

IDENTIFICATION/CONFIRMATION OF DESIGNER BENZODIAZEPINES BY LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRY

46.1 METHOD

This test method may be used to identify or confirm the presence of select designer benzodiazepines in whole blood, for qualitative reporting. 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 tandem mass spectrometer (MS-MS) detector equipped with a jet stream ionization source.

46.2 SPECIMENS

The specimen volume is 0.5 mL whole blood. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

Analysis of other matrices may be performed, with negative and all positive control levels matrix-matched to the specimen (see 46.4.3.3).

46.3 REAGENTS, MATERIALS AND EQUIPMENT

46.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H₂O) is specified, laboratory general-use DI H₂O is used in reagent preparation. Organic solvents are reagent grade, 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 (or equivalent from a high purity filtration system)
- Ethyl acetate (EtAC)
- Formic acid, concentrated
- 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H₂O in a 1 L flask. 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.

- Methanol (MeOH), 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. 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 a glass bottle at room temperature for up to 6 months.

Adjustments to final volume are permitted as long as the proportions are maintained.

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

46.3.2 MATERIALS

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

46.3.3 EQUIPMENT

- Agilent HPLC (1290 series, or equivalent)
- Agilent MS-MS with AJS source (Ultivo 6465 or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heated stir plate, rotary mixer, vortex mixer)

46.4 STANDARDS, CALIBRATORS AND CONTROLS

46.4.1 STANDARDS

All compounds

- Working standard: 1 ng/ μL
- Working control standard: 1 ng/ μL
- Working internal standard: 0.5 ng/ μL

46.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 46.5 SAMPLE PREPARATION.

46.4.3 CONTROLS

- 46.4.3.1 At least one negative blood control and three positive blood controls are tested with each batch, as described in 46.5.
- 46.4.3.2 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimens samples), with case specimens bracketed by positive controls.

- 46.4.3.3 All controls (one negative, three positive) must be included for each alternate matrix type tested in the batch, with the positive controls bracketing the specimen(s) in that matrix.

46.5 SAMPLE PREPARATION

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

- 46.5.1 Label a clean extraction tube for each member of the test batch (i.e., calibrator, control).
- 46.5.2 Add 0.5 mL 0.13M sodium borate solution into each tube.
- 46.5.3 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).
- 46.5.4 Prepare a 1:10 dilution of the working standard. (0.1 ng/μL)
- Using a calibrated pipette, combine 100 μL of the working standard with 900 μL of ACN or MeOH in a labeled tube.
 - Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 46.5.5 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilution.

Calibrator Description	Volume (μL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 5.0 ng/mL	25	0.1 ng/μL	1:10
Calibrator 2 – 10 ng/mL	50	0.1 ng/μL	1:10
Calibrator 3 – 25 ng/mL	125	0.1 ng/μL	1:10
Calibrator 4 – 50 ng/mL	25	1 ng/μL	WS
Calibrator 5 – 100 ng/mL	50	1 ng/μL	WS
Calibrator 6 – 200 ng/mL	100	1 ng/μL	WS

- 46.5.6 Prepare a 1:10 dilution of the working control standard. (0.1 ng/μL)
- Using a calibrated pipette, combine 100 μL of the working control standard with 900 μL of ACN or MeOH in a labeled tube.
 - Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 46.5.7 Using a calibrated pipette, spike the controls according to the following table, using the working control standard and the prepared dilution.

Control Description	Volume (μ L) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 – 15 ng/mL	75	0.1 ng/ μ L	1:10
Control 2 – 80 ng/mL	40	1 ng/ μ L	QC
Control 3 – 160 ng/mL	80	1 ng/ μ L	QC

- 46.5.8 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.
- 46.5.9 Using a calibrated pipette or verified repeater-pipette, add 80 μ L of the working internal standard solution to each tube. Final concentration of the internal standard is 80 ng/mL.
- 46.5.10 Vortex mix tubes until homogenous.
- 46.5.11 Add 4 mL ethyl acetate to each tube.
- 46.5.12 Cap the tubes and place on a rotary mixer for at least 5 minutes.
- 46.5.13 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).
- 46.5.14 Transfer the ethyl acetate layer to a clean, labeled centrifuge or screw-cap tube.
- 46.5.15 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.
- 46.5.16 Immediately after evaporation, reconstitute the extracts with the addition of 500 μ L HPLC grade MeOH to each tube and briefly vortex mix. Centrifuge the tubes for 2 minutes at 2000 rpm to obtain a clear extract.
- 46.5.17 Transfer the extracts to labeled glass autosampler vials and cap.

