CONFIRMATION OF ZOLPIDEM BY LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

9.1 METHOD

This test method may be used to confirm the presence of zolpidem in biological specimens. Zolpidem (ZOL) and internal standard diazepam-d_5 (DZP-d_5) are isolated from biological matrices by solid phase extraction (SPE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source.

9.2 SPECIMENS

The specimen volume is 0.2 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

The presence of diazepam in a specimen may cause interference with qualifier m/z 154 for DZP-d_5 internal standard, affecting ion ratios (see NOTE in 9.5 and 9.7).

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in liver (tissue) homogenate or serum/plasma specimens (see 9.4.3.2).

9.3 REAGENTS, MATERIALS AND EQUIPMENT

9.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H_2O) is specified, laboratory general-use DI H_2O is used in reagent preparation. Organic solvents are reagent grade unless otherwise specified.

- Acetic acid (glacial)
- 0.1M Acetic acid

  Add 5.72 mL glacial acetic acid to 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Store the solution in a glass bottle at room temperature for up to six months.
- Acetonitrile (ACN), reagent grade and LC-MS grade
- Ammonium hydroxide (concentrated)
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade (or equivalent from a high-purity filtration system)
- Elution solvent

  To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store the solvent in a glass flask/bottle at room temperature and use on date of preparation only.

- Formic acid (concentrated)
- 0.1% Formic acid

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade DI H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the acid in a glass bottle at room temperature for up to one year.
- Isopropanol (IPA)
- Methanol (MeOH)
- Methylene chloride (dichloromethane, CH_2Cl_2)
- Sodium acetate trihydrate ($NaC_2H_3O_2 \cdot 3H_2O$)
- 0.1M Sodium acetate buffer (pH 4.5)

  Dissolve 2.93 g sodium acetate trihydrate in 400 mL DI H_2O. Add 1.62 mL glacial acetic acid. Dilute to 500 mL with DI H_2O and mix. Check pH and, if necessary, adjust to 4.5 ± 0.2 with glacial acetic acid or NaOH. Store the buffer in a glass or plastic bottle at room temperature for up to one year.

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

9.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200 mg/3 mL), or equivalent
- HPLC Column, Agilent Zorbax Eclipse Plus C8, 50 mm x 2.1 mm ID, dp = 1.8 µM, or equivalent
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

9.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Agilent MS with API-ES source (6130 model, or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter or pH paper, solvent filtration apparatus, vacuum manifold, vortex mixer)

9.4 STANDARDS, CALIBRATORS AND CONTROLS

9.4.1 STANDARDS

- Working standard: 10 ng/µL

- Working control standard: 10 ng/µL
- Working internal standard (DZP-d_5): 1 ng/µL

9.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 9.5 SAMPLE PREPARATION.

9.4.3 CONTROLS

9.4.3.1 At least one negative whole blood control and two positive whole blood controls are included in the batch, prepared as described in 9.5.

9.4.3.2 One positive and one negative control must be included for each alternate matrix type tested in the batch, for qualitative or quantitative analysis.

9.4.3.3 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls.

9.4.3.4 Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both whole blood and alternate matrix controls apply towards 10% of the batch.

9.5 SAMPLE PREPARATION

NOTE: Abundance of DZP-d_5 qualifier ion (m/z 154) in case specimen samples must be carefully evaluated upon review of the testing batch. If it is determined that the DZP-d_5 qualifier is affected (ion ratio failure and/or chromatography), an alternative test method must be used for qualitative/quantitative analysis of zolpidem (see 9.7).

NOTE: Laboratory general use DI H_2O is used in sample preparation. 0.1% Formic acid used in reconstitution (9.5.22) is prepared using LC-MS grade DI H_2O (or equivalent). Organic solvents used in sample preparation are reagent grade.

9.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample).

9.5.2 Add 2 mL 0.1M sodium acetate buffer pH 4.5 into each tube.

9.5.3 Using a calibrated pipette, add 0.2 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

9.5.4 Prepare a 1:10 dilution of the working standard. (1 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

9.5.5 Prepare a 1:100 dilution of the working standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

9.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the prepared working standard dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS
Calibrator 1 – 10 ng/mL	20	0.1 ng/µL	1:100
Calibrator 2 – 25 ng/mL	50	0.1 ng/µL	1:100
Calibrator 3 - 50 ng/mL	100	0.1 ng/µL	1:100
Calibrator 4 - 100 ng/mL	20	1 ng/µL	1:10
Calibrator 5 - 250 ng/mL	50	1 ng/µL	1:10
Calibrator 6 - 500 ng/mL	100	1 ng/µL	1:10

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

a. Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

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

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

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

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC
Control 1 – 30 ng/mL	60	0.1 ng/µL	1:100
Control 2 - 400 ng/mL	80	1 ng/µL	1:10

9.5.10 Using a calibrated pipette, sample 0.2 mL of each case sample into its respective tube.

9.5.11 Using a calibrated pipette or verified repeater-pipette, add 100 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 500 ng/mL.

