Section Six
Urine and Blood Toxicology

6.1 Extraction Methods for LCMS-QQQ Confirmation

6.1.1 Confirmation of Benzodiazepines and Z drugs in Blood and Urine

6.1.1.1 BACKGROUND

Benzodiazepines continue to be the most prescribed group of therapeutic agents. Approximately 20 benzodiazepines are approved for use in the US.[2] Benzodiazepines were first introduced in the 1960s in pursuit of the perfect sedative hypnotic agent, and have replaced barbiturates as the major class of central nervous system (CNS)-depressant drugs.[2] In 1962, Chlordiazepoxide (Librium®) was introduced, followed by the introduction of Diazepam (Valium®) in 1968. There are four main classes of benzodiazepines, the 1,4-benzodiazepines, the triazolobenzodiazepines, the diazolobenzodiazepines, and the 7-nitrobenzodiazepines.

Benzodiazepines are used primarily as antiepileptics in the treatment of seizure disorders, as anxiolytics for the short-term relief of anxiety disorders, as sedative-hypnotics for the treatment of sleep disorders, and as muscle relaxants to relieve spasticity. The primary side effects that accompany their use include dose-related extensions of the intended actions, including sedation and sleepiness/drowsiness. In addition, other undesired effects that will influence the outcome of field sobriety tests include ataxia, a blocked ability to coordinate movements, a staggering walk and/or poor balance, lethargy/apathy, indifference or sluggishness, mental confusion, disorientation, slurred speech, and amnesia. Impairment of motor abilities, especially a person's ability to drive an automobile, is common. This impairment is compounded by the drug-induced suppression of one's' ability to assess their own level of physical and mental impairment. Alcohol combined with other CNS depressants (e.g., barbiturates antidepressants, etc.) will increase CNS depressant effects, such as impairment of psychomotor function and sedation, in an additive manner.[4-6]

Z drugs (zolpidem, zopiclone), prescribed as sleep aids, and quetiapine which is used in the treatment of mental disorders act in a similar manner to benzodiazepines, but are not included in that particular class of drugs.

The benzodiazepines are lipid soluble and are absorbed well from the GI tract with good distribution to the brain. They are metabolized primarily in the liver. Their CNS active metabolites extend their duration of action. The benzodiazepines work by enhancing, facilitating or potentiating the action of the inhibitory neurotransmitter GABA. They serve to increase the frequency of GABA-mediated chloride ion channel opening.

Benzodiazepines are metabolized primarily in the liver via several different microsomal enzyme systems.[6] Many products of their metabolism are active. Since many of the active metabolites have been marketed as therapeutic agents, it may be difficult to ascertain which drug was ingested based solely upon the results of analysis. Current drug therapy will assist in determining the source of a particular compound. The detection of a particular agent is determined partly by whether its metabolism yields active metabolites. Excretion of the benzodiazepines is predominantly in the urine. Depending upon the particular benzodiazepine, the urine may contain parent compounds, N-dealkylation and oxidative (hydroxylation) metabolism products and/or glucuronide conjugates.

6.1.1.2 SCOPE

This method is used for the confirmation of 7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam in blood and urine. The words *calibrator* and *calibration* are used to coincide with the terminology in instrument software and manufacturer manuals. The manufacturer's term *calibrator* refers to what is considered by ISP-FS as reference material that has a certified concentration of drug present

6.1.1.3 EQUIPMENT AND SUPPLIES

6.1.1.3.1 Agilent 6410B LC/MS/MS system and MassHunter software
6.1.1.3.2 De-Tox A Tubes (or equivalent Toxi A tubes)
6.1.1.3.3 Tapered glass tubes for evaporation and reconstitution
6.1.1.3.4 Transfer pipettes
6.1.1.3.5 Pipettes for accurate dispensing of volumes 10 µL to 4 mL
6.1.1.3.6 Auto-sampler vials with snap-caps for Agilent 1260 ALS
6.1.1.3.7 Test tube rocker or rotator
6.1.1.3.8 Centrifuge capable of 3000 rpm
6.1.1.3.9 Oven capable of 60 ^{o}C

6.1.1.4 REAGENTS

Refer to manual section 5.12 for preparation instructions.

