From: Black, Amanda (WSP)
To: Capron, Brian (WSP); Chandler, Amanda (WSP); Couper, Fiona (WSP); Daniel, Kelly (WSP); Flaherty, Rebecca (WSP); Gingras, Andrew (WSP); Harris, Katie (WSP); Johnston, Christopher S. (WSP); Knoy, Justin (WSP); Knoy, Lyndsey (WSP); Louis, Asa (WSP); Mitchell-Mata, Christie (WSP); Nguyen, David (WSP); Nuwayhid, Naziha (WSP); O"Neill, Kari (WSP); O"Reilly, Brianne (WSP); Peterson, Brianna (WSP); Sachs, Aaron (WSP); Sklerov, Dawn (WSP); Thomas, Brittany (WSP); Valencia, Darlene (WSP); Wehner, Elizabeth (WSP)
Subject: Etizolam and Estazolam
Date: Wednesday, May 16, 2018 7:50:00 AM

Good morning,

Following up my discussion in yesterday's laboratory meeting, below is the information on qualitative testing for etizolam and estazolam using the benzodiazepine LC-MSMS test method:

When extracting benzos, include one positive control prepared from the etizolam/estazolam working standard. Target concentration is 100 ng/mL (add 10 µL of the etizolam/estazolam working standard to the positive control tube).

The benzodiazepine method now includes transitions for etizolam and estazolam. The everyday benzodiazepine data analysis method does not include these compounds. When no case samples in the batch appear to have etizolam (indicated by peaks appearing in the trizolam window not identified as triazolam), and estazolam has not been previously identified in any cases (estazolam will not show up as non-identified peaks in a window), the etizolam/estazolam control is processed as a sample with the rest of the batch, using the regular benzo DA method (it should only identify internal standards). No samples need to be processed using the etizolam/estazolam specific DA method.

If a case (or cases) has etizolam (more likely than estazolam), the negative control, case(s), positive etizolam/estazolam control and matrix blank only are processed with the ETIZ DA method. The DA method is set up to create a two point calibration curve, with the 100 ng/mL control point (designated as L1) and the origin. This will provide an estimated concentration for the case(s), which may be helpful if a decision needs to be made on whether to send the case out for quantitation.

Etizolam RT – 10.6
Estazolam RT – 8.8

Thanks to Justin for running test batches and the first set of cases. The development data (identification of RT, transitions and parameters), specificity data (verifying selectivity for these compounds when included with all the other benzos in the method), test runs and Justin's case batch were reviewed by Fiona. This data will be filed with the benzo validation/verification folder.

A copy of this email will be filed with the current benzodiazepine SOP, pending the next revision.

Amanda M. Black
Quality Assurance Manager
Washington State Patrol
Toxicology Laboratory Division
2203 Airport Way South, Suite 360

Seattle, WA 98134
206-262-6100

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 a member of TLD Management, 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; 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.

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

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 4,400000 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 piston 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 (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.

13.6.1.4 Working internal standard (1 ng/µL)

a. Using a calibrated pipette, measure 250 µL each of oxazepam-d_5 and temazepam-d_5 stock internal standards into a 250 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 (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.

13.6.1.5 Control working standard

a. Using a calibrated pipette, measure 25 µL each of midazolam and lorazepam stock standards, 100 µL of the nordiazepam stock standard, 200 µL each of chlordiazepoxide, quetiapine and diazepam stock standards and 50 µL each of all other target compound stock standards into a 50 mL class-A volumetric flask. Add acetonitrile or methanol to the flask to the designated volume. NOTE: 7-aminoclonazepam, 7-aminoflunitrazepam and zopiclone are reported qualitatively from this assay and are not included in the positive control.
b. The final concentration of the control working standard is 0.5 ng/µl (midazolam, lorazepam), 2 ng/µL (nordiazepam), 4 ng/µL (chlordiazepoxide, quetiapine, diazepam) and 1 ng/µL for all other target compounds. The control 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.

13.6.4.6 Glucuronide urine control working standard

a. 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. Add acetonitrile or methanol to the flask to the designated volume.
b. 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 (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.

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 Controls

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. The total number of controls must make up at least 10% of the batch (based on number of case specimen samples), including one negative.
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. If the same lot must be used, the control working standard must be prepared by someone other than the person that prepared the working standard.

d. The control working standard is prepared as described in 13.6.1.5.
e. 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.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

Urine

a. Two urine calibrators are included in the batch, and a glucuronide (process) control is prepared at time of extraction to verify successful hydrolysis of any glucuronides present in the batch.

