

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

### 13.1 METHOD

This test method may be used to confirm the presence of select benzodiazepines, quetiapine and zopiclone in biological matrices. Target compounds and internal standards oxazepam-d<sub>5</sub> and temazepam-d<sub>5</sub> are isolated from biological specimens by liquid-liquid extraction (LLE). 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.

### 13.2 SPECIMENS

The specimen volume is 1 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.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in serum/plasma specimens (see 13.4.3.4). Matrix-matching of the full calibration curve and all positive control levels is required for quantitation in liver (tissue) homogenate specimens (see 13.4.2 and 13.4.3).

NOTE: Quetiapine is quantitated using a specimen volume of 0.5 mL using quetiapine-specific sample preparation, as described in 13.7. Qualitative results for quetiapine may be reported from testing performed as described in 13.5 and 13.6 (all benzodiazepine compounds, quetiapine and zopiclone).

### 13.3 REAGENTS, MATERIALS AND EQUIPMENT

#### 13.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H<sub>2</sub>O) is specified, laboratory general-use DI H<sub>2</sub>O is used in reagent preparation. Organic solvents are reagent grade, unless otherwise specified.

- 1% Acetic acid  
Add 10 mL glacial acetic acid to 800 mL LC-MS grade DI H<sub>2</sub>O. Dilute to 1 L with LC-MS grade DI H<sub>2</sub>O and mix. Store the solution in a glass bottle at room temperature for up to one year.
- Acetic acid (glacial)
- Acetonitrile (ACN), reagent and LC-MS grade
- Ammonium hydroxide, concentrated
- $\beta$ -glucuronidase solution, commercially prepared (e.g., *Helix pomatia*). Consult certificate of analysis for solution concentration (FU/mL).

Example:

Certificate of analysis lists solution activity of  $\geq 80000$  FU/mL, equivalent to 80 FU/ $\mu$ L. 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):

$6000 \text{ FU} / 80 \text{ FU}/\mu\text{L} = 75 \mu\text{L}$  added to each tube

$8000 \text{ FU} / 80 \text{ FU}/\mu\text{L} = 100 \mu\text{L}$  added to each tube

Add 75 – 100  $\mu$ L of the aqueous solution to each tube for final activity of 3000 – 4000 FU/mL

- Boric acid ( $\text{H}_3\text{BO}_3$ )
- 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  $9.0 \pm 0.5$  with additional solution B or strong base (e.g.,  $\text{NH}_4\text{OH}$ ). Store the solution in a glass or plastic bottle at room temperature for up to one year.

Solution A: In a 1 L flask, dissolve 61.8 g  $\text{H}_3\text{BO}_3$  and 74.6 g KCl in DI  $\text{H}_2\text{O}$ . Dilute to with DI  $\text{H}_2\text{O}$  and mix until dissolved (may require low heating).

Solution B: In a 500 mL flask, dissolve 53 g  $\text{Na}_2\text{CO}_3$  in DI  $\text{H}_2\text{O}$ . Dilute to 500 mL with DI  $\text{H}_2\text{O}$  and mix until dissolved (may require low heating).

- Certified blank blood and/or other biological matrices
- DI  $\text{H}_2\text{O}$ , laboratory general-use and LC-MS grade (or equivalent from a high purity filtration system)
- Ethyl acetate (EtAC)
- Methanol (MeOH)
- Monobasic potassium phosphate ( $\text{KH}_2\text{PO}_4$ )
- 0.1M Potassium phosphate buffer pH6.8

In a 250 mL flask, dissolve 3.4 g  $\text{KH}_2\text{PO}_4$  in approximately 100 mL DI  $\text{H}_2\text{O}$ . Dilute to 250 mL with DI  $\text{H}_2\text{O}$  and mix thoroughly. Check the pH and, if necessary, adjust to  $6.8 \pm 0.5$  with 10N KOH. Store the solution in refrigerator in a glass bottle for up to one year.

