

CONFIRMATION OF SELECT BENZODIAZEPINES AND ZOPICLONE BY LIQUID CHROMATOGRAPHY/AJS – TANDEM MASS SPECTROMETRY

45.1 METHOD

This test method may be used to confirm the presence of select benzodiazepines and zopiclone in biological matrices. Target compounds and internal standards are isolated from biological specimens by liquid-liquid extraction (LLE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) detector equipped with a jet stream atmospheric pressure electrospray ionization source.

45.2 SPECIMENS

The specimen volume is 0.5 mL. For any matrix other than whole blood (serum, tissue homogenate), matrix-matching of all calibrators and controls is required. Alternatively, test method TCb12713 may be used for analysis of these matrices, and is required for analysis of urine specimens. Dilutions of specimens may be analyzed, at the Forensic Scientist's discretion.

NOTE: Specimens analyzed at less than standard sample volume (dilution for high-concentrations, low sample volume available) are not brought to standard volume in sample preparation. Where a specimen is analyzed at less than standard volume, a dilution control is included in the batch (see 45.3.10).

Method validation studies indicate the presence of desmethylclomipramine interferes with temazepam; peaks for both transitions were integrated within the acceptable retention time range however, qualifier ratios did not meet acceptability criteria.

45.3 REAGENTS, MATERIALS AND EQUIPMENT

45.3.1 REAGENTS

NOTE: Laboratory general-use deionized water (DI H₂O) and reagent grade organic solvents are used in reagent preparation, unless otherwise specified.

- Acetonitrile (ACN), reagent and LC-MS grade
- Certified blank blood and/or other biological matrices
- DI H₂O, laboratory general-use and LC-MS grade H₂O (or equivalent from a high purity filtration system)
- Ethyl acetate (EtAC)
- Formic acid, concentrated
- 0.1% Formic acid in LC-MS grade H₂O

Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H₂O in a 1 L flask and mix. Dilute to 1 L with LC-MS grade H₂O and mix. Store the acid in an amber glass bottle at room temperature for up to one year.

NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H₂O must be used in place of LC-MS grade H₂O.

- Methanol (MeOH), reagent and HPLC grade
- Sodium borate decahydrate (Na₂B₄O₇ • 10H₂O)
- 0.13M Sodium borate solution (saturated)

In a 100 mL volumetric flask, dissolve 4.9 g Na₂B₄O₇ • 10H₂O in approximately 75 mL DI H₂O and mix. Dilute to 100 mL with DI H₂O and mix thoroughly (may require low heating). The weighed contents may not go completely into solution. This is normal. Store the solution in an amber glass bottle at room temperature for up to 6 months.

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

45.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Disposable transfer pipettes (glass)
- Glass autosampler vials with caps
- HPLC column (Agilent Poroshell Phenyl Hexyl 100 mm x 2.1 mm ID, d_p=2.7 μm, or equivalent)
- Laboratory glassware (graduated cylinders, flasks)

45.3.3 EQUIPMENT

- Agilent HPLC (1200 series, or equivalent)
- Agilent MS-MS with AJS source (Ultivo or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heating plate, rotary mixer, vortex mixer)

45.4 STANDARDS, CALBRATORS AND CONTROLS

45.4.1 STANDARDS

- Working standard: 2/5/10 ng/μL
- Working control standard: 2/5/10 ng/μL
- High-concentration control standards: 20/50/100 ng/μL
- Working internal standard: 1 ng/μL

45.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 45.5. For analysis of alternate matrices, all calibrators are prepared in that matrix.

45.4.3 CONTROLS

- 45.4.3.1 At least one negative blood control and three positive blood controls are tested with each batch, as described in 45.5. For analysis of alternate matrices, all controls (positive and negative) are prepared in that matrix.
- 45.4.3.2 Controls (positive or negative) must make up at least 10% of the extracted batch (by number of case specimen samples), with case specimens bracketed by positive controls.
- 45.4.3.3 Positive controls prepared in the alternate matrix must make up at least 10% of the extracted batch, and bracket specimen(s) in that matrix, as is described for blood controls in 45.4.3.2.

45.5 SAMPLE PREPARATION

NOTE: Organic solvents used in sample preparation are reagent grade, with the exception of HPLC grade MeOH as reconstitution solvent.

