Idaho State Police
Forensic Services

Approval for Quality System Controlled Documents

Discipline/Name of Document: Toxicology
2.4.3 Qualitative Benzodiazepines and Ancillary Compounds in Urine

Revision Number: 3

Issue Date: 5/07/2007

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5/7/07
Date Signed

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 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. 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, 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 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 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 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] 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
2.4.3.3.2 Laboratory oven or waterbath
2.4.3.3.3 Laboratory Centrifuge

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 Drybath
2.4.3.3.6 Evaporative Concentrator equipped with nitrogen tank.
2.4.3.3.7 16X100mm 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 Microinsert
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, 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 5.12 for preparation instructions.

2.4.3.4.1 Glucuronidase
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 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 the working solution described below and/or obtained commercially.

2.4.3.5.1.1 **Positive Control Stock Solution**

Obtain 1mg/mL stock benzodiazepine class drug reference material solutions through Cerilliant, Alltech, Sigma or other appropriate vendor. Benzodiazepine reference material mixes may be employed.

2.4.3.5.1.2 **10ng/µL Positive Control Working Solution**

Add 100µL stock solution to 10mL methanol. A minimum of two benzodiazepine compounds must be included in the control. At least one of the compounds must form a TMS derivative.

Suitable pair includes alprazolam and α-Hydroxyalprazolam (forms TMS).

2.4.3.5.2 Non-extracted Reference Material

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

2.4.3.5.2.2 Dilute 1.0mg/mL reference material solution to 250ug/mL with methanol.

2.4.3.5.3 Non-extracted Derivatized Reference Material

2.4.3.5.3.1 Derivatize reference material as necessary based on current drug therapy and examination of GC/MSD data.

2.4.3.5.3.2 Add 50µL of working solution to labeled tapered bottom centrifuge tube. Derivatize as described in 2.4.3.6.9.

2.4.3.5.4 Internal Standard

2.4.3.5.4.1 **Stock Solutions**
1 mg/mL Prazepam

2.4.3.5.4.2 **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.

2.4.3.5.5 Conjugated Controls

2.4.3.5.5.1 Control is used to verify the β-glucuronidase enzyme's ability to cleave glucuronide conjugated compounds.

2.4.3.5.5.2 Urinary Oxazepam Glucuronide or Morphine Glucuronide can either be spiked into urine with working solution or commercially obtained.

2.4.3.5.5.3 Glucuronide conjugated drug must be at a minimum of 375ng/mL.

2.4.3.5.5.4 **Conjugated Stock Solution**
Obtain 1mg/mL stock oxazepam glucuronide or morphine glucuronide drug reference material solution through appropriate vendor.

2.4.3.5.5.5 **Conjugated Working Solution – 10ng/µL**
Add 100µL stock solution to 10mL methanol.

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

2.4.3.6 PROCEDURE

2.4.3.6.1 Initial set-up
Label extraction tubes and ALS vials with microinserts for the negative control, positive controls, Glucuronide controls (with and without glucuronidase) and appropriate laboratory numbers. Label tubes and GC/MS vials with microinserts for non-extracted derivatized reference material.

2.4.3.6.2 Positive Control Sample

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

2.4.3.6.2.2 For a prepared 500ng/mL spiked positive control, pipette 300µL mixed working control solution to 6mL negative urine.

2.4.3.6.3 Conjugated Reference Material Controls

2.4.3.6.3.1 For a 500ng/mL spiked control, pipette 300µL of conjugated working control solution into two 6mL aliquots of negative urine.

2.4.3.6.3.2 Prepare one control with and one control without the addition of glucuronidase.

2.4.3.6.4 Casework Samples
Transfer 6mL casework samples to screw top extraction tube.

2.4.3.6.5 Negative Control Sample
Transfer 6mL negative urine to extraction tube.

2.4.3.6.6 Internal Standard Addition

2.4.3.6.6.1 To each prepared sample, add 300µL of internal standard. Vortex to mix.

2.4.3.6.6.2 Allow samples to stand 10 minutes.

2.4.3.6.7 Sample Hydrolysis

2.4.3.6.7.1 Add 200µL 2M acetate buffer to each tube.

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

2.4.3.6.7.3 Place all tubes in 60°C laboratory oven or waterbath for two hours.

3.4.3.6.7.4 Allow samples to cool before proceeding with solvent extraction.

2.4.3.6.8 Extraction

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

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

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

2.4.3.6.8.4 Rock for 15 minutes.

2.4.3.6.8.5 Centrifuge at 3200 - 3400 rpm for 15 minutes.

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

2.4.3.6.8.7 Evaporate solvent to dryness under a gentle stream of nitrogen at ≤37°C.

2.4.3.6.9 Derivatization

2.4.3.6.9.1 To one set of tapered-bottom tubes add 20µL ethyl acetate and 30µL of silylating agent.

2.4.3.6.9.2 Cap tubes. Vortex.

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

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

2.4.3.6.10 Reconstitution with Ethyl Acetate

2.4.3.6.10.1 To remaining set of extraction tubes, add 50µL ethyl acetate. Vortex.

2.4.3.6.10.2 Transfer extract to labeled ALS vial with microinsert.

2.4.3.6.11 Preparation for Analysis Run

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

2.4.3.6.11.2 Load samples, reference material, blank and controls into the quadrant rack as noted in the sequence table.

2.4.3.6.12 GC-MSD Analysis Parameters

2.4.3.6.12.1 Refer to instrument METHOD for current analysis parameters.

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

2.4.3.6.13 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 APPLICATION OF METHOD 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 and hydrocodone.

2.4.3.7.3 Appropriate standards should be prepared as required.

2.4.3.8 QUALITY ASSURANCE REQUIREMENTS

2.4.3.8.1 General

2.4.3.8.1.1 Urine samples are to be stored frozen until allowed to thaw prior to analysis.

2.4.3.8.1.2 Urine samples are to be stored under refrigeration after aliquots are removed for analysis.

2.4.3.8.1.3 Post analysis, urine samples are to be stored frozen until appropriate disposal date.

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

2.4.3.9.2 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.

Revision History

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Revision #	Issue Date	Revisions
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. Reformatted.