- Potassium chloride (KCl)
- Potassium hydroxide (KOH, concentrated)
- Sodium carbonate ( $\text{Na}_2\text{CO}_3$ )

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

### 13.3.2 MATERIALS

- Disposable extraction tubes (16 x 125mm recommended) and screw-cap or centrifuge tubes with closures
- Disposable transfer pipettes (polypropylene or glass)
- Glass autosampler vials with caps
- HPLC column (Agilent Zorbax Eclipse Plus XDB-C18 150 mm x 4.6 mm ID,  $d_p=5.0\ \mu\text{m}$ , or equivalent)
- Laboratory glassware (graduated cylinders, flasks)
- Solvent filters (0.45  $\mu\text{m}$  pore size; reduced cellulose, other)

### 13.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Agilent MS-MS with API-ES source (6410/6420 or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heated stir plate, pH meter/paper, rotary mixer, solvent filtration apparatus, vortex mixer)

## 13.4 STANDARDS, CALIBRATORS AND CONTROLS

### 13.4.1 STANDARDS

All compounds

- Working standard: 10 ng/ $\mu\text{L}$  (20 ng/ $\mu\text{L}$  quetiapine)
- Working control standard: 0.5, 1, 2, 4 ng/ $\mu\text{L}$
- Working internal standard: 1 ng/ $\mu\text{L}$
- Etizolam/estazolam working standard: 0.1 ng/ $\mu\text{L}$

Quetiapine only

- Working standard: 10 ng/ $\mu\text{L}$
- Working control standard: 10 ng/ $\mu\text{L}$
- Working internal standard (high concentration): 10 ng/ $\mu\text{L}$

### 13.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 13.5 SAMPLE PREPARATION (13.7 for quetiapine quantitation). For urine analysis, preparation is described in 13.6. Quantitation in liver (tissue) homogenate specimens requires that a calibration curve be prepared in blank matrix. If testing only liver (tissue) homogenate specimens, a whole blood calibration curve is not required.

The etizolam/estazolam positive control is used to establish a two-point calibration (including origin), for qualitative identification.

### 13.4.3 CONTROLS

- 13.4.3.1 At least one negative whole blood control and two positive whole blood controls are included in the batch, prepared as described in 13.5 (three positive whole blood controls are used for quetiapine quantitation – see 13.7). For quantitative analysis of liver homogenate specimens only, whole blood controls are not required.
- 13.4.3.2 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.
- NOTE: The etizolam/estazolam positive control does not apply toward the 10% requirement, as it is only analyzed as a positive control where specimens in the batch are positive for the compound(s).
- 13.4.3.3 For qualitative analysis of any alternate matrices, one negative control and one positive control must be included for each alternate matrix type tested in the batch (see 13.4.3.6 for urine analysis).
- 13.4.3.4 For quantitative analysis of serum/plasma specimens, matrix-matching of the full calibration curve and positive controls is not required. One negative control and one positive control must be included in the batch. Positive controls in both whole blood and/or serum serve to bracket serum/plasma case specimens.
- 13.4.3.5 For quantitative analysis of liver (tissue) homogenate specimens, matrix-matching of the full calibration curve and positive controls (to meet 10% and bracket specimens in that matrix) is required.
- 13.4.3.6 For urine analysis, calibrators 1 and 4 and the glucuronide process control (included to verify successful hydrolysis of glucuronides) serve as positive controls for that matrix.

### 13.5 SAMPLE PREPARATION (BLOOD, SERUM, PLASMA, TISSUE HOMOGENATE)

NOTE: Organic solvents used in sample preparation are reagent grade.

- 13.5.1 Label a clean extraction tube for each member of the test batch (i.e., calibrator, control, case sample).
- 13.5.2 Add 1 mL pH9 borate buffer into each tube.
- 13.5.3 Using a calibrated pipette, add 1 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).
- 13.5.4 Prepare a 1:10 dilution of the working standard. (1 ng/μL)
- Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.
  - Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

- 13.5.5 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.
- 13.5.6 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	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 10/20 ng/mL	100	0.1 ng/μL	1:100
Calibrator 2 – 25/50 ng/mL	25	1 ng/μL	1:10
Calibrator 3 – 50/100 ng/mL	50	1 ng/μL	1:10
Calibrator 4 – 125/250 ng/mL	125	1 ng/μL	1:10
Calibrator 5 – 250/500 ng/mL	25	10 ng/μL	WS
Calibrator 6 – 500/1000 ng/mL	50	10 ng/μL	WS
Calibrator 7 – 1000/2000 ng/mL	100	10 ng/μL	WS

NOTE: Quetiapine standard/standard dilution concentrations are 20 ng/μL (WS), 2 ng/μL (1:10) and 0.2 ng/μL (1:100).