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

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 Using a calibrated pipette, add 100 µL of the working control standard to the positive control tube(s).

13.7.1.8 Using a calibrated pipette, sample 1 mL of each case sample into its respective tube.

13.7.1.9 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 100 ng/mL.

13.7.1.10 Vortex tubes until homogeneous.

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 glass 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 Using a calibrated pipette, add 1 mL negative urine to each of the control tubes.

13.7.2.3 Prepare urine calibrators at calibrator 1 and calibrator 4 concentrations, as described in 13.7.1.6.

13.7.2.4 Using a calibrated pipette, add 20 µL glucuronide working control solution to the glucuronide (process) control tube.

13.7.2.5 Using a calibrated pipette, add 1 mL of each case sample to its respective tube.

13.7.2.6 Using a calibrated pipette, 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 Using a calibrated pipette or verified repeater-pipette, 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 glass 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.

NOTE: For urine batches, case specimens are considered bracketed when analyzed between the urine calibrators and the glucuronide process control.

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/x (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

For urine data analysis, a two-point calibration curve is generated, with equal weighting, origin excluded.

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% for internal standards and target compounds 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.

For calibrator 1 (target concentration 10 ng/mL), result comparisons will use whole integer values 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 (including quetiapine in calibrator 1), result comparisons will use whole integer values in units of ng/mL.

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

13.10.1.5 For urine, calibrators must meet criteria in 13.10.1.1 - 13.10.1.2. All members of the urine testing batch are analyzed against the urine calibration curve.

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% for internal standards and target compounds 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. All positive controls in the batch must meet acceptability criteria for a target compound in order to report quantitative results for that compound in a case specimen.
f. The glucuronide conjugate (process) control is used to evaluate the effectiveness of the enzymatic hydrolysis, and the results shall be at or above 60 ng/mL. Criteria in 13.10.2.2.a and 13.10.2.2.b must be met when compared to urine calibrator 4.

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 with a calculated concentration is ≥ 10 ng/mL (20 ng/mL quetiapine) are suitable for qualitative reporting if criteria in 13.11.1 and 13.11.2 are met, when compared to urine calibrator 4, and the glucuronide process control meets criteria described in 13.10.2.2.e.

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 two significant figures for reporting.

a. Example: diazepam is measured at 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 0.25 mg/L (two significant figures) and reported.

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

13.12.5 7- aminoclonazepam, 7-aminoflunitrazepam and zopiclone are reported qualitatively from this test method for all specimen types.

13.12.6 Urine results for all target compounds are reported qualitatively.

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	EMV	+400V
Peakwidth	0.07 min	Nebulizer pressure	50 psi
Resolution	Unit	Nebulizer pressure	50 psi
Time segment 1 (0 min)	To waste	Drying gas	Nitrogen
Time segment 2 (2.5 min)	To MS	Drying gas flow	12 L/min
Time segment 3 (5.8 min)	To MS	Drying gas temp	350° C
Time segment 4 (8.0 min)	To MS	Capillary voltage	4kV
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
10/7/15	Changed policy in 13.1 to reflect that deviations may be approved by any member of TLD Management. Changed wording in 13.6.3.2.a to describe that 10% of the batch (number of specimens) must consist of control samples (including one negative). Added wording to 13.10.2.2.e to indicate that urine hydrolysis is determined effective if the compounds in the urine glucuronide control are at or above the LLOQ.	1, 6, 9
4/17/17	Changed wording in 13.4.3 to allow scientist discretion when running samples that require dilution. Added clarification to 13.6.3.2.c for use of same CRM in preparation of working standard and working control standard and note regarding CRM expiration dates in 13.6.1.3 and 13.6.1.4. Added preparation of control working standard and glucuronide urine working control standard in 13.6.1.5 and 13.6.1.6. Changed 13.7.2.3 for preparation of two urine calibrators in place of one positive urine control. Added additional criteria in 13.10.1 and 13.10.2 for calibrator/control acceptability and noted in 13.10.2.2.e that all controls must pass for a target compound to report quantitative results. Added wording to 13.11.5 to indicate urine concentrations must be ≥ 10 ng/mL (20 ng/mL quetiapine) for qualitative reporting. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 13.7.	1, 5-13