- 45.5.1 Label a clean extraction tube for each member of the test batch (i.e., calibrator, control, case sample).
- 45.5.2 Add 0.5 mL 0.13M sodium borate buffer into each tube.
- 45.5.3 Using a calibrated pipette, add 0.5 mL of certified blank blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).
- 45.5.4 Prepare a 1:10 dilution of the working standard. (0.2/0.5/1 ng/μL)
 - a. Using a calibrated pipette, combine 100 μL of the working standard with 900 μL of ACN or MeOH in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 45.5.5 Prepare a 1:100 dilution of the working standard. (0.02/0.05/0.1 ng/μL)
 - a. Using a calibrated pipette, combine 100 μL of the 1:10 dilution with 900 μL of ACN or MeOH in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 45.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 2/5/10 ng/mL	50	0.02/0.05/0.1 ng/µL	1:100
Calibrator 2 – 5/12.5/25 ng/mL	125	0.02/0.05/0.1 ng/µL	1:100
Calibrator 3 – 10/25/50 ng/mL	25	0.2/0.5/1 ng/µL	1:10
Calibrator 4 – 20/50/100 ng/mL	50	0.2/0.5/1 ng/µL	1:10
Calibrator 5 – 50/125/250 ng/mL	125	0.2/0.5/1 ng/µL	1:10
Calibrator 6 – 100/250/500 ng/mL	25	2/5/10 ng/µL	WS
Calibrator 7 – 200/500/1000 ng/mL	50	2/5/10 ng/µL	WS

45.5.7 Prepare a 1:10 dilution of the working control standard. (0.2/0.5/1 ng/µL)

- a. Using a calibrated pipette, combine 100 µL of the working control standard with 900 µL of ACN or MeOH in a labeled tube.
- b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

45.5.8 Prepare a 1:100 dilution of the working control standard. (0.02/0.05/0.1 ng/µL)

- a. Using a calibrated pipette, combine 100 µL of the 1:10 dilution with 900 µL of ACN or MeOH in a labeled tube.
- b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

45.5.9 Using a calibrated pipette, spike the positive controls according to the following table, using the working control standard and prepared dilutions.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 – 6/15/30 ng/mL	150	0.02/0.05/0.1 ng/µL	1:100
Control 2 – 80/200/400 ng/mL	20	2/5/10 ng/µL	QC
Control 3 – 160/400/800 ng/mL	40	2/5/10 ng/µL	QC

45.5.10 If one or more case specimens are analyzed at less than standard sample volume, a dilution control is included in the batch, prepared to a dilution factor matched to the specimen(s). If all specimens were analyzed at full volume, continue at 45.5.14.

NOTE: If more than one dilution factor is used for specimens in the batch, a dilution control is prepared at each dilution factor.

45.5.11 For dilution control only: Using a calibrated pipette, sample 1 mL of blank blood into the dilution control preparation tube.

45.5.12 For dilution control only: Using a calibrated pipette, spike the dilution control preparation tube according to the following table, using each of the high-

concentration working control standards. Choose the dilution factor most similar to that for the case specimen(s).

Control Description	Volume (µL) Added	High QC Standard Concentrations
Dilution control 1:2 – 300/750/1500 ng/mL	15 (each high QC)	20/50 and 100 ng/µL
Dilution control 1:4 – 360/900/1800 ng/mL	18 (each high QC)	20/50 and 100 ng/µL
Dilution control 1:5 – 400/1000/2000 ng/mL	20 (each high QC)	20/50 and 100 ng/µL
Dilution control 1:10 – 1000/2500/5000 ng/mL	50 (each high QC)	20/50 and 100 ng/µL

45.5.13 For dilution control only: Using a calibrated pipette, sample the appropriate volume from the dilution control preparation tube into its respective tube.

NOTE: Dilution control and diluted specimens are not brought to standard sample volume.

45.5.14 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.

45.5.15 Using a calibrated pipette or verified repeated pipette, add 100 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 200 ng/mL.