- 13.5.7 Using a calibrated pipette, add 100 μL of the working control standard to the positive control tube(s). Target positive control concentrations are found in APPENDIX A.
- 13.5.8 Using a calibrated pipette, add 100 μL of the etizolam/estazolam working standard to the etizolam/estazolam positive control tube. Target positive control concentration is 10 ng/mL.
- 13.5.9 Using a calibrated pipette, sample 1 mL of each case specimen into its respective tube.
- 13.5.10 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.
- 13.5.11 Vortex mix tubes until homogenous.
- 13.5.12 Add 6 mL ethyl acetate to each tube.
- 13.5.13 Cap the tubes and place on a rotary mixer for at least 5 minutes.
- 13.5.14 Centrifuge the tubes for 5 minutes at 2500 rpm (recommended for 16 x 125 mm tubes).

- 13.5.15 Transfer the ethyl acetate layer to a clean, labeled centrifuge or screw-cap tube.
- 13.5.16 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.
- 13.5.17 Immediately after evaporation, reconstitute the extracts with the addition of 500 µL MeOH to each tube and briefly vortex mix. As needed, centrifuge the tubes for 2 minutes at 2000 rpm to obtain a clear extract.
- 13.5.18 Transfer the extracts to labeled glass autosampler vials and cap.

### 13.6 SAMPLE PREPARATION (URINE)

NOTE: Organic solvents used in sample preparation are reagent grade.

- 13.6.1 Label a clean extraction tube for each member of the test batch (i.e. calibrator, control, case sample).
- 13.6.2 Using a calibrated pipette, add 1 mL negative urine to each of the calibrator and control tubes.
- 13.6.3 Prepare urine calibrators at calibrator 1 and calibrator 4 concentrations, as described in 13.5.6.
- 13.6.4 Using a calibrated pipette, add 100 µL of the etizolam/estazolam working standard to the etizolam/estazolam positive control tube. Target positive control concentration is 10 ng/mL.
- 13.6.5 Using a calibrated pipette, add 20 µL glucuronide working control solution to the glucuronide (process) control tube.
- 13.6.6 Using a calibrated pipette, add 1 mL of each case sample to its respective tube.
- 13.6.7 Using a calibrated pipette, add the appropriate volume of β-glucuronidase solution to achieve approximately 3000 - 4000 FU of activity per mL buffered urine to each tube (see example in 13.3.1).
- 13.6.8 Add 1 mL phosphate buffer pH6.8 to each tube.
- 13.6.9 Using a calibrated pipette or verified repeater-pipette, add 100 µL working internal standard solution to each tube. Final concentration is 100 ng/mL.
- 13.6.10 Cap tubes and vortex briefly.
- 13.6.11 Incubate tubes for at least two hours at 60°C.
- 13.6.12 Following hydrolysis, add 1 mL borate buffer pH9 to each tube and vortex briefly.

- 13.6.13 Add 6 mL ethyl acetate to each tube.
- 13.6.14 Cap the tubes and place on a rotary mixer for at least 5 minutes.
- 13.6.15 Centrifuge the tubes for 5 minutes at 2500 rpm (recommended for 16 x 125 mm tubes) to achieve separation.
- 13.6.16 Transfer the ethyl acetate layer to a clean, labeled 10 mL centrifuge or screw-cap tube.
- 13.6.17 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.
- 13.6.18 Immediately after evaporation, reconstitute samples in 500 µL methanol and vortex briefly. As needed, centrifuge the tubes for 2 minutes at 2000 rpm to obtain a clear extract.
- 13.6.19 Transfer the extracts to labeled glass autosampler vials and cap.

### **13.7 QUETIAPINE QUANTITATION - SAMPLE PREPARATION (BLOOD, SERUM, PLASMA, TISSUE HOMOGENATE)**

NOTE: Organic solvents used in sample preparation are reagent grade.

- 13.7.1 Label a clean extraction tube for each member of the test batch (i.e., calibrator, control, case sample).
- 13.7.2 Add 1 mL pH9 borate buffer into each tube.
- 13.7.3 Using a calibrated pipette, add 0.5 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).
- 13.7.4 Prepare a 1:10 dilution of the quetiapine working standard. (1 ng/µL)
  - c. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.
  - d. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 13.7.5 Prepare a 1:100 dilution of the quetiapine working standard. (0.1 ng/µL)
  - c. 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.
  - d. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 13.7.6 Using a calibrated pipette, spike the calibrators according to the following table, using the quetiapine working standard and the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 20 ng/mL	100	0.1 ng/µL	1:100
Calibrator 2 – 50 ng/mL	25	1 ng/µL	1:10
Calibrator 3 – 100 ng/mL	50	1 ng/µL	1:10
Calibrator 4 – 250 ng/mL	125	1 ng/µL	1:10
Calibrator 5 – 500 ng/mL	25	10 ng/µL	WS
Calibrator 6 – 1000 ng/mL	50	10 ng/µL	WS
Calibrator 7 – 1500 ng/mL	75	10 ng/µL	WS
Calibrator 8 – 2000 ng/mL	100	10 ng/µL	WS