6.1.1.4.1 β-Glucuronidase Solution (obtained commercially ≥10000 units per ml)
6.1.1.4.2 2M Acetate buffer, pH 4.8
6.1.1.4.3 0.1% formic acid in water (mobile phase A)
6.1.1.4.4 0.1% formic acid in acetonitrile (mobile phase B)
6.1.1.4.5 Deionized water
6.1.1.4.6 LC/MS grade water
6.1.1.4.7 LC/MS grade acetonitrile
6.1.1.4.8 LC/MS grade methanol
6.1.1.4.9 LC/MS grade formic acid
6.1.1.4.10 Extract reconstitution solvent: 9:1 mobile phase A to mobile phase B

6.1.1.5 QUALITATIVE ASSURANCE: REFERENCE MATERIALS AND CONTROLS

6.1.1.5.1 **Calibrator and Control Solutions**

Corresponding calibrator and control reference material must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. *NOTE: Stock solution concentrations other than those listed here may be obtained, but appropriate addition volume adjustments must be made when direct spiking or preparing working solutions. Stock solutions should be stored as recommended by vendor.*

6.1.1.5.1.1 **Reference Material Stock Solutions**

1mg/mL single component benzodiazepine-class reference solutions. A multi-component benzodiazepines mix (250 µg/mL) may be obtained for use in controls.

6.1.1.5.1.2 **Reference Material Working Solutions**
Refer to Appendix 1 for the preparation instructions and stability of the working solutions.

6.1.1.5.1.3 **Internal Standard Solutions**

6.1.1.5.1.3.1 **Stock Solution (100 µg/mL)**
7-Aminoflunitrazepam-D7
Alphahydroxyalprazolam-D5
Oxazepam-D5
Nordiazepam-D5
Clonazepam-D4
Temazepam-D5
Diazepam-D5

6.1.1.5.1.3.2 **Working Solution**
Refer to Appendix 1 for the preparation instructions and stability of the working solution.

Required Extracted Controls for all options contained in this method:

6.1.1.5.2 **Extracted Negative Control**
An extracted negative control will be run for each matrix that is included in the run. The controls may be commercially obtained or in-house urine or blood verified to be negative for drugs of interest. The extracted negative matrix control will be run directly preceding each case sample to rule out carryover. The response of the negative control preceding a sample must be at least 100 times less than any compound confirmed in the case sample, and must be below the limit of confirmation.

6.1.1.5.3 **Extracted Positive Control**
An extracted positive control will be run for each matrix that is included in a run. Positive Controls can be prepared with single or multi-component working solutions and/or obtained commercially. The positive control must have at least two compounds in it that are included in the scope of the method. Controls should contain an approximate concentration between 75 and 400 ng/mL. *The compounds in the controls **cannot** be the same lots as were used for the calibrators*. For the control to be considered passing, it should give a response greater than 50 ng/mL for each intended analyte.

6.1.1.5.4 **Extracted Glucuronide Controls (URINE ONLY).**
Positive and negative glucuronide controls are required for any run that includes urine samples. These controls may be obtained commercially or prepared in-house by spiking negative urine. The same lot of negative urine must be used to prepare both the positive and negative glucuronide controls. Oxazepam-glucuronide or Lorazepam-glucuronide may be used; approximate concentration of controls should be 300ng/mL.

