CONFIRMATION OF SELECT BENZODIAZEPINES, QUETIAPINE AND ZOPICLONE BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

13.1 POLICY

This test method may be used to confirm the presence of select benzodiazepines, quetiapine and zopiclone in biological samples. Quantitative results obtained through the use of this method will only be reported within the 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 either the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch file.

13.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and/or quantitation of select benzodiazepines, quetiapine and zopiclone 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 compounds.

13.3 PRINCIPLE

The targeted compounds and internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, 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 tandem mass spectrometer (MS-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 and multiple-reaction monitoring are 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 target compounds identified in a sample is determined from its calibration curve.

13.4 SPECIMENS

13.4.1 The specimen volume is 1 mL.

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

13.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

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

13.5 REAGENTS, MATERIALS AND EQUIPMENT

13.5.1 REAGENTS

13.5.1.1 1% Acetic acid (filter prior to use on the HPLC)

Add 10 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 one year. Adjustments to final volume are permitted as long as the proportions are maintained.

13.5.1.2 Acetic acid (Glacial)

13.5.1.3 Acetonitrile (filter prior to use on the HPLC)

13.5.1.4 Ammonium hydroxide, concentrated

13.5.1.5 ß-glucuronidase lyophilized powder from *Escherichia coli*, *Patella vulgata*, abalone or equivalent

Prepare to a minimum 25,000 Fishman Units/mL (FU/mL) in pH6.8 phosphate buffer, based on activity per gram solid (consult certificate of analysis, as this varies by supplier and lot number).

a. Determine total number of units per container (total weight in g × % protein × FU/g protein)
b. Determine total volume needed to achieve target concentration of ≥25,000 FU/mL.
c. Dispense 2 mL aliquots of prepared solution into labeled (include preparation date and activity) microcentrifuge tubes and store in freezer for up to six months.
d. Example:

Manufacturer bottle lists total solid weight as 405.6 mg with 4400000 FU/g protein. Certificate of analysis indicates that protein is 41% of the total weight.

0.40 g × 0.41 × 4400000 FU/g protein = 721600 FU

Total volume needed to achieve ≥25000 FU/mL:

721600 FU / 25000 FU/mL = 28.86 mL

Alternatively, a commercially prepared aqueous solution of ß-glucuronidase may be used (ex. *Helix pomatia)*. Consult certificate of analysis for solution concentration (FU/mL).

a. Example:

Certificate of analysis lists solution activity of ≥80000 FU/mL, equivalent to 80 FU/µL. For urine sample preparation, total volume added to each tube is 2 mL (1 mL urine, 1 mL phosphate buffer pH6.8). For target concentration of 3000 – 4000 FU/mL (or 6000 – 8000 FU/2 mL):

6000 FU / 80 FU/µL = 75 µL added to each tube
8000 FU / 80 FU/µL = 100 µL added to each tube

Add 75 – 100 µL of the aqueous solution to each tube for final activity of 3000 – 4000 FU/mL

13.5.1.6 Boric acid (H_3BO_3)

13.5.1.7 Borate buffer pH9

Add 630 mL solution A and 370 mL solution B in a 1 L beaker. Mix thoroughly. Check the pH and, if necessary, adjust to 9.0 ±0.5 with additional solution B or strong base (e.g. NH_4OH). Store the solution in a glass or plastic bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

Solution A: In a 1 L flask, dissolve 61.8 g H_3BO_3 and 74.6 g KCl in DI H_2O. Dilute to with DI H_2O and mix until dissolved (may require low heating).

Solution B: In a 500 mL flask, dissolve 53 g Na_2CO_3 in DI H_2O. Dilute to 500 mL with DI H_2O and mix until dissolved (may require low heating).

13.5.1.8 Certified blank blood

13.5.1.9 Deionized water (DI H_2O)

13.5.1.10 Ethyl acetate

13.5.1.11 Methanol

13.5.1.12 Monobasic potassium phosphate (KH_2PO_4)

13.5.1.13 0.1M Potassium phosphate buffer pH6.8

In a 250 mL flask, dissolve 3.4 g KH_2PO_4 in approximately 100 mL DI H_2O. Dilute to 250 mL with DI H_2O and mix thoroughly. Check the pH and, if necessary, adjust to 6.8 ±0.5 with 10N KOH. Store the solution in refrigerator in a glass bottle for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

