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 60ºC

6.1.1.4 REAGENTS

Refer to manual section 5.12 for preparation instructions.

6.1.1.4.1 BG100 β-Glucuronidase Solution (Kura Biotec)
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.

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 ng/mL 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 300 ng/mL.

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

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

6.1.1.5.4.3 Working Glucuronide Solution (10 ng/µL)
Add 1 mL 100 µg/mL Stock Solution to 10 mL 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
5 ng/mL Cal 1	5 µL
10 ng/mL Cal 2	10 µL
25 ng/mL Cal 3	25 µL
50 ng/mL Cal 4	50 µL
100 ng/mL Cal 5	100 µL

Sample type	10.0 µg/mL Target mix
500 ng/mL Cal 6	50 µL
1000 ng/mL Cal 7	100 µL
3000 ng/mL Cal 8	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 Non-extracted Blank

The non-extracted blank will be run directly preceding each case sample to rule out carryover. The area response of the blank preceding a sample must be at least 10 times less than any compound confirmed in the case sample, and must be below the limit of confirmation for any analyte

confirmed in the case sample. If confirmation criteria (e.g. ion ratios) are not met, the analyte is not considered present.

6.1.1.6.2.1 Multiple non-extracted blanks may be prepared if the batch has greater than 5 samples. To prepare, spike a tapered bottomed tube with the necessary amount of internal standard *(ie 100 µL if reconstituted in 100 µL reconstitution solvent or 200 µL internal standard if reconstituted in 200 µL reconstitution solvent, etc.*)

6.1.1.6.2.2 Evaporate the organic phase to dryness under nitrogen at ~ 40 degrees C. **It is critical that the extracts are evaporated completely to dryness, but *DO NOT* over-dry.**

6.1.1.6.2.3 Reconstitute the dry extract in 100 µL 1:1 Acetonitrile:Water. (*NOTE: The reagents for this step shall be LC/MS grade.*) Transfer the reconstituted sample from the evaporation tube into to the corresponding autosampler vial and cap.

6.1.1.6.3 Casework sample and control preparation

6.1.1.6.3.1 Casework and Control Samples (Blood or Urine)

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

6.1.1.6.3.2 Internal Standard Addition

6.1.1.6.3.2.1 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.

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

6.1.1.6.3.3.1 Enzyme hydrolysis: add 20µL 2M acetate buffer to all controls and case samples, and 76 µl BG100 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 30 minutes. Remove from oven and allow to cool.

6.1.1.6.3.4 Extraction

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

6.1.1.6.3.4.2 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).

6.1.1.6.3.4.3 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.*)

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

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

6.1.1.6.3.4.6 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.*

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

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

6.1.1.6.3.5 Reconstitution

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

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

6.1.1.6.4 Instrument and run set up

Refer to method 5.13 for general instrument operation and maintenance.

6.1.1.6.5 Data Analysis

Refer to AM 5.13 for general instructions on Data Analysis and report generation.

6.1.1.6.6 **Analytical Method Specific Batch Review**

6.1.1.6.6.1 The lab criterion for acceptable calibration curve R^2 is $\geq$0.975. If the 25 ng/mL confirmation decision point is removed from the curve, the new administrative cutoff will be the lowest calibrator that meets quality assurance requirements (excluding the 10 ng/mL data point). If the 10 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.

6.1.1.6.6.2 The method specific criteria for a positive result are:

6.1.1.6.6.2.1 The sample must have a concentration greater than the 25 ng/mL calibrator (or the lowest calibrator that meets quality assurance requirements).

6.1.1.6.6.2.2 Inconclusive samples are those that meet all other criteria for identification but fall between the 10 ng/mL calibrator and the administrative cutoff (See section 6.1.1.6.6.1 for cutoff requirements and 6.1.1.9.1 and 6.1.1.9.3 for the exceptions regarding Diazepam and Nitrazepam). Samples with concentrations exceeding the highest calibrator may be reported without dilution/re-extraction provided that retention time and ion ratio requirements are met.

6.1.1.7 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 Reports for the batch and controls, if printed, 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 60-70 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: *(Document reagents on a prep sheet with an expiration of one year, store under refrigeration)*

1.0 µg/mL Target mix in methanol

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

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

Fill a 10 mL volumetric flask about half full with methanol, add 100 µl of 100 *µg*/mL stock solution of the following compounds. (If the stock solution is a different concentration you will need to adjust addition volumes.) Alternate total volumes may be prepared, as long as concentration ratios remain the same (*ie. 250 µL of 100 µg//mL in 25 mL*)

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).
5	06/10/2015	Updated to enzyme hydrolysis procedure (validated shorter incubation time in conjunction with use of new reagent). Updated reagent list for new enzyme. Confirmation cutoff changed from 50 ng/mL to 25 ng/mL. "Inconclusive" cutoff changed. Cutoff changes validated through data review. Updated efficiency percentage to reflect new enzyme validated for this method. Clarified central file storage requirements. Added 2 calibration levels (5 & 10 ng/mL). Provided way to ensure enough negative control is present for use as matrix blanks with larger batches.
6	07/26/2016	Minor formatting, addition of use of non-extracted blanks to replace use of negative matrix control. Removed Instrument operation parameters and added reference to AM 5.13.