Idaho State Police
Forensic Services
Toxicology Section

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 1960s in the pursuit of the perfect sedative hypnotic agent and have replaced barbiturates as the major class of CNS-depressant drugs.[2] Chlordiazepoxide (Librium®) was originally in 1962 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. 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. These include 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, indifferent or sluggish, metal 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 his or her own level of physical and mental impairment. Alcohol and other CNS depressants (e.g., barbiturates antidepressants, etc.) will increase CNS depressant effects, such as impairment of psychomotor function and sedation, in an addition 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 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.

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] This procedure is to prepare benzodiazepines in urine for either qualitative or quantitative GC/MSD analysis. Two urine aliquots are subjected to a Glucuronidase hydrolysis followed by extraction with chloroform-isopropanol. Following evaporation, one extract is reconstituted with ethyl acetate while the other is derivatized with a silylating agent. Each of the resulting extracts is analyzed by GC/MSD.

2.4.3.3 EQUIPMENT AND SUPPLIES

2.4.3.3.1 Tube Rocker (Fisher Scientific or equivalent)

2.4.4.3.2 Laboratory oven or waterbath capable of achieving 60°C (Fisher or equivalent)

2.4.3.3.3 Laboratory Centrifuge (Fisher Marathon or equivalent)

2.4.3.3.4 Drybath (Fisher or equivalent)

2.4.3.3.5 Evaporative Concentrator (Zymark TurboVap or equivalent) equipped with nitrogen tank.

2.4.3.3.6 Glassware

16X100mm tubes (Fisher 14-959-35AA or equivalent)
Screw caps (Fisher 14-930-15E or equivalent)
16X144mm tapered tip centrifuge tubes (Fisher 05-538-41C or equivalent)
Snap caps (Fisher 05-538-41N or equivalent)
GC/MS ALS vials (HP 5182-0865 or equivalent)
GC/MS vial microinsert (HP 5183-2088 or equivalent)

2.4.3.3.7 Gas Chromatograph equipped with a mass selective detector (HP 6890/5973 or equivalent) and a nonpolar capillary column with a phase composition capable of efficiently separating amines, alkaloids, drugs 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 2.6 for preparation instructions.

2.4.3.4.1 Glucuronidase (Sigma G-0876 or equivalent)

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

2.4.3.4.5 Ethyl Acetate (Ansys #203 or Fisher #E145-1 or equivalent)

2.4.3.4.6 Silylating Agents

MSFTA (Pierce #48910 or equivalent) or
BSTFA with 1%TMCS (Pierce #38831 or equivalent)

2.4.3.5 CONTROLS

2.4.3.5.1 Liquid Urine Control containing a minimum of Oxazepam or Nordiazepam (BioRad 443, Utak 88121 or equivalent)

2.4.3.5.2 Drug Mix (Alltech 601826 {Medazepam, Oxazepam, Lorazepam, Diazepam, Temazepam, and Bromazepam}or similar)

2.4.3.6 STANDARDS

Obtain as required.

Standards (in methanol)	*Potential Vendors*
Alprazolam	Cerilliant A-903, Alltech 01427
α-Hydroxyalprazolam	Cerilliant A-907, Alltech 01545
Bromazepam	Cerilliant B-903, Alltech 6013563
Chlordiazepoxide	Cerilliant C-022
Norchlordiazepoxide	Alltech 6013433
Clonazepam	Cerilliant C-907, Alltech 017943
7-Aminoclonazepam	Cerilliant A-916
Diazepam	Cerilliant D-907, Alltech 017213
Estazolam	Cerilliant E-901, Alltech 601560
Flurazepam	Cerilliant F-003, Alltech 017953
Flunitrazepam	Cerilliant F-907, Alltech 6015123
7-aminoflunitrazepam	Cerilliant A-911
Lorazepam	Cerilliant L-901, Alltech 013583
Medazepam	Alltech 013578
Midazolam	Cerilliant M-908
4-hydroxymidazolam	Cerilliant H-902
Nitrazepam	Cerilliant N-906, Alltech 017933
Nordiazepam	Cerilliant N-905, Alltech 013453
Oxazepam	Cerilliant O-902, Alltech 013703
Temazepam	Cerilliant T-907, Alltech 013833
Triazolam	Cerilliant T-910, Alltech 014283
α-Hydroxytriazolam	Cerilliant T-911
Oxazepam Glucuronide	Alltech 01541

Refer to an opiate SOP for opiate standards.

2.4.3.7 **PROCEDURE**

2.4.3.7.1 Standard Preparation

Prepare a minimum of the following non-extracted standards. Additional standards should be prepared as necessary indicated by *current drug therapy*.

- TMS derivative: Oxazepam, temazepam, nordiazepam and lorazepam. Add 10µL of stock solution to labeled tapered bottom centrifuge tube. Derivatize as described in 2.4.3.7.6.

2.4.3.7.2 Initial set-up

Label extraction tubes, tapered-bottom derivatization tubes and GC/MS vials with microinserts as follows for both the underivatized/ethyl acetate (EA) and derivatized extractions (TMS) for the negative control (NC), positive control (PC) and appropriate laboratory numbers without prefix.

2.4.3.7.3 Sample Preparation

- Transfer 6-mL of urine specimen, negative urine or positive control to extraction tube.
- If a drug mix is used as an additional control, add drug mix solution to 6-mL of negative urine. For a 0.1mg/mL solution, use 100µL.

2.4.3.7.4 Sample Hydrolysis

- To each extraction tube add:
  - 200µL 2M acetate buffer
  - 100µL β-Glucuronidase
- Cap and vortex *gently* to mix.
- Place in 60°C laboratory oven or waterbath for two hours.
- Allow samples to cool before proceeding with solvent extraction.

2.4.3.7.5 Extraction

- To each tube add:
  - 2mL 50mM sodium bicarbonate to each tube.
  - 4mL of chloroform/isopropanol {9:1}.
- Rock for 15 minutes.
- Centrifuge at 3500 rpm for 15 minutes.
- Transfer lower organic phase from tube into labeled tapered bottom tube.
- Evaporate solvent to dryness, under a gentle stream of nitrogen, in TurboVap at 37°C.

2.4.3.7.6 Derivatization

- To one set of tapered-bottom tubes add:
  - 20µL ethyl acetate
  - 30µL of MSTFA.
- Cap tubes with snap caps.
- Vortex.
- Heat tube for 15 minutes in 75°C dry bath.
- Remove from heat and allow to cool.
- Transfer derivative to labeled GC/MS ALS vial with microinsert.

2.4.3.7.7 Reconstitution with Ethyl Acetate

- To remaining set of tapered bottom tubes, add:
  - 50µL ethyl acetate.
  - Vortex.
  - Transfer extract to labeled GC/MS ALS vial with microinsert.

2.4.3.8 GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) ANALYSIS

2.4.3.8.1 Analysis Parameters

2.4.3.8.1.1 Inject 1 µL into GC/MS using the ALS.

2.4.3.8.1.2 Analyze sample extract in full scan acquisition.

2.4.3.8.1.3 Refer to attached GC/MSD method printout for current analysis parameters.

2.4.3.8.2 Detection and Identification Criteria

2.4.3.8.2.1 The presence of a drug compound can be established if there are no significant differences in the retention time and mass spectra for the sample versus standards.

- Acceptable retention time window is +/- 5%.

2.4.3.9 APPLICATION OF METHOD TO OTHER ANALYTES

2.4.3.9.1 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.

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

2.4.3.9.3 Appropriate standards should be prepared as required.

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.