CONFIRMATION OF ZOLPIDEM BY LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

9.1 POLICY

This test method may be used to confirm the presence of zolpidem (ZOL), with diazepam-d_5 (DZP-d_5) internal standard, in biological samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

9.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of zolpidem present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compound.

9.3 PRINCIPLE

The targeted compound and internal standard are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of solid-phase extraction (SPE). Following SPE, the specimens, now termed extracts, are injected into a high performance liquid chromatograph (HPLC) where they are separated between a liquid mobile and liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time.

The HPLC is coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion-monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any zolpidem identified in a sample is determined from its calibration curve.

9.4 SPECIMENS

9.4.1 The specimen volume is 0.2 mL.

9.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

9.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; in addition, the specimen may be analyzed at standard volume, as dictated by screening results, to ensure that concentrations of all target compounds present are within the dynamic range of the test method.

9.4.4 Analysis of larger specimen volumes must be approved and documented.

9.5 REAGENTS, MATERIALS AND EQUIPMENT

9.5.1 REAGENTS

9.5.1.1 0.1M sodium acetate buffer (pH4.5)

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. Store the buffer in a glass or plastic bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.

9.5.1.2 Acetic acid (Glacial)

9.5.1.3 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 for up to six months. Adjustments to final volume are permitted as long as proportions are maintained.

9.5.1.4 Acetonitrile (ACN)

9.5.1.5 Ammonium hydroxide (concentrated)

9.5.1.6 Certified blank blood

9.5.1.7 Deionized water (DI H_2O)

9.5.1.8 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. Adjustments to final volume are permitted as long as the proportions of the elution solvent are maintained.

9.5.1.9 Formic acid (concentrated)

9.5.1.10 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the solution in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

9.5.1.11 Isopropanol (IPA)

9.5.1.12 Methanol (MeOH)

9.5.1.13 Methylene chloride (dichloromethane, CH_2Cl_2)

9.5.1.14 Sodium acetate trihydrate

9.5.2 MATERIALS

9.5.2.1 Autosampler vials (polypropylene), inserts and caps

9.5.2.2 Disposable 16 x 100mm tubes with safety closures

9.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

9.5.2.4 Disposable pipette tips

9.5.2.5 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent

9.5.2.6 HPLC column (Agilent Zorbax Eclipse Plus C8 50 mm x 2.1 mm ID, d_p=1.8 μm, or equivalent)

9.5.2.7 Laboratory glassware (graduated cylinders, flasks)

9.5.2.8 Solvent filters (0.45 μm pore size; reduced cellulose, other)

9.5.2.9 Volumetric glassware (flasks)

9.5.3 EQUIPMENT

9.5.3.1 Agilent HPLC (1100/1200 series or equivalent)

9.5.3.2 Agilent MS with API-ES source (6410 or equivalent)

9.5.3.3 Calibrated, adjustable piston pipettes

9.5.3.4 Centrifuge

9.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

9.5.3.6 pH Meter and/or indicating pH paper

9.5.3.7 Solvent filtration apparatus

9.5.3.8 Verified, adjustable repeater-pipettes

9.5.3.9 Vortex mixer

9.5.3.10 Vacuum manifold

9.6 STANDARDS, CALIBRATORS AND CONTROLS

9.6.1 STANDARDS

9.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards (which in turn are used to produce calibrators and positive controls) and the working internal standard.

9.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a. Zolpidem: 1.0 mg/mL
b. Diazepam-d_5: 1.0 mg/mL

9.6.1.3 Working standard (10 ng/μL)

a. Using a calibrated pipette, measure 250 µL of ZOL stock standard into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working standard is 10 ng/µL. The working standard is stored in the freezer in an amber bottle and

expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

9.6.1.4 Working internal standard (1 ng/µL)

a. Using a calibrated pipette, measure 25 µL of DZP-d_5 into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working internal standard is 1 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

9.6.2 CALIBRATORS

9.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 9.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure. If the matrix has not been verified as negative, a matrix blank must be included in the batch.

9.6.3 CONTROLS

9.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

9.6.3.2 Positive Controls

a. At least two positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.

d. The control working standard (10 ng/μL) is prepared as described in 9.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 9.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

9.7 SAMPLE PREPARATION

NOTE: The presence of diazepam in case samples may cause interference with DZP-d_5 internal standard, affecting chromatography and transition ratios. If this occurs, an alternative test method may be used for confirmation/quantitation of zolpidem, with relevant documentation retained in the batch record.

9.7.1 Label a clean 16 x 100mm tube for each member of the test batch. (i.e. Calibrator, control, case sample)

9.7.2 Place 2 mL of 0.1M sodium acetate buffer pH4.5 into each tube.

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

9.7.4 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 acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

9.7.5 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 acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

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

Calibrator Description	Volume (μL) Added	Working Standard
Calibrator 1 (10 ng/mL)	20	0.1 ng/μL
Calibrator 2 (25 ng/mL)	50	0.1 ng/μL
Calibrator 3 (50 ng/mL)	100	0.1 ng/μL
Calibrator 4 (100 ng/mL)	20	1 ng/μL
Calibrator 5 (250 ng/mL)	50	1 ng/μL
Calibrator 6 (500 ng/mL)	100	1 ng/μL

9.7.7 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 acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

9.7.8 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 acetonitrile or methanol in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

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

Control Description	Volume (μL) Added	Control Working Standard
Control 1 (30 ng/mL)	60	0.1 ng/μL
Control 2 (400 ng/mL)	80	1 ng/μL

9.7.10 If in-house positive controls are being used, transfer 0.2 mL of each into their labeled tubes, using a calibrated pipette.