6.1.1.5.4.1 Stock Solution
100µg/mL Oxazepam- or Lorazepam-Glucuronide

6.1.1.5.4.2 Direct spiking
Spike negative urine with 30µL of 100µg/mL stock solution or 300 µL working solution.

6.1.1.5.4.3 Working Glucuronide Solution (10ng/µL)
Add 1mL 100µg/mL Stock Solution to 10mL MeOH. *Solution is stable for one year when stored under refrigeration.*

6.1.1.6 PROCEDURE

6.1.1.6.1 Calibrator preparation (calibrators may be prepared in advanced and re-run if they were prepared with the same internal standard as the samples)

6.1.1.6.1.1 Label a conical glass tube for each calibrator. Add 100 µL of 1.0 µg/mL ISTD mix to each tube, as well as the following volumes of reference material. Evaporate to dryness.

Sample Type	1.0 µg/mL Target mix
Blank	-
25 ng/mL Cal 1	25 µL
50 ng/mL Cal 2	50 µL
100 ng/mL Cal 3	100 µL

Sample type	10.0 µg/mL Target mix
500 ng/mL Cal 4	50 µL
1000 ng/mL Cal 5	100 µL
3000 ng/mL Cal 6	300 µL

6.1.1.6.1.2 Reconstitute in 100 µL 9:1 mobile phase A to mobile phase B.

6.1.1.6.1.3 Label autosampler vials to correspond to the evaporation tubes.

6.1.1.6.1.4 Transfer most of the reconstituted sample from the evaporation tube into to the corresponding autosampler vial and cap the vials.

6.1.1.6.2 Casework sample and control preparation

6.1.1.6.2.1 Casework and Control Samples (Blood or Urine)

6.1.1.6.2.1.1 Transfer 1.0 mL casework and controls to labeled conical tubes.

6.1.1.6.2.2 Internal Standard Addition

6.1.1.6.2.2.1 Add 100 µL of 1.0 µg/mL ISTD mix to labeled conical glass tube for each blank, QC and case sample. Vortex to mix.

6.1.1.6.2.3 Sample Hydrolysis ***(Urine Samples Only)***

6.1.1.6.2.3.1 Enzyme hydrolysis: add 40µL 2M acetate buffer to all controls and case samples, and 15µl β-glucuronidase to calibrators, controls and casework samples (except the negative glucuronidase control sample). Cap and gently vortex the samples. Incubate at approximately 60°C in an oven for 2 hours. Remove from oven and allow to cool.

6.1.1.6.2.4 Extraction

6.1.1.6.2.4.1 Label a De-Tox Tube A for each QC, blank, and case sample.

6.1.1.6.2.4.2 To the De-Tox Tubes, add ~4 mL of deionized water to each tube (or add the 4 mLs to the conical tubes with the samples).

6.1.1.6.2.4.3 Transfer the casework and control samples with added ISTD from the labeled conical tube to the corresponding De-Tox Tube (*for blood samples, the ISTD and sample may be added directly to the De-Tox tube. There is no requirement to place it in a conical tube first.*)

6.1.1.6.2.4.4 Cap the De-Tox Tubes and mix by inverting.

6.1.1.6.2.4.5 Rotate or rock the tubes gently for ~ 5 minutes.

6.1.1.6.2.4.6 Centrifuge the tubes at approximately 2000-2500 rpm for ~ 5 minutes. *NOTE: If an emulsion occurs, it may be broken up with a disposable transfer pipette and the tube re-centrifuged at approximately 3000 rpm for ~5 minutes. Care should be taken that no solvent is lost to the disposable pipette when the emulsion is broken up.*

6.1.1.6.2.4.7 Transfer most (~2 mL) of the upper organic layer from each De- Tox Tube to the corresponding labeled evaporation tube. **Avoid transferring any solids.**

6.1.1.6.2.4.8 Evaporate to dryness under nitrogen at ~ 40 degrees C. **It is critical that the extracts are evaporated completely to dryness.**

6.1.1.6.2.5 Reconstitution

6.1.1.6.2.5.1 Reconstitute in 100 µL 9:1 mobile phase A to mobile phase B.

6.1.1.6.2.5.2 Transfer the reconstituted sample from the evaporation tube into to the corresponding autosampler vial and cap.

