



Standard Operating Procedure  
Screening Test for Benzodiazepines in Urine

1 Background\*:

The benzodiazepines are a structurally-related class of drugs that have a wide spectrum of sedative-hypnotic, muscle-relaxant, anxiolytic and anti-convulsant effects.<sup>2</sup> The metabolism of barbiturates is complicated. Benzodiazepines with N1 alkyl substituents suffer N-dealkylation resulting in N-desmethyldiazepam as a common metabolite for several of the benzodiazepines. Hydroxylation at the number 3 carbon follows, and the drugs are then excreted as glucuronide conjugates. Oxazepam is a common metabolite of temazepam, diazepam, clorazepate, prazepam, halazepam and chlordiazepoxide.<sup>2</sup> Other benzodiazepines are also metabolized by hydroxylation and glucuronidation<sup>2,3</sup>.

Administration of benzodiazepines alone, even in overdose, does not seem to be associated with prolonged CNS depression<sup>4,5,6</sup>. The use of alcohol together with benzodiazepines is associated with a danger of drug-related death<sup>4-9</sup>. Overdose of benzodiazepines in pregnancy does not seem to be associated with impaired fetal outcome.<sup>10</sup>

Perhaps because of their wide availability, the abuse of benzodiazepines has become a widespread problem. Evidence points to two distinct patterns of abuse, that associated with prolonged prescription of benzodiazepines alone, and that of benzodiazepines as a part of multiple drug abuse.<sup>11</sup> This may reflect possible interactions of benzodiazepine and opiate agonists in the CNS.<sup>12</sup>

\*Taken from DPC Coat-A-Count Benzodiazepines in Urine Instruction Manual, with permission.

2 References:

- 2.1 National Institute on Drug Abuse (NIDA) Urine testing for drugs of abuse Research Monograph 1986; 73.
- 2.2 Harvey SC. Hypnotics and Sedatives In: Gilman AG, Goodman LS, Rall TW, Murad F, editors. The Pharmacological Basis of Therapeutics, 7th ed. New York: Macmillan, 1985: 339-51.
- 2.3 Abernathy DR, et al. Pharmacokinetics of alprazolam. J Clin Psychiatry 1983; 44: 45-7.
- 2.4 Greenblatt DJ, et al. Acute overdosage with benzodiazepine derivatives. Clin Pharmacol Ther 1977; 21: 497-514.
- 2.5 Divoll M, et al. Benzodiazepine overdosage: plasma concentrations and clinical outcome. Psychopharmacol 1981; 73: 381-3.
- 2.6 Jatlow P, et al. Serum diazepam derivatives in overdosage. Am J Clin Pathol 1979; 72: 571-7.
- 2.7 Kelley RC, et al. Association of benzodiazepines with death in a major metropolitan area. J Anal Toxicol 1982; 6: 91-6.
- 2.8 Piesiur-Strehlow B, et al. Mortality of patients dependent on benzodiazepines. Acta Psychiatr Scand 1986; 73: 330-55.
- 2.9 Sellers EM, Busto U. Benzodiazepines and ethanol: assessments of the effects and consequences of psychotropic drug interactions. J Clin Psychopharmacol 1982; 2: 249-62.
- 2.10 Cerqueira MI, et al. Intoxication by benzodiazepines during pregnancy. Lancet 1988; i: 1341.
- 2.11 Busto U, et al. Patterns of benzodiazepine abuse and dependence. Br J Addict 1986; 81: 87-94.
- 2.12 Moreau J-L, Pieri L. Effects of intrathecally administered benzodiazepine receptor agonist, antagonist and inverse agonist on morphine-induced spinal nociceptive reflex. Br J Pharmacol 1988; 93:964-8.
- 2.13 Coat-A-Count Benzodiazepines In Urine, Diagnostic Products Corporation, 1997.



2.14 Instruction Sheet, Benzodiazepines Direct RIA Kit, Immunalysis Corporation, June 2001.

### 3 Scope and Application:

- 3.1 This procedure describes the use of the Immunalysis Benzodiazepines Direct RIA Kit system as a screening test (qualitative determination) for the presence of benzodiazepines in both ante- and post-mortem urine samples.
- 3.2 If required, blood may be used in place of urine.
- 3.3 This procedure is intended strictly for *in vitro* diagnostic use in the context of a program involving an established confirmatory test for benzodiazepines. Gas Chromatography/mass spectrometry (GC/MS) is the confirmatory method employed by the SASL.
- 3.4 Clinical considerations and professional judgement must be applied to any drug of abuse test result, particularly when preliminary positive results are used.
- 3.5 The detection limit (or "minimum detectable dose") of this procedure is approximately 5 ng/mL
- 3.6 The threshold for signaling a "Positive" test has been set to 100 ng/mL.