13.5.1.14 Potassium chloride (KCl)

13.5.1.15 Potassium hydroxide (KOH, concentrated)

13.5.1.16 Sodium carbonate (Na_2CO_3)

13.5.2 MATERIALS

13.5.2.1 Autosampler vials and caps

13.5.2.2 Disposable 16 x 125mm tubes with closures

13.5.2.3 Disposable centrifuge or screw-top tubes with closures

13.5.2.4 Disposable transfer pipettes (glass or polypropylene)

13.5.2.5 Disposable pipette tips

13.5.2.6 HPLC column (Agilent Zorbax Eclipse Plus XDB-C18 150 mm x 4.6 mm ID, d_p=5.0 μm, or equivalent)

13.5.2.7 Laboratory glassware (graduated cylinders, flasks)

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

13.5.2.9 Volumetric glassware (flasks)

13.5.3 EQUIPMENT

13.5.3.1 Agilent HPLC (1100/1200 series or equivalent)

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

13.5.3.3 Calibrated, adjustable air-displacement pipettes

13.5.3.4 Centrifuge

13.5.3.5 Evaporator (Caliper LS, formerly Zymark TurboVap)

13.5.3.6 pH Meter and/or indicating pH paper

13.5.3.7 Rotary mixer

13.5.3.8 Solvent filtration apparatus

13.5.3.9 Vortex mixer

13.6 STANDARDS, CALIBRATORS AND CONTROLS

13.6.1 STANDARDS

13.6.1.1 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, positive controls and the working internal standard.

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

a.	7-aminoclonazepam:	1.0 mg/mL
b.	7-aminoflunitrazepam:	1.0 mg/mL
c.	α-hydroxyalprazolam:	1.0 mg/mL
d.	Alprazolam:	1.0 mg/mL
e.	Chlordiazepoxide:	1.0 mg/mL
f.	Clonazepam:	1.0 mg/mL
g.	Desalkylflurazepam	1.0 mg/mL
h.	Diazepam:	1.0 mg/mL
i.	Flunitrazepam:	1.0 mg/mL
j.	Flurazepam:	1.0 mg/mL
k.	Lorazepam:	1.0 mg/mL
l.	Lorazepam glucuronide:	0.1 mg/mL
m.	Midazolam:	1.0 mg/mL
n.	Nordiazepam:	1.0 mg/mL
o.	Oxazepam:	1.0 mg/mL

p. Oxazepam glucuronide: 0.1 mg/mL
q. Oxazepam-d_5: 1.0 mg/mL
r. Quetiapine: 1.0 mg/mL
s. Temazepam: 1.0 mg/mL
t. Temazepam-d_5: 1.0 mg/mL
u. Triazolam: 1.0 mg/mL
v. Zopiclone: 1.0 mg/mL

13.6.1.3 Working standard (10 ng/µL)

a. Using a calibrated pipette, measure 1 mL of each target compound stock standard (2 mL for quetiapine) into a 100 mL class-A volumetric flask.
b. Add acetonitrile or methanol to the flask to the designated volume.
c. The final concentration of the working standard is 10 ng/µL (20 ng/µl quetiapine). The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

13.6.1.4 Working internal standard (1 ng/µL)

a. Using a calibrated pipette, measure 100 µL each of oxazepam-d_5 and temazepam-d_5 stock internal standards into a 100 mL class-A volumetric flask. Add acetonitrile or methanol to the flask to the designated volume.
b. 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. Volumes may be adjusted provided that proportions remain constant.

13.6.2 CALIBRATORS

13.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 13.7 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.

13.6.3 CONTROLS

13.6.3.1 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.)

13.6.3.2 Positive Controls

Blood, Serum, Plasma, Tissue Homogenate

a. At least one positive whole blood control is tested with every batch. If the batch is in excess of twenty (20) case samples, an additional positive control is included for every 20-case increment.
b. Control stock standards used to prepare controls 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. Alternatively, the same manufacturer lot number may be used provided that the controls are prepared by an analyst who did not prepare the working standard.
d. Preparation of the spiked positive whole blood control is detailed in 13.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
e. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

Urine

a. At least two positive urine controls are tested with every batch.
b. A free benzodiazepine control is spiked with the working standard solution at time of extraction. A glucuronide control is also prepared at time of extraction to verify successful hydrolysis of any glucuronides present in the batch.
c. A glucuronide working control solution is prepared to spike the positive urine glucuronide control.
    i. Using a calibrated pipette, measure 1 mL each of the lorazepam glucuronide and oxazepam glucuronide stock standards into a 10 mL class-A volumetric flask.
    ii. Add acetonitrile or methanol to the flask to the designated volume.
    iii. The final concentration of the glucuronide working control solution is 10 ng/µL. The solution is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

13.7.1 SAMPLE PREPARATION (Blood, Serum, Plasma, Tissue Homogenate)

13.7.1.1 Label a clean 16 x 125mm tube for each member of the test batch. (i.e. calibrator, control, case sample)

13.7.1.2 Place 1 mL of borate buffer pH9 into each tube.

13.7.1.3 Add 1 mL of certified blank whole blood into each of the seven calibrator tubes and the negative control tube(s).