45.5.16 Vortex mix tubes until homogenous.

45.5.17 Add 4 mL ethyl acetate to each tube.

45.5.18 Cap the tubes and place on a rotary mixer for at least 5 minutes.

45.5.19 Centrifuge the tubes for 5 minutes at 3000 rpm (recommended for 16 x 100 mm tubes).

45.5.20 Transfer the ethyl acetate layer to a clean, labeled centrifuge or screw-cap tube.

45.5.21 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.

45.5.22 Immediately after evaporation, reconstitute the extracts with the addition of 500 µL HPLC grade MeOH to each tube and briefly vortex mix. Centrifuge the tubes for 2 minutes at 2000 rpm to obtain a clear extract.

45.5.23 Transfer the extracts to labeled glass autosampler vials and cap.

45.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – ULTBENZO (instrumental parameters in Appendix B)
- Calibration curve – linear or quadratic, 1/x or 1/x² weighting factor (see Appendix A for curve specifics for each target compound)

- Updating calibrator (retention times $\pm 3\%$, ion ratios $\pm 20\%$) – Cal 4
- Result comparisons –
Cal 1 ($\pm 25\%$ from target): target 2 or 5 ng/mL – truncated to two decimal places (acceptable ranges 1.50 – 2.50, 3.75 – 6.25); target 10 ng/mL – truncated to one decimal place, 7.5 – 12.5 ng/mL)
Cals 2-7, Ctls ($\pm 20\%$ from target): truncated to one decimal place for target value ≤ 10 ng/mL, truncated to whole integer values for values > 10 ng/mL

45.7 REPORTING

Quantitative results are reported for specimen concentrations \geq the calibrator 1 concentration (5/10 ng/mL); midazolam, with calibrator 1 concentration of 2 ng/mL, is also reported ≥ 5 ng/mL.

Reported results are truncated to two significant figures; target compounds with LLOQ of 5 ng/mL are reported in units of ng/mL and target compounds with LLOQ of 10 ng/mL are reported in units of mg/L.

7-aminoflunitrazepam, zopiclone and triazolam are reported qualitatively from this test method, where specimen concentrations are \geq LLOQ (5 ng/mL).

NOTE: Pending sample stability evaluation, 7-aminoclonazepam is reported qualitatively if the batch is injected more than 24 hours after extraction.

45.8 METHOD PERFORMANCE

- Lower limit of quantification: 5 ng/mL (5 – 500 ng/mL compounds and midazolam)
10 ng/mL (10 – 1000 ng/mL dynamic range)
- Dynamic range: 2 – 200, 5 – 500, 10 – 1000 ng/mL
- Upper limit of quantitation: 200/500/1000 ng/mL
- Limit of detection, upper limit of linearity: see table in Appendix A

45.9 REFERENCES

- A. Black, in-house method development.
- J. Hudson, J. Hutchings, C. Harper, R. Wagner, P. Friel, Benzodiazepine and Z-Drug Quantitation Using an Agilent 6430 LC/MS/MS, *Agilent Application Note 5991-2291EN*, June 2013.
- H. Rivera, G. Stewart Walker, P. Stockham, D. Sims, J. Hughes, Analysis of Benzodiazepines in Blood by LC/MS/MS, *Agilent Application Note 5989-4737EN*, March 2006.
- K. Simonsen, S. Hermansson, A. Steentoft, K. Linnet, A Validated Method for Simultaneous Screening and Quantification of Twenty-Three Benzodiazepines and Metabolites Plus Zopiclone and Zaleplon in Whole Blood by Liquid-Liquid Extraction and Ultra-Performance Liquid Chromatography – Tandem Mass Spectrometry, *J Anal Tox.* 34:332-341 (2010).

APPENDIX A

Target Compounds, Internal Standards, Curve Fit and Dynamic Range (ng/mL)
 All calibration curves are weighted 1/x, unless otherwise specified.

