Section Two
Urine Toxicology

2.4 Liquid-Liquid Extraction Methods for GC/MSD Confirmation
2.4.3 Qualitative Benzodiazepines and Ancillary Compounds in Urine

2.4.3.1 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] Chlordiazepoxide (Librium®) was introduced in 1962 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. Refer to the following chart for a compilation of benzodiazepines currently prescribed in the US or ones that are commonly encountered.

1,4-Benzodiazepines	***Trade Name***	***Major Metabolite(s)***
Diazepam	Valium®	Nordiazepam, Oxazepam, Temazepam
Nordiazepam	---	Oxazepam
Oxazepam	Serax®	Glucuronide conjugate
Temazepam	Restoril®	Oxazepam
Clorazepate	Tranxene®	Nordiazepam, Oxazepam
Chlordiazepoxide	Librium®	Demoxepam, Nordiazepam, Oxazepam
Halazepam	Paxipam®	3-Hydroxy-Halazepam, Nordiazepam, Oxazepam
Quazepam	Dormalin®, Doral®	2-Oxoquazepam
Flurazepam	Dalmane®	Desalkylflurazepam
Lorazepam	Ativan®	3-Glucuronide
7-Nitrobenzodiazepines		
Clonazepam	Klonopin®	7-Aminoclonazepam
Flunitrazepam	Rohypnol® *Not Prescribed in US*	7-Aminoflunitrazepam
Triazolobenzodiazepines		
Alprazolam	Xanax®	α-Hydroxy-alprazolam, 4-Hydroxy-alprazolam
Triazolam	Halcion®	α-Hydroxy-triazolam
Estazolam	ProSom®	---
Diazolobenzodiazepine		
Midazolam	Versed (Parenteral)	α-Hydroxymidazolam

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 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 ones' ability to assess their own level of physical and mental impairment. Alcohol and other CNS depressants (e.g., barbiturates, antidepressants, etc.) will increase CNS depressant effects, such as sedation and impairment of psychomotor function, in an additive manner.[4-6]

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 is difficult to ascertain which drug was ingested solely upon the basis of 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 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.

2.4.3.2 SCOPE

This extraction method is a modification of the method developed by Valentine, et al., for the extraction of benzodiazepines from urine.[1] The method has also been found to be effective in the extraction of opiates and various other drugs such as Zolpidem, Buprenorphine and trazodone.

At the analyst's discretion, the samples may be extracted with or without derivatizing, and there are two options in the method for derivatization.

2.4.3.3 EQUIPMENT AND SUPPLIES

2.4.3.3.1 Tube Rocker
2.4.3.3.2 Laboratory oven or water bath
2.4.3.3.3 Laboratory Centrifuge capable of 3500 rpm
2.4.3.3.4 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.
2.4.3.3.5 Dry bath

2.4.3.3.6 Evaporative Concentrator equipped with nitrogen tank.
2.4.3.3.7 Threaded-end 16X100 Round Bottom Glass Tubes and/or 16X114 Tapered Bottom Glass Centrifuge Tubes
2.4.3.3.8 Screw caps for 16mm O.D. Tubes
2.4.3.3.9 pH Indicator Strips
2.4.3.3.10 ALS Vials
2.4.3.3.11 ALS Vial Microinserts
2.4.3.3.12 Gas Chromatograph equipped with a mass selective detector and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drug compounds and other analytes encountered in toxicological specimens (e.g. 100%-dimethylpolysiloxane or 95%-dimethyl-polysiloxane with 5% diphenyl).

2.4.3.4 REAGENTS

Refer to manual section 5.12 for preparation instructions.

2.4.3.4.1 β-Glucuronidase Solution
2.4.3.4.2 2M Acetate buffer, pH 4.8
2.4.3.4.3 50mM Sodium Bicarbonate, pH 11
2.4.3.4.4 Chloroform/Isopropanol 9:1 (Each Certified ACS Grade)
2.4.3.4.5 Ethyl Acetate (Certified ACS Grade)
2.4.3.4.6 Silylating agent: MSFTA or BSTFA with 1% TMCS

2.4.3.5 QUALITATIVE REFERENCE MATERIAL AND CONTROLS

2.4.3.5.1 Positive Control

Positive Control can be prepared with single or multicomponent working solutions and/or obtained commercially.

2.4.3.5.1 Non-extracted Reference Material

Run necessary reference material as indicated by examination of GC/MSD data. Reference material mixes may be employed.

2.4.3.5.2 Non-extracted Derivatized Reference Material

2.4.3.5.2.1 Prepare derivatized reference material as necessary based on current drug therapy and examination of GC/MSD data.