6.1.1.6.3 Instrument and run set up

6.1.1.6.3.1 Before analysis, make sure a successful check tune has been run that week, clean the electrospray ion source if necessary, and add fresh solvent to the solvent bottles (be sure to reset the solvent levels in the acquisition software). Turn the LC/MS/MS ON and run the system using the background check method to evaluate the system. The maximum intensity for any background ion should be < 100,000 area counts, and ideally < 10,000 area counts.

6.1.1.6.3.2 In MassHunter Acquisition, load the Benzos_Z-Drugs_ACN_FA method. Allow column temperature and LC pressure to stabilize. Verify that the binary pump ripple is <1%.

6.1.1.6.3.3 Open or start a worklist in MassHunter Acquisition. Enter the calibrators, blanks, controls and samples as needed.

6.1.1.6.3.4 Select Worklist/Worklist Run Parameters, and create a Data path for this Batch (e.g. 110808BZ).

6.1.1.6.3.5 Also in Worklist Run Parameters, select Acquisition Cleanup/Standby, to put the instrument in Standby after the Worklist, or if a Not Ready Timeout occurs.

6.1.1.6.3.6 Save the Worklist

6.1.1.6.3.7 Allow the instrument to stabilize for at least 15 minutes from the time it is turned ON.

6.1.1.6.3.8 Begin the Worklist by clicking on the Multiple Vial icon on the top center of the MassHunter Acquisition screen. The cycle time for each injection is ~15 minutes.

6.1.1.6.4 Data Analysis

6.1.1.6.4.1 Open MassHunter Quantitative Analysis.

6.1.1.6.4.2 Select File/New Batch.

6.1.1.6.4.3 Navigate to the MassHunter/Data directory, and open the folder containing the data files for the current Batch. Assign a name to the Batch (e.g. 110808BZ), and select Open.

6.1.1.6.4.4 Select File/Add Samples, Select All, and OK to add all the samples to the Batch. Any column rinse injections will not contain meaningful results, and can be removed from the Add Samples list.

6.1.1.6.4.5 Select Method/Open/Open and Apply from Existing File.

6.1.1.6.4.6 Navigate to the location of the Quantitative Analysis Data Analysis Method (Ex – benzos.quantmethod), select it, and select Open. In this example, the benzo.quantmethod is stored in the MassHunter/data analysis methods directory.

6.1.1.6.4.7 When the method has been opened and applied, the Batch Table appearance will change, but the results will not yet be populated.

6.1.1.6.4.8 Select Analyze Batch, or F5, to complete the Batch analysis, and Save the Batch.

6.1.1.6.4.9 The Batch Table view will show the Batch Table with results, Compound Information, and the Calibration Curve. Navigation by Compound can be accomplished by using either the arrows or the drop-down menu in the Compound section of the Batch Table.

6.1.1.6.4.10 To update the retention times and qualifier ion ratios for the current Batch, go to Method/Edit, or use F10, to enter the Method Editor view of MassHunter Quantitative Analysis. Review the retention times and qualifier ion ratios from the calibrators, and make updates as appropriate.

6.1.1.6.4.11 To return to the Batch Table and apply the updated retention times and qualifier ion ratios, select the Exit button, answer Yes, and in the Batch Table select Analyze Batch, or F5.

6.1.1.6.5 **Batch Review**

6.1.1.6.5.1 The lab criterion for acceptable calibration curve R^2 is $\geq$0.975. A minimum of four calibration points are required for a valid curve. If the confirmation decision point (50 ng/mL) is removed from the curve, the new administrative cutoff will be the lowest calibrator that meets quality assurance requirements (excluding the 25 ng/mL data point). If the 25 ng/mL calibration point for a compound is removed from the curve, no results of "inconclusive" may be reported for that compound in that batch.

6.1.1.6.5.2 Outliers are highlighted in the Batch Table with the color codes blue and red, for below or above acceptable limits (respectively).

6.1.1.6.5.3 The default criterion for Accuracy is that each calibrator result should agree with the target value $\pm$20%.