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

9.7.12 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.

9.7.13 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500rpm.

9.7.14 Place new, labeled SPE columns into the vacuum manifold.

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

a. 3 mL methanol
b. 3 mL DI H_2O
c. 2 mL 0.1M acetate buffer (pH4.5)

Do not let columns dry out between each conditioning step.

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

9.7.17 Wash the SPE columns by passing each of the following 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 methanol

9.7.18 Dry the columns for 10 minutes under vacuum.

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

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

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

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

9.7.23 Transfer the extracts to labeled polypropylene autosampler vials and cap.

9.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation or OpenLab to the date of the test. After entering all vial locations and sample descriptions in the sequence table, ensure that the method listing in the table is ZOLPIDEM.M for each line. As needed, the sequence may conclude with an injection that rinses the column and puts the instrument in standby (e.g., using method RINSE.M), or this may be done manually.

9.9 DATA ANALYSIS

9.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation or OpenLab.

9.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

9.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves and data analysis parameters (calibration report).

9.9.4 Technical review of the batch is conducted according to the criteria listed below.

9.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

9.10.1 Calibrators and calibration curves

9.10.1.1 Chromatographic peaks for ZOL and internal standard shall appear symmetrical (i.e., no co-elution, split peaks, or shoulders).

9.10.1.2 Retention times for ZOL and internal standard shall be within ±2%, and ion ratios shall be within ±20%, of those in calibrator 4. These are inclusive ranges.

9.10.1.3 Quantitative results for ZOL in each calibrator shall be within ±20% of the target value with the exception of calibrator 1 which shall be within ±25% of the target. These are inclusive ranges.

For calibrator 1 (target concentration 10 ng/mL), result comparison will use the value truncated after the first decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL).

For target concentrations >10 ng/mL, result comparisons will use the truncated, whole integer values in units of ng/mL.

9.10.1.4 The calibration curve for ZOL shall have a correlation coefficient ≥0.99.

9.10.2 Controls

9.10.2.1 The negative control(s) shall not identify ZOL above its limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

9.10.2.2 Positive controls

a. Chromatographic peaks for ZOL and internal standard shall appear symmetrical.
b. Retention times for ZOL and internal standard shall be within ±2%, and ion ratios shall be within ±20%, of those in calibrator 4. These are inclusive ranges.
c. Quantitative results for ZOL in each control shall be within ±20% of the target value. These are inclusive ranges. Result comparison will use whole integer, truncated results in units of ng/mL.
d. All positive controls in the batch must meet acceptability criteria in order to report quantitative results in a case specimen.

9.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

9.11.1 Chromatographic peaks for ZOL and internal standard shall appear symmetrical.

9.11.2 The retention times for ZOL and internal standard are within ±2%, and the ion ratios are within ±20%, of those in calibrator 4. These are inclusive ranges.

9.11.3 The quantitative results for ZOL must be within the dynamic range of the test method. Results greater than the upper limit of quantitation may be reported qualitatively, provided that all other criteria for acceptance are met.

9.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

9.12 REPORTING

9.12.1 Results are reported in units of milligrams per liter (mg/L).

9.12.2 The whole integer, truncated results are converted from ng/mL to mg/L.

9.12.3 Converted results are truncated to two significant figures for reporting.

a. For example: zolpidem is measured as 206.51 ng/mL.

b. The unit conversion step truncates the result to 206 ng/mL and then represents the result as 0.206 mg/L.

c. The result is truncated to 0.20 mg/L (two significant figures) and reported.

9.12.4 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

9.13 METHOD PERFORMANCE

9.13.1 Limit of detection: 1 ng/mL (0.001 mg/L)

9.13.2 Lower limit of quantification: 10 ng/mL (0.01 mg/L)

9.13.3 Dynamic range: 10 – 500 ng/mL

9.13.4 Upper limit of quantitation: 500 ng/mL (0.50 mg/L)

9.14 TRACEABILITY

9.14.1 Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.50 mL/min
Solvent A	0.1% Formic Acid
Solvent B	Acetonitrile
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	15 sec
Flush-port solvent	Acetonitrile

MASS SPECTROMETER

Ion mode	(+) SIM	Nebulizer gas	Nitrogen
Peakwidth	0.5 min	Nebulizer pressure	30 psi
Dwell time	50 msec	Drying gas	Nitrogen
		Drying gas flow	12 L/min
		Drying gas temp	350° C
		Capillary voltage	4kV

Signals	Ions	Ion Ratios
Zolpidem	236, 263, 308	236/308, 263/308
Diazepam-d5	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