2.4.3.5.2.2 Add ≅ 3-5uL (1mg/mL) stock reference material to labeled centrifuge tube. Derivatize as described in 2.4.3.6.2.3

2.4.3.5.3 Internal Standard

2.4.3.5.3.1 **Stock Solutions**

1 mg/mL Prazepam

2.4.3.5.3.2 **Working Internal Standard Solution [10ng/µL]**

Add 100µL Prazepam stock solution to 10mL volumetric ball flask. QS with methanol.

Solution is stable for one year when stored under refrigeration.

2.4.3.5.4 Required Extracted Controls (inclusive of all options for method)

2.4.3.5.4.1 Extracted Negative Control

Commercially obtained or in-house urine verified to be negative for drugs of interest.

2.4.3.5.4.2 Positive Control

Positive Control can be prepared with single or multi-component working solutions and/or obtained commercially. The positive control must have at least one compound in it that is appropriate for demonstrating that each chosen extraction and derivatization (if applicable) is working. The preferred concentration range of this control is 300–600ng/mL. Examples of preparation and dilutions for an in-house control are given at the end of this method in Appendix 1.

2.4.3.5.4.3 Positive and Negative Glucuronide Controls.

These controls may be obtained commercially or prepared in-house by spiking negative urine. The same negative urine must be used to prepare both the positive and negative glucuronide controls. Oxazepam glucuronide, lorazepam glucuronide or morphine glucuronide may be used for these controls and must be at a minimum concentration of 375ng/mL. Examples of preparation are given in Appendix 1. Derivatization will be required for the controls prepared in-house, even if there are no case samples requiring derivatization. The positive and negative glucuronide controls are used to demonstrate the glucuronidase was effective (if these samples are run in conjunction with samples that are not derivatized or are derivatized using option 2, one set of glucuronide controls can be used for both.)

2.4.3.6 PROCEDURE

This method provides three options for the analyst. The method describes the preparation of an ethyl acetate extract and two options for a derivatized extract. Based on compounds of interest, both extracts need not be prepared and only the corresponding control material must be included.

2.4.3.6.1 Non-derivatized Ethyl Acetate Samples

2.4.3.6.1.1 Casework and Control samples

2.4.3.6.1.1.1 Transfer 6mL casework samples and controls to screw top extraction tubes.

2.4.3.6.1.2 Internal Standard Addition

2.4.3.6.1.2.1 To each prepared sample, add 300µL of internal standard (10ng/uL working solution) or 3uL of 1mg/mL stock solution. Vortex to mix.

2.4.3.6.1.3 Sample Hydrolysis

2.4.3.6.1.3.1 Add 200µL 2M acetate buffer to each tube, vortex.

2.4.3.6.1.3.2 To all but the glucuronidase negative, add 100µL β-Glucuronidase Solution. Cap and vortex *gently* to mix.

2.4.3.6.1.3.3 Place all tubes in 60°C laboratory oven or water bath for two hours.

2.4.3.6.1.3.4 Allow samples to cool before proceeding with solvent extraction.

2.4.3.6.1.4 Extraction

2.4.3.6.1.4.1 Add 2mL 50mM sodium bicarbonate to each sample tube. Vortex.

2.4.3.6.1.4.2 Check pH. If necessary, adjust pH to approximately pH 9 with 1N NaOH or KOH.

2.4.3.6.1.4.3 Add 4mL of chloroform/isopropanol {9:1}.

2.4.3.6.1.4.4 Rock for approximately 15 minutes.

2.4.3.6.1.4.5 Centrifuge (~3300-3500 for about 10-15 minutes)

2.4.3.6.1.4.6 Transfer lower organic phase from tube into labeled tapered bottom tube.

2.4.3.6.1.4.7 Evaporate solvent to dryness under a gentle stream of nitrogen at ≤37°C. Proceed to 2.4.3.6.1.5 if not derivatizing, or to 2.4.3.6.2 if derivatizing before running on GC/MS.

2.4.3.6.1.5 Reconstitution with Ethyl Acetate (No Derivatization)

2.4.3.6.1.5.1 Add 50µL ethyl acetate. Vortex.

2.4.3.6.1.5.2 Transfer extract to labeled ALS vial with microinsert.

2.4.3.6.1.6 Preparation for Analysis Run

2.4.3.6.1.6.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

2.4.3.6.1.6.2 Load samples, reference material, blanks and controls into the quadrant rack(s) as noted in the sequence table.

2.4.3.6.2 Derivatization of Samples: Option 1

2.4.3.6.2.1 Follow Ethyl acetate sample preparation steps 2.4.3.6.1.2 - 2.4.3.6.1.4

2.4.3.6.2.2 Derivatization

2.4.3.6.2.2.1 To the tapered-bottom tubes add 20µL ethyl acetate and 30µL of silylating agent.

2.4.3.6.2.2.2 Cap tubes. Vortex.

2.4.3.6.2.2.3 Heat tube for 15 minutes in 75°C dry bath.

2.4.3.6.2.2.4 Remove from heat and allow to cool. Transfer derivative to labeled ALS vial with microinsert.

2.4.3.6.2.3 Preparation for Analysis Run

2.4.3.6.2.3.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

2.4.3.6.2.3.2 Load samples, reference material, blanks and controls into the quadrant rack(s) as noted in the sequence table.

