution).

8.6.7.8 Drug concentrations greater than the highest calibration point where the results are not necessary for interpretation in the case may be reported as “greater than . . . “the highest calibration point.

8.6.7.9 Results shall be reported in ng/ml and truncated to the whole number.

8.6.7.10 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.