7-aminoclonazepam	d4-7-aminoclonazepam	linear	5-500
7-aminoflunitrazepam	d7-7-aminoflunitrazepam	quadratic (1/x ²)	5-500
zopiclone	d5-oxazepam	quadratic	5-500
chlordiazepoxide	d5-chlordiazepoxide	linear	10-1000
midazolam	d5-diazepam	quadratic	2-200
flurazepam	d7-flunitrazepam	quadratic	5-500
α-hydroxyalprazolam	d5-α-hydroxyalprazolam	linear	5-500
oxazepam	d5-diazepam	quadratic (1/x ²)	10-1000
estazolam	d5-alprazolam	quadratic	5-500
nordiazepam	d5-diazepam	quadratic	10-1000
lorazepam	d5-diazepam	quadratic	5-500
alprazolam	d5-alprazolam	linear	5-500
clonazepam	d5-alprazolam	quadratic (1/x ²)	10-1000
desalkylflurazepam	d5-oxazepam	linear	5-500
etizolam	d5-diazepam	quadratic	5-500
triazolam	d5-diazepam	quadratic	5-500
temazepam	d5-diazepam	linear	10-1000
flunitrazepam	d7-flunitrazepam	linear	5-500
diazepam	d5-diazepam	quadratic	10-1000

Limits of Detection, Upper Limits of Linearity

Target Compound	LOD (ng/mL)	ULOL (ng/mL)
7-aminoclonazepam	1	1000
7-aminoflunitrazepam	2.5	POS
zopiclone	0.2	POS
chlordiazepoxide	2	1500
midazolam	0.4	1000
flurazepam	2	1000
α-hydroxyalprazolam	3.75	750
oxazepam	6	1500
estazolam	2	2500
nordiazepam	4	5000
lorazepam	3	2500
alprazolam	2	750
desalkylflurazepam	2.5	750
clonazepam	4	1500
etizolam	1.5	2500
triazolam	5	POS
temazepam	2	2000
flunitrazepam	1.5	2500
diazepam	1	2000

APPENDIX B - INSTRUMENTAL PARAMETERS

Agilent Ultivo System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.500 mL/min
Solvent A	0.1% Formic acid in LCMS H ₂ O
Solvent B	Acetonitrile (LC-/MS grade)
Initial Composition	85% (A), 15% (B)
0 – 1.5 min	35% B
Hold to 5.0 min	35% B
5.0 – 5.5 min	90% B
Hold to 7.0 min	90% B
7.0 – 7.5 min	15% B
Hold to 10.0 min	15% B
Column Temp	30° C
Autosampler	
Injection Volume	1.0 µL
Injection flush-port	Active
Flush-port time	15 sec
Flush-port solvent	75:25 HPLC grade MeOH:LC-MS grade H ₂ O

MASS SPECTROMETER

Ion mode	(+) dMRM	EMV	Set in tune
Time filter width	0.07 min	Capillary voltage	4kV
Resolution	Unit	Nebulizer pressure	40 psi
Sheath gas flow	12 L/min	Drying/sheath gas	Nitrogen
Sheath gas temp	400° C	Drying gas flow	13 L/min
Nozzle voltage	1500V	Drying gas temp	350° C
Compounds	MRM Transitions	Compounds	MRM Transitions
7-aminoclonazepam	286 → 222, 121	Nordiazepam	271 → 208, 139.9
7-aminoflunitrazepam	284 → 237.1, 135.1	Oxazepam	287 → 241.1, 103.9
α-hydroxyalprazolam	325 → 297.1, 205	Temazepam	301 → 255, 177
Alprazolam	309 → 281, 205	Triazolam	343 → 308, 239.1
Chlordiazepoxide	300 → 282, 226.9	Zopiclone	389 → 245, 217.1
Clonazepam	316 → 270, 214	d4-7-aminoclonazepam	291 → 255, 227.2
Desalkylflurazepam	289 → 226.2, 139.9	d7-7-aminoflunitrazepam	291.3 → 263.2, 138
Diazepam	285 → 193, 153.9	d5-α-hydroxyalprazolam	330 → 302, 210.1
Estazolam	295 → 267.1, 205.1	d5-alprazolam	314 → 286.1, 210.1
Etizolam	343 → 314.1, 289.1	d5-chlordiazepoxide	305.5 → 286.1, 232.1
Flunitrazepam	314 → 268.1, 239	d5-diazepam	291 → 263.1, 228.1
Flurazepam	388 → 315.1, 133.9	d7-flunitrazepam	321.3 → 275.2, 246.2
Lorazepam	321 → 275.1, 229.1	d5-oxazepam	292 → 274, 246.1
Midazolam	326 → 291, 249.1		

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