2.4.3.6.3 Derivatization of Samples: Option 2
(Complete ethyl acetate sample extraction procedure, run samples on GC/MS, then complete derivatization).

2.4.3.6.3.1 Derivatization

2.4.3.6.3.1.1 Once the ethyl acetate extracts have run on the GC/MS, add 20µL of silylating agent to remaining extract in the autosampler insert and vortex.

2.4.3.6.3.1.2 Heat vials for 15 minutes at 75°C.

2.4.3.6.3.1.3 Remove from heat and allow to cool.

2.4.3.6.3.2 Preparation for Analysis Run

2.4.3.6.3.2.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

2.4.3.6.3.2.2 Load samples, reference material, blanks and controls into the quadrant rack(s) as noted in the sequence table.

2.4.3.6.3.3 GC/MSD Analysis Parameters

2.4.3.6.3.3.1 Refer to instrument METHOD for current analysis parameters.

2.4.3.6.3.3.2 Current analysis method must be stored centrally as a hard or electronic copy.

2.4.3.6.4 Detection and Identification Criteria
The presence of a drug compound is indicated if the retention time for the sample, versus applicable reference material, does not differ by more than ±0.2 minutes and there are no significant differences in the mass spectral data.

2.4.3.7 METHOD LIMITATIONS AND APPLICATION TO OTHER ANALYTES

2.4.3.7.1 This method is applicable to other compounds, which require an enzymatic hydrolysis to liberate the compound of interest. Both the ethyl acetate extraction and the TMS derivative can be applied toward the identification of these compounds.

2.4.3.7.2 This method has proven useful in the identification of opiate class compounds such as codeine, morphine, 6-monoacetylmorphine, hydrocodone, and buprenorphine.

2.4.3.7.3 Care should be taken when estazolam is detected, particularly in samples containing alprazolam and/or alpha-hydroxyalprazolam. For samples containing alprazolam and/or alpha-hydroxyalprazolam, estazolam must be detected in both underivatized and derivatized GC/MSD data to be considered reportable. Estazolam shall not be reported if alprazolam and/or alpha-hydroxyalprazolam are detectable in the sample *and* derivatized estazolam is not detected.

2.4.3.8 QUALITY ASSURANCE REQUIREMENTS

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

2.4.3.9 ANALYSIS DOCUMENTATION

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

2.4.3.9.2 Original data for controls will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

2.4.3.9.3 A copy of controls need not be included in individual case files. When necessary, a copy of control printouts can be prepared from the centrally stored document.

2.4.3.10 REFERENCES

2.4.3.10.1 Valentine, J.L., Middleton, R., Sparks, C. *Identification of Urinary Benzodiazepines and their Metabolites: Comparison of Automated HPLC and GC-MS after Immunoassay Screening of Clinical Specimens*. J. Anal. Tox. **20**:416-424, 1996.

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

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

2.4.3.10.4 *Drug Facts and Comparisons* Prescription Drug Information Binder, Updated monthly.

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

2.4.3.10.6 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:

Positive Control Sample

Use the lot of negative urine that will be used to prepare negative control.

Pipette 6mL of commercially obtained positive control or prepare positive control as described below.

Use the table below as a guide for spiked positive control preparation. Prepare control for a concentration between 300 to 600ng/mL.

Spiking Solution Concentration	Amount to Add (µL)	Resulting ng/mL
100ng/µL (100µg/mL)	30	500
10ng/µL	300	500
1mg/mL	3	500
250ng/µL (250µg/mL)	12	500

Revision History

Section Two
Urine Toxicology

2.4 Liquid-Liquid Extraction Methods for GC/MSD Confirmation
2.4.3 Qualitative Benzodiazepines and Ancillary Compounds in Urine

Revision No.	Issue Date	Revision/Comments
1	02-05-2002	Original Issue in SOP format
2	10-19-2002	Refinements
3	05-07-2007	Addition of internal standard and updated QA measures and reformatting.
4	07-28-2008	Clarified that negative urine used to prepare positive control is the same lot as used for negative control.
5	03-07-2011	Clarified expiration dates for working solutions
6	3-7-2012	Added Option 2 for derivatization, moved instructions for control prep to appendix, reworked control requirements.
7	04/02/2015	Addition of 2.4.3.9.1 in accordance to LIMS System requirements. Minor formatting changes. Addition of limitation regarding reporting of estazolam (2.4.3.7.3).