9.5.12 Cap the tubes and briefly vortex mix.

9.5.13 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

9.5.14 Place new SPE columns into the vacuum manifold.

9.5.15 Condition the SPE columns by passing each of the following reagents/solvents completely through under force of gravity.

a. 3 mL MeOH
b. 3 mL DI H_2O
c. 2 mL 0.1M acetate buffer (pH 4.5)

Do not let columns dry out between each conditioning step.

9.5.16 Transfer the contents of each extraction tube to its respective SPE column and allow to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

9.5.17 Wash the SPE columns by passing each of the following reagents/solvents completely through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

a. 3 mL DI H_2O
b. 2 mL 0.1M acetic acid
c. 3 mL MeOH

9.5.18 Dry the columns for 10 minutes under vacuum.

9.5.19 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.

9.5.20 Pass 3 mL of elution solvent through each SPE column and collect the extracts.

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

9.5.22 Reconstitute the extracts with the addition of 100 µL 0.1% formic acid to each tube and briefly vortex mix. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

9.5.23 Transfer the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

9.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – ZOLPIDEM (instrumental parameters in Appendix A)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4
- Result comparisons –

Cal 1: truncated to one decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL)

Cals 2-6, Ctls 1-2: truncated, whole integer values in units of ng/mL

9.7 REPORTING

Results are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L), and truncated to two significant figures for reporting.

Where interference with the DZP-d_5 qualifier is observed in case specimens (see 9.2 and NOTE in 9.5), an alternative test method must be used for qualitative or quantitative analysis of zolpidem.

9.8 METHOD PERFORMANCE

- Limit of detection: 1 ng/mL (0.001 mg/L)
- Lower limit of quantification: 10 ng/mL (0.01 mg/L)
- Dynamic range: 10 – 500 ng/mL (0.010 – 0.50 mg/L)
- Upper limit of quantitation: 500 ng/mL (0.50 mg/L)

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
Solvent B	ACN (LC-MS grade)
Initial composition	85% A, 15% B
0 – 4.0 min	% B increased to 55%
Hold time	4.0 min (55% B)
Re-equilibration	9.0 min
Column temp	30°C
Autosampler	
Injection volume	2.0 µL
Injection flush-port	Active
Flush-port time/volume	15 sec
Flush-port solvent	ACN (LC-MS grade)

MASS SPECTROMETER

Ion mode	(+) SIM	Nebulizer gas	Nitrogen
EM gain	1.0	Nebulizer pressure	30 psi
Peak width	0.05 min	Drying gas	Nitrogen
		Drying gas flow	12 L/min
		Drying gas temp	350 °C
		Capillary voltage	4 kV

Compound	Ions	Ion Ratios
Zolpidem	236, 263, 308	236/308, 263/308
Diazepam-d_5	154, 290	154/290

LIST OF CHANGES

Revision Date	Description	Page Number
3/01/12	Method approved by Washington State Toxicologist. See DRA dated 2/13/12. Method released for use in evidentiary testing on 3/01/12.	All
2/01/14	HPLC column description in section 9.5.2.7 changed to Agilent Zorbax Eclipse Plus C8 (50 x 2.1 mm; 1.8um I.D.) or equivalent.	3
10/01/15	Changed wording in 9.1 to reflect that deviations are approved by a member of TLD Management. Added note to 9.7 with information regarding possible interference with DZP-d_5 in cases containing diazepam. Other minor edits throughout.	1, 4-5, 7
4/6/16	Wording was added to for adjustment of prepared volumes in 9.5.1.1, 9.5.1.3, 9.5.10, 9.6.1.3 and 9.6.1.4. Added clarification to 9.6.3.2.c for use of same CRM in preparation of working standard and working control standard and note regarding CRM expiration dates in 9.6.1.3 and 9.6.1.4. Edited 9.12.3 to reflect that only two significant figures are used for reporting. Other minor edits throughout.	2-4, 8
5/8/17	Wording added to 9.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 9.7. Specified calibrator concentration criteria/ranges in 9.10.1.3. Edited 9.10.2.2d to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-8
7/23/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix matching and testing of specimens that contain diazepam in 9.2 SPECIMENS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 9.3.1. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Formatting and minor edits throughout.	All