13.7.1.4 Prepare a 1:10 dilution of the working standard. (1.0 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.

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

a. Using a calibrated pipette, combine 0.1 mL of the 1.0 ng/µL 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.

13.7.1.6 Using the working standard and the prepared dilutions, spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Working Standard
Calibrator 1 (10 ng/mL)	100	0.1 ng/µL
Calibrator 2 (25 ng/mL)	25	1.0 ng/µL
Calibrator 3 (50 ng/mL)	50	1.0 ng/µL
Calibrator 4 (125 ng/mL)	125	1.0 ng/µL
Calibrator 5 (250 ng/mL)	25	10 ng/µL
Calibrator 6 (500 ng/mL)	50	10 ng/µL
Calibrator 7 (1000 ng/mL)	100	10 ng/µL

13.7.1.7 Add 100 µL of the working control standard to the positive control tube.

13.7.1.8 Sample 1 mL of each case sample into its respective tube.

13.7.1.9 Add 100 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 100 ng/mL.

13.7.1.10 Vortex tubes until homogenous.

13.7.1.11 Add 6 mL ethyl acetate to each tube.

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

13.7.1.13 Centrifuge the tubes for 5 minutes at 2000-2500 rpm to achieve separation.

13.7.1.14 Transfer the ethyl acetate layer to clean, labeled 10 mL centrifuge tube.

13.7.1.15 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

13.7.1.16 Immediately after evaporation, reconstitute samples in 500 µL methanol and vortex briefly.

13.7.1.17 Transfer the extracts to labeled autosampler vials and cap.

13.7.2 URINE SAMPLE EXTRACTION

13.7.2.1 Label a clean 16 x 125mm tube for each member of the test batch. (i.e. control, case sample)

13.7.2.2 Add 1 mL negative urine to each of the control tubes.

13.7.2.3 Add 50 µL working standard solution to the free benzodiazepine (unconjugated) control tube.

13.7.2.4 Add 20 µL glucuronide working control solution to the glucuronide control tube.

13.7.2.5 Add 1 mL of each case sample to its respective tube.

13.7.2.6 Add 300 µL prepared ß-glucuronidase solution to each tube.

Note: If commercially available aqueous solution of ß-glucuronidase is used, adjust added volume to achieve approximately 3000 - 4000 FU of activity per mL buffered urine.

13.7.2.7 Add 1 mL phosphate buffer pH6.8 to each tube.

13.7.2.8 Add 100 µL working internal standard solution to each tube. Final concentration is 100 ng/mL.

13.7.2.9 Cap tubes and vortex briefly.

13.7.2.10 Incubate tubes for at least two hours at 60°C.

13.7.2.11 Following hydrolysis, add 1 mL borate buffer pH9 to each tube and vortex briefly.

13.7.2.12 Add 6 mL ethyl acetate to each tube.

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

13.7.2.14 Centrifuge the tubes for 5 minutes at 2000 rpm to achieve separation.

13.7.2.15 Transfer the ethyl acetate layer to clean, labeled 10 mL centrifuge tube.

13.7.2.16 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

13.7.2.17 Immediately after evaporation, reconstitute samples in 500 µL methanol and vortex briefly.

13.7.2.18 Transfer the extracts to labeled autosampler vials and cap.

13.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a batch worklist and set the data path in MassHunter to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the worklist, ensure that the method listing in the table is BENZO.M for each line. As needed, the sequence may conclude with an injection that rinses the column (e.g. using method RINSE.M), or this may be done manually.

13.9 DATA ANALYSIS

13.9.1 Analysis of the batch data is conducted using the MassHunter Quantitative instrumental data analysis software.

13.9.2 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.

13.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.

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

13.10 CRITERIA FOR BATCH ACCEPTANCE

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

13.10.1 Calibrators and calibration curves

13.10.1.1 Chromatographic peaks for all target compounds and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

13.10.1.2 Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4 for all target compounds. These are inclusive ranges.

13.10.1.3 Quantitative results for all target compounds in each calibrator shall be within ±20% of their target values with the exception of calibrator 1 which shall be within ±25% of their targets. These are inclusive ranges. Result comparisons will use whole integer, truncated results in units of ng/mL.

13.10.1.4 The calibration curves for all target compounds shall have a correlation coefficient ≥0.99.

13.10.2 Controls

13.10.2.1 The negative control(s) shall not identify any target compound above its limit of quantitation. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios. Negative urine control(s) shall not identify any target compound above its limit of quantitation, based on above criteria.