- 13.7.7 Prepare a 1:10 dilution of the quetiapine control working standard. (1 ng/µL)
- 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.
  - Cap and vortex mix. This dilution shall be disposed of after control preparation.
- 13.7.8 Using a calibrated pipette, spike the positive controls according to the following table, using the quetiapine working control standard and dilution.

Control Description	Volume Added (µL)	Standard Concentration	Dilution of QC (or QC)
Control 1 – 60 ng/mL	30	1 ng/µL	1:10
Control 2 – 800 ng/mL	40	10 ng/µL	QC
Control 3 – 1600 ng/mL	80	10 ng/µL	QC

- 13.7.9 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.
- 13.7.10 Using a calibrated pipette or verified repeater-pipette, add 25 µL of the high concentration (10 ng/µL) working internal standard solution to each tube. Final concentration of the internal standard is 500 ng/mL.
- 13.7.11 Continue with sample preparation as described in 13.5.11 – 13.5.18.

### 13.8 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – BENZO (instrumental parameters in Appendix B)
- Calibration curve – all compounds (including qualitative quetiapine): linear, 1/a weighting factor; for quetiapine only quantitation: quadratic, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4  
 Updating calibrator – urine (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); truncated to whole integer value in units of ng/mL for quetiapine (acceptable range 15 – 25 ng/mL)  
Cals 2-7, Pos Ctl: truncated, whole integer values in units of ng/mL
- For etizolam and estazolam, the calibration curve is generated (two-point curve, with positive control and origin) and reports are generated for the positive control, negative control and blank matrix (if run). Reports are also generated for any specimens in the batch that contain etizolam or estazolam. Retention time/transition ratios are compared to those in the positive control (applies also to urine analysis).
- 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.
- Urine specimens with a calculated concentration of  $\geq 10$  ng/mL (20 ng/mL quetiapine) are suitable for qualitative reporting if urine calibrators and glucuronide process control meet acceptability criteria.

### 13.9 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. Qualitative results are reported for urine specimens.

Results for compounds not included in the positive control (7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone) are reported qualitatively. Results for etizolam/estazolam are reported qualitatively.

Quetiapine is reported qualitatively from benzo analysis (13.5, 13.6), with quantitative results reported from the quetiapine-only procedure (13.7).

### 13.10 METHOD PERFORMANCE

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

APPENDIX A

Target Compounds and Internal Standards

7-aminoclonazepam	Lorazepam glucuronide
7-aminoflunitrazepam	Midazolam
α-hydroxyalprazolam	Nordiazepam
Alprazolam	Oxazepam
Chlordiazepoxide	Oxazepam glucuronide
Clonazepam	Oxazepam-d <sub>5</sub>
Desalkylflurazepam	Quetiapine
Diazepam	Temazepam
Estazolam	Temazepam-d <sub>5</sub>
Etizolam	Triazolam
Flunitrazepam	Triazolam
Flurazepam	Zopiclone
Lorazepam	

Positive Control Target Concentrations (ng/mL)

α-hydroxyalprazolam	100
Alprazolam	100
Chlordiazepoxide	400
Clonazepam	100
Desalkylflurazepam	100
Diazepam	400
Flunitrazepam	100
Flurazepam	100
Lorazepam	50
Midazolam	50
Nordiazepam	200
Oxazepam	100
Quetiapine	400
Temazepam	100
Triazolam	100
Quetiapine-only (quantitation)	60, 800, 1600

Glucuronide (Process) Control Target Compounds

Lorazepam glucuronide  
Oxazepam glucuronide

APPENDIX B - INSTRUMENTAL PARAMETERS

LC-MSMS 1- Agilent 6410

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	1.00 mL/min
Solvent A	1% Acetic Acid
Solvent B	Acetonitrile (LC-/MS grade)
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 <sub>2</sub> O