46.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – DESBZ (instrumental parameters in Appendix B)
- Calibration curve – quadratic, 1/a weighting factor
- Updating calibrator (retention times \pm 2%, ion ratios \pm 20%) – Cal 4
- Result comparisons –

Cal 1 (\pm 25% from target): truncated to one decimal place in units of ng/mL

Cals 2-6, Controls 1-3 (\pm 20% from target): truncated, whole integer values in units of ng/mL

NOTE: Although only qualitative results are reported, calibrators/calibration curves are evaluated using criteria described in Quality Assurance Principles 4.5.6.4, to assess data/performance for inclusion in future quantitative validation. The positive controls must meet acceptability criteria for chromatography, retention time and ratios. When control values for the compounds are not within \pm 20% of target, qualitative results are still reportable.

46.7 REPORTING

Qualitative results are reported for concentrations at or above the calibrator 1 (5.0 ng/mL) concentration, as “positive.” Specimens with concentrations above calibrator 6 (200 ng/mL) are eligible for positive reporting, provided all acceptance criteria are met.

46.8 METHOD PERFORMANCE

- Limit of detection: 2.0 ng/mL
- Lower limit of qualitative reporting: 5.0 ng/mL
- Dynamic range: 5.0 – 200 ng/mL

APPENDIX A

<u>Target Compounds</u>	<u>Internal Standards</u>
7-aminonimetazepam	d7-7-aminoflunitrazepam
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bromazolam	d5-alprazolam
clobazam	d5-diazepam
clonazolam	d5-alprazolam
clotiazepam	d5-oxazepam
delorazepam	d5-oxazepam
demoxepam	d5-diazepam
deschloroetizolam	d5-oxazepam
desmethylflunitrazepam	d7-flunitrazepam
diclazepam	d5-diazepam
flualprazolam	d5-alprazolam
flubromazepam	d5-nitrazepam
flubromazolam	d5-alprazolam
flunitrazolam	d5-oxazepam
lormetazepam	d5-diazepam
meclonazepam	d5-diazepam
methylclonazepam	d5-diazepam
N-desmethylclobazam	d5-oxazepam
nifoxipam	d5-oxazepam
nimetazepam	d5-diazepam
nitrazepam	d5-nitrazepam
nitrazolam	d5-alprazolam
phenazepam	d7-flunitrazepam
pyrazolam	d5-chlordiazepoxide

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	3.0 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-aminonitrazepam	252.0 → 93.9, 77	nimetazepam	296.0 → 250.1, 221.1
7-aminonimetazepam	266.0 → 135.1, 209.0	lormetazepam	335.0 → 288.9, 176.9
pyrazolam	354.0 → 206.0, 166.9	clobazam	301.0 → 259.0, 224.2
nifoxipam	316.0 → 270.0, 194.1	phenazepam	349.0 → 206.0, 184.0
demoxepam	287.0 → 269.1, 242.0	meclonazepam	330.0 → 238.2, 214.1
deschloroetizolam	309.0 → 280.0, 255.1	methylclonazepam	330.0 → 205.1, 164.9
flunitrazolam	338.0 → 292.2, 264.2	diclazepam	319.0 → 154.0, 227.1
nitrazolam	320.0 → 292.1, 246.1		
clonazolam	354.0 → 308.1, 280.1		
nitrazepam	282.0 → 180.1, 207.0		
flualprazolam	327.0 → 223.1, 299.0		
desmethylflunitrazepam	300.0 → 225.1, 198.1	d7-7aminoflunitrazepam	291.3 → 138.0, 263.2
N-desmethyloclobazam	287.0 → 210.0, 181.1	d5-chlordiazepoxide	305.5 → 286.1, 232.1
flubromazolam	371.0 → 292.2, 223.1	d5-oxazepam	292.0 → 274.0, 246.1
bromazolam	353.0 → 325.0, 205.1	d5-nitrazepam	287.3 → 241.1, 185.2
flubromazepam	333.0 → 226.2, 104.8	d5-alprazolam	314.0 → 286.1, 210.1
clotiazepam	319.0 → 154.1, 218.1	d7-flunitrazepam	321.3 → 275.2, 246.2
delorazepam	305.0 → 139.9, 76.9	d5-diazepam	291.0 → 263.1, 228.1



LIST OF CHANGES

Revision Date	Description	Page Number
1/25/21	Method approved by Washington State Toxicologist. See DRA dated 1/15/21. Method released for use in evidentiary testing on 1/25/21.	All