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: Laboratory general-use deionized water (DI H_2O) and reagent-grade organic solvents are used in reagent preparation, unless otherwise specified.

- Acetic acid (glacial)
- 0.1M Acetic acid

  Add 5.72 mL glacial acetic acid to 800 mL DI H_2O and mix. 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 H_2O (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 in LC-MS grade H_2O

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask and mix. Dilute to 1 L with LC-MS grade H_2O 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 H_2O must be used in place of LC-MS grade H_2O.

- Isopropanol (IPA)
- Methanol (MeOH), reagent grade and HPLC grade
- Methylene chloride (dichloromethane, CH_2Cl_2)
- Sodium acetate trihydrate ($NaC_2H_3O_2 \bullet 3H_2O$)
- 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 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/6 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

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, 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 blood control and two positive 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. When the batch contains more than 20 specimens, a third positive control (low or high) must be extracted and analyzed mid-run.

9.4.3.4 Positive controls in both blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both 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).

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 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 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.

9.5.5 Prepare a 1:100 dilution of the working standard. (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.

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 working control standard. (1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the control 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 control preparation.

9.5.8 Prepare a 1:100 dilution of the working control standard. (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.

9.5.9 Using a calibrated pipette, spike the positive controls according to the following table, using the prepared dilutions of the working control 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 specimen 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 in LC-MS H_2O to each tube and briefly vortex mix. Centrifuge the tubes for 2 minutes at 2000 rpm (recommended) 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 ±3%, ion ratios ±20%) – Cal 4
- Result comparisons –

  Cal 1: truncated to one decimal place in units of ng/mL (acceptable range ±25%; 7.5 – 12.5 ng/mL)

  Cals 2-6, Ctls 1-2: truncated, whole integer values in units of ng/mL (acceptable range ±20%)

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)

9.9 REFERENCES

- A. Black, in-house method development.
- OCME Toxicology Laboratory, Washington D.C., Quantitation of Zolpidem by LC-MS (2007).

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid in LC-MS grade H_2O
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
Autosampler	
Column temp	30°C
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 9.10.2.2.d 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
9/5/18	Corrected document ID in footer to TCz12709.	All
5/16/20	Edited NOTE in section 9.3.1; moved filtration information to NOTE in prep of 0.1% formic acid (no filtration required for prep with LC-MS grade H_2O). Changed references for "LC-MS grade DI H_2O" to "LC-MS grade H_2O." NOTE regarding specific grade of H_2O and solvents used was removed from 9.5 (covered in 9.3.1). Use of mid-run control added in 9.4.3.3. Changed pipetted volumes in 9.5.4 – 9.5.5 and 9.5.7 – 9.5.8 from mL to µL. Added 9.9, list of references used in method development. Retention time criteria changed from ±2% to ±3% in section 9.6. Other minor edits throughout.	All