6.1.1.6.5.4 The default criteria for a positive result are:

6.1.1.6.5.4.1 Retention time within $\pm$5% of the average of the calibrators.

6.1.1.6.5.4.2 Qualifier ion ratios within +20% of the average of the calibrators.

6.1.1.6.5.4.3 The sample must have a concentration greater than the 50 ng/mL calibrator; samples that meet all other criteria for identification but fall between the 25 ng/mL calibrator and 50 ng/mL calibrator can be reported as inconclusive. (See section 6.1.1.9.3 for the exceptions regarding nitrazepam reporting). Samples with concentrations exceeding the highest calibrator may be reported without dilution/re-extraction provided that retention time and ion ratio requirements are met.

6.1.1.6.5.5 Manual integration should not be needed frequently. When it is needed, it is enabled with the Start/End Manual Integration Tool in the Compound Information section of the Batch Table.

6.1.1.6.5.6 Manual integration is accomplished by left-clicking and dragging on the black boxes at peak start and end. Spurious peaks can be deleted by selecting the Start/End Manual Integration tool, right clicking in Compound Information, and selecting Zero Peak.

6.1.1.6.5.7 Review the results for each analyte in the Batch. Check for outliers, R^2 values, and check QC values.

6.1.1.6.5.7 When Batch review is complete, Save the Batch a second time.

6.1.1.6.5.8 <u>To generate a report</u>

6.1.1.6.5.8.1 Select Report/Generate and navigate to the report template (Ex – ISP_Summary_07_ LCMS_1Qual), select it, then select OK. Once the report has generated, print it, then select the ISTD template report (Ex – Quant Report_ISTD_Calibration_B_05_00) and print it. Alternatively, the generated reports may be saved as electronic files (Ex – pdfs) and stored electronically per any requirements in the ISP-FS Quality Manual.

6.1.1.6.5.9 The Queue Viewer, which allows you to track the report generation process, will open automatically. Depending on the size of the Batch, report generation may take approximately 5-20 minutes.

6.1.1.7 QUALITY ASSURANCE REQUIREMENTS

6.1.1.7.1 Refer to toxicology analytical methods 5.8 and 5.10 for additional quality assurance and reference material authentication requirements.

6.1.1.8 ANALYSIS DOCUMENTATION

6.1.1.8.1 Case results are to be recorded in the LIMS system.

6.1.1.8.2 The printed reports for the batch and controls will be stored centrally in the lab in which the analysis was performed. A copy of data for controls may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

6.1.1.8.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

6.1.1.9 LIMITATIONS OF METHOD

6.1.1.9.1 The hydrolysis process for glucuronides in urine has limited efficiency; based on the validation study, the estimated conversion is about 30-50 percent. There is potential for a small amount of temazepam to convert to diazepam in the hydrolysis process. If both diazepam and temazepam are detected in a urine sample, the diazepam will not be reported unless it has a response that is greater than 5% of the temazepam response.

6.1.1.9.2 Currently, this method has only been evaluated for qualitative identification of the listed compounds in urine and blood. The uncertainty associated with the quantitative values has not been established; therefore, no values shall be referenced or reported.

6.1.1.9.3 Nitrazepam has been found to have significant variability in concentration responses with this method, though no false positives have been observed. If a case sample gives a nitrazepam response that is >5 ng/mL and <50 ng/mL, it will be reported as "inconclusive for nitrazepam due to method limitations."

6.1.1.10 REFERENCES

6.1.1.10.1 This method was developed in conjunction with Agilent. Patrick Friel from Agilent came to the Idaho State Police Forensic lab located in Coeur d'Alene and provided application training July 23-26, 2012.

6.1.1.10.2 Williamson S.C. ISP Toxicology Analytical Method 2.4.3

6.1.1.10.3 Levine, B. *Central Nervous System Depressants*. pp. 191-197. *in:* Principles of Forensic Toxicology. Levine, B. ed., AACC, 1999.