MASS SPECTROMETER

Ion mode	(+) MRM	EMV	+400V
Peakwidth	0.07 min	Capillary voltage	4kV
Resolution	Unit	Nebulizer pressure	50 psi
Time segment 1	To waste	Drying gas	Nitrogen
Time segment 2	To MS	Drying gas flow	12 L/min
Time segment 3	To MS	Drying gas temp	350° C
Time segment 4	To MS		
Time segment 5	To MS		
Time segment 6	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	
Estazolam	4	295 → 267, 205	
Etizolam	4	343 → 314, 259	
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 <sub>5</sub>	4	292 → 246	
Quetiapine	3	384.1 → 253.2, 221.1	
Temazepam	4	301 → 255, 177	
Temazepam-d <sub>5</sub>	4	306 → 260	
Triazolam	4	343 → 308, 239	
Zopiclone	2	389.1 → 245.1, 217.1	

LC-MSMS 5- Agilent 6420

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	1.00 mL/min
Solvent A	1% Acetic Acid
Solvent B	Acetonitrile (LC-/MS grade)
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 <sub>2</sub> O

MASS SPECTROMETER

Ion mode	(+) MRM	EMV	+200V
Time filter width	0.07 min	Capillary voltage	4kV
Resolution	Unit	Nebulizer pressure	50 psi
Time segment 1	To waste	Drying gas	Nitrogen
Time segment 2	To MS	Drying gas flow	12 L/min
Time segment 3	To MS	Drying gas temp	350° C
Time segment 4	To MS		
Time segment 5	To MS		
Time segment 6	To waste		
Signals	Time Segment	MRM Transitions	
7-aminoclonazepam	2	286 → 222.1, 121.2	
7-aminoflunitrazepam	2	284 → 226.1, 135.1	
α-hydroxyalprazolam	4	325 → 297.1, 216.1	
Alprazolam	4	309 → 281.1, 274.2	
Chlordiazepoxide	2	300 → 283.1, 227.1	
Clonazepam	4	316 → 270.1, 214.1	
Desalkylflurazepam	4	289 → 226.1, 140	
Diazepam	5	285 → 257.1, 222.1	
Estazolam	4	295.1 → 267.1, 205.1	
Etizolam	4	343 → 314.1, 259.1	
Flunitrazepam	4	314 → 268.2, 239.2	
Flurazepam	3	388.1 → 315.1, 288.1	
Lorazepam	4	321 → 275, 229.1	
Midazolam	3	326 → 291.2, 249.1	
Nordiazepam	4	271 → 165.1, 140	
Oxazepam	4	287 → 269., 241.1	
Oxazepam-d <sub>5</sub>	4	292 → 246.1	
Quetiapine	3	384.1 → 253.1, 221.2	
Temazepam	4	301 → 255.1, 177.1	
Temazepam-d <sub>5</sub>	4	306 → 260.1	
Triazolam	4	343 → 308.1, 239.1	
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
10/07/15	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
04/17/17	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
7/23/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 13.2 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 13.3.1 and removed glucuronidase preparation from powder. Added step in 13.5 and 13.6 for preparation of the etizolam/estazolam positive control. 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. Target compound/internal standard list and target positive control concentrations added in APPENDIX A. Instrument Parameters moved to APPENDIX B. Formatting and minor edits throughout.	All



3/13/19	Updated 13.7 and 13.9 to reflect quetiapine curve with 1/a <sup>2</sup> weighting and dynamic range of 10 – 1000 ng/mL. Added instrument parameters for LC-MSMS 5 in APPENDIX B. Added estazolam and etizolam transitions MRM list for LC-MSMS 1.	7, 10-11
5/28/19	Note added in 13.2 to specify 0.5 mL sample volume for quetiapine-only quantitation. Added quetiapine-specific standard concentrations to 13.4.1 (including high concentration internal standard). Specified use of three positive controls for quetiapine quantitation in 13.4.3.1. Added procedure for quetiapine-only sample preparation in 13.7. Updated 13.8, 13.9 and 13.10 to reflect quetiapine-only analysis (dynamic range 20 – 2000 ng/mL, quadratic curve with 1/x weighting, positive control target concentrations of 60, 800 and 1600 ng/mL). Wording added to 13.8 for etizolam/estazolam data analysis and reports. Other minor edits throughout.	1, 3-5, 7-10
9/30/19	In 13.2 and 13.9, clarified that qualitative reporting for quetiapine testing described in 13.5 and 13.6. Added detailed description for curve fit for quetiapine in all-compound testing (qualitative for quetiapine) and in quetiapine-only testing in 13.8.	1, 8-9