13.10.2.2 Positive controls

a. Chromatographic peaks for all target compounds and internal standards shall appear symmetrical.
b. Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4 for all target compounds in the positive control. These are inclusive ranges.
c. Quantitative results for all target compounds in each control shall be within ±20% of their target values. These are inclusive ranges. Result comparison will use whole integer, truncated results in units of ng/mL.
d. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
e. For positive urine control(s): For a urine result to be reported, the free benzodiazepine (unconjugated) control must also identify that compound. The glucuronide conjugate control is used to evaluate the effectiveness of the enzymatic hydrolysis.

13.11 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.

13.11.1 Any chromatographic peak for target compounds shall appear symmetrical.

13.11.2 The retention times for target compounds are ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

13.11.3 The quantitative results for target compounds must be within the dynamic range of the test method.

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

13.11.5 Urine samples are suitable for qualitative reporting if criteria in 13.11.1 are met and retention times for identified compounds are ±2% and the ion ratios are within ±20% of those in the positive urine control.

13.12 REPORTING

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

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

13.12.3 Converted results are truncated to no more than two significant figures for reporting.

a. Example 1: diazepam is measured as 258.6 ng/mL.

b. The unit conversion step truncates the result to 258 ng/mL and then represents the result as 0.258 mg/L.

c. The result is truncated to 0.26 mg/L (two significant figures) and reported.

d. Example 2: alprazolam is measured as 21.9 ng/mL.

e. The unit conversion step truncates the result to 21 ng/mL and then represents the result as 0.021 mg/L.

f. The result is truncated to 0.02 mg/L (one significant figure) and reported.

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

13.13 METHOD PERFORMANCE

13.13.1	Lower limit of quantification:	10 ng/mL (0.01 mg/L) 20 ng/mL (0.02 mg/L) quetiapine
13.13.2	Dynamic range:	10 - 1000 ng/mL 20 – 2000 ng/mL quetiapine
13.13.3	Upper limit of quantification:	1000 ng/mL (1.0 mg/L) 2000 ng/mL (2.0 mg/L) quetiapine

13.14 TRACEABILITY

13.14.1 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	1.00 mL/min
Solvent A	1% Acetic Acid
Solvent B	Acetonitrile
Initial Composition	80% (A), 20% (B)
0 - 6.00 min	40% B
6.00 – 16.00 min	60% B
16.00 – 17.00 min	90% B
17.00 – 20.00 min	20%B
Post Time	5.00 min
Column Temp	40° C
Autosampler	
Injection Volume	5.0 µL
Injection flush-port	Active
Flush-port time	10 sec
Flush-port solvent	75:25 Methanol:DI H_2O

MASS SPECTROMETER

Ion mode	(+) MRM	Nebulizer gas	Nitrogen
Peakwidth	0.07 min	Nebulizer pressure	50 psi
Resolution	Unit	Drying gas	Nitrogen
Time segment 1 (0 min)	To waste	Drying gas flow	12 L/min
Time segment 2 (2.5 min)	To MS	Drying gas temp	350° C
Time segment 3 (5.8 min)	To MS	Capillary voltage	4kV
Time segment 4 (8.0 min)	To MS		
Time segment 5 (12.5 min)	To MS		
Time segment 6 (15 min)	To waste		

Signals	Time Segment	MRM Transitions
7-aminoclonazepam	2	286 → 222, 121
7-aminoflunitrazepam	2	284 → 256, 226
α-hydroxyalprazolam	4	325 → 297, 216
Alprazolam	4	309 →281, 274
Chlordiazepoxide	2	300 → 283, 227
Clonazepam	4	316 → 270, 214
Desalkylflurazepam	4	289 →261, 226
Diazepam	5	285 →257, 222
Flunitrazepam	4	314 →268, 239
Flurazepam	3	388 → 315, 288
Lorazepam	4	321 → 275, 229
Midazolam	3	326 → 291, 249
Nordiazepam	4	271 → 165, 140
Oxazepam	4	287 → 269, 241
Oxazepam-d_5	4	292 → 246
Quetiapine	3	384.1 → 253.2, 221.1
Temazepam	4	301 → 255, 177
Temazepam-d_5	4	306 → 260
Triazolam	4	343 → 308, 239
Zopiclone	2	389.1 → 245.1, 217.1

LIST OF CHANGES

Revision Date	Description	Page Number
02/25/13	Method approved by Washington State Toxicologist. See DRA dated 02/20/13. Method released for use in evidentiary testing on 02/25/13.	All
07/01/13	Changed working standard concentration to 10 ng/µL (20 ng/µL quetiapine). Incorporated the use of a spiked positive control in lieu of a pooled whole blood control. Modified sample preparation to reflect changes.	5 – 7