6.1.1.10.4 Huang, W. and Moody, D.E. *Immunoassay Detection of Benzodiazepines and Benzodiazepine Metabolites in Blood.* J. Anal. Tox. **19**:333-342, 1995.

6.1.1.10.5 Fu, S. Molnar, A. Bowen, P. Lewis J. Wang H. *Reduction of Temazepam to Diazepam and Lorazepam to Delorazepam During Enzyme Hydrolysis.* Anal Bioanal Chem 400: 153-164, 2011.

6.1.1.10.6 Julien, R.M. *A Primer of Drug Action*. pp. 95-107, W.H. Freeman and Company: NewYork, 1998.

6.1.1.10.7 Hobbs, W.R., Rall, T.W. and Verdoorn, T.A. *Hypnotics and Sedatives*. pp. 362-373. *in:* Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th edition, Hardman, J.G. ed., McGraw-Hill, 1996.

Appendix 1:

1.0 µg/mL Target mix in methanol

(Document on a prep sheet with an expiration of one year, store under refrigeration)

In a 10 mL volumetric flask fill the flask about half full with methanol, add 10 µL of 1mg/mL stock solution of the following compounds. (If the stock solution is a different concentration, you will need to adjust addition volumes.)

7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam

QS with methanol and ensure it is thoroughly mixed.

10.0 µg/mL Target mix in methanol

(Document on a prep sheet with an expiration of one year, store under refrigeration)

In a 25 mL volumetric flask fill the flask about half full with methanol add 250 µl of 1mg/mL stock solution of the following compounds.

7-aminoclonazepam, 7-aminoflunitrazepam, zopiclone, zolpidem, chlordiazepoxide, quetiapine, midazolam, flurazepam, nitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, oxazepam, nordiazepam, clonazepam, lorazepam, alprazolam, flunitrazepam, temazepam, and diazepam

QS with methanol and ensure it is thoroughly mixed.

1.0 µg/mL ISTD mix in methanol

(*Document on a prep sheet with an expiration of one year, store under refrigeration)*

Fill a 10 mL volumetric flask about half full with methanol, add 100 µl of 100ug/mL stock solution of the following compounds. (If the stock solution is a different concentration you will need to adjust addition volumes.)

7-aminoflunitrazepam-D7, alphahydroxyalprazolam-D5, oxazepam-D5, nordiazepam-D5, clonazepam-D4, temazepam-D5, diazepam-D5

QS with methanol and ensure it is thoroughly mixed.

Revision History

Section Six
Urine and Blood Toxicology

6.1 Extraction Methods for LCMS-QQQ Confirmation
6.1.1 Confirmation of Benzodiazepines and Z drugs in blood and urine

Revision No.	Issue Date	Revision/Comments
0	4/9/2013	Original Issue in SOP format.
1	9/6/2013	Replaced Toxi A tube with De-Tox tube A. Clarified when calibrators needed to be prepared. Increased centrifuge rpm speed from 2000 to 2000-2500 rpm. Made it optional to add blood and ISTD to conical tube before placing in De-Tox tube.
2	1/16/2014	Amendment to 6.1.1.8 in accordance with new LIMS system. Minor formatting changes.
3	3/9/2014	Some formatting and grammatical corrections. Addition of Calibrator and Control Solutions section. Amendment to 6.1.1.5.2 defining what constitutes a passing control; addition of requirement that calibrator and control lots be different. Amendment to 6.1.1.6.2.4.6 for dealing with emulsions. Amendment to 6.1.1.6.5.4.3 referencing newly-added section to method limitations; added provision for reporting results for samples with concentrations exceeding highest calibrators. Added additional method limitation statement (6.1.1.9.3). Changed required report template names to examples of names. Added statement allowing for printing electronically.
4	04/03/2015	Minor changes to sentence structure to be more concise. Removed screen shots from data analysis sections to minimize file size; these will be covered in QQQ training. Clarification of calibration curve acceptance criteria. Reconstitution volume change (validated).