### 4 Summary of the Analytical Method

The Immunalysis Benzodiazepines Direct RIA Kit procedure is a solid-phase radioimmunoassay, wherein <sup>125</sup>I-labeled benzodiazepine competes for a fixed time with benzodiazepines in the sample for antibody sites. The sample is mixed in a scintillation vial with a solution containing benzodiazepine antibody, and radio-labeled benzodiazepine. Any benzodiazepines present in the sample compete with the labeled benzodiazepine for the added antibody, decreasing the amount of labeled benzodiazepine that subsequently precipitates out. The antibody which has been bound to the benzodiazepine present in the sample and the added benzodiazepine is precipitated through the addition of a second antibody-PEG complex. After centrifugation, the supernatant is decanted to terminate the competition and to isolate the antibody-bound fraction of the radio-labeled benzodiazepine. The presence or absence of benzodiazepines is determined by comparing the number of counts obtained for the sample, to that for a standard with a known benzodiazepine concentration.

### 5 Sample Handling and Preservation:

- 5.1 Store the unused portions of the Immunalysis Benzodiazepines Direct RIA Kit at 2-8 °C, in a secured refrigerator designated for radioactive materials.
- 5.2 Collect urine without preservative. The specimen can be refrigerated or frozen. If the specimen is cloudy, it should be cleared by filtration or centrifugation before use, and mixed by gentle swirling.
- 5.3 If adulteration of the specimen is suspected, do not analyze the sample until after speaking with the submitting laboratory.
- 5.4 The sample must be analyzed within 14 days of receipt.
- 5.5 Samples containing radioactive contamination from previous *in vivo* diagnostic procedures are not compatible with this method.

### 6 Safety:

- 6.1 The toxicity or carcinogenicity of all of the reagents and standards used in this method has not been fully established. All of the chemicals should be regarded as potential health hazards and exposure to these compounds should be avoided.



- 6.2 The samples, and some of the components of the Immunoanalysis Benzodiazepines Direct RIA Kit contain human source material, or other potentially bio-hazardous materials. All standards and samples must be handled according to the accepted procedures laid out in the SASL document entitled "Blood Borne Pathogen and Bio-Safety".
- 6.3 This procedure involves the use of isotopically labeled reagents. These reagents must be handled in accordance with the procedures described in the SASL radiation safety protocol.
- 6.4 Samples and standards considered to be hazardous must be prepared for disposal by the accepted procedures laid out by the SASL.
- 6.5 Since this procedure has potential exposure to both radioisotopes and bio-hazards, the use of both the Personal Protective Equipment (PPE) and the Engineering Controls (EC) are necessary. Follow the SASL guidelines for the use of the Personal Protective Equipment and Engineering Controls.
- 6.6 Sodium azide has been added to certain components of the Immunoanalysis Benzodiazepines Direct RIA Kit as an antibacterial agent. The concentration of sodium azide is below the regulatory limit (0.1 g/dL) in all lyophilized reagents. To prevent buildup of explosive metal azides in lead and copper plumbing, reagents should be discarded into sewage only if diluted and flushed with large volumes of water.

## 7 Interferences:

- 7.1 The anti-serum is broadly specific for benzodiazepines including:  
  
Alprazolam, Alpha-OH Alprazolam, Chlordiazepoxide, Chlorazepate, Demoxepam, Diazepam, Flurazepam, Flunitrazepam, Halazepam, Lorazepam, Medazepam, Nitrazepam, Prazepam, Temazepam, Triazolam.
- 7.2 Based on a review of the literature and other studies, the manufacturer of the Immunoanalysis RIA system has found that there does not appear to be any substances, other than related drugs, that could cause false positive reactions.
- 7.3 The anti-serum is highly specific for benzodiazepines, with an extremely low cross-reactivity to other compounds that may be present in patient samples. The following compounds were found to be non-detectable by the double antibody benzodiazepines procedure at a level of 10,000 ng/mL.  
  
Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Ascorbic acid, Atropine, Benzoylcegonine, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydro-carbamazepine, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Methamphetamine, Mephenytoin, "-Methyl-"propylsuccinimide, Methyl PEMA, Methsuximide, 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phenuximide, PEMA, Primidone, Phencyclidine, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, THC-COOH.
- 7.4 Other substances and/or factors not listed in the above, e.g.. technical or procedural errors, may interfere with the test and cause a false positive result

## 8 Equipment and Supplies:

- 8.1 Immunoanalysis Benzodiazepines Direct RIA Kit. Immunoanalysis Part Number 114-0100.
- 8.2 Gamma counter, Logic Systems Model 111 (or equivalent).
- 8.3 Vortex mixer.
- 8.4 Centrifuge.



- 8.5 12x75 mm polypropylene scintillation vials.
- 8.6 Micro-pipettes: 25  $\mu$ L, 100  $\mu$ L, and 200  $\mu$ L.
- 8.7 Class A Volumetric Pipettes.
- 8.8 Class A Volumetric Flasks.
- 8.9 Foam decanting rack.

## 9 Reagents:

- 9.1 Type II water.
- 9.2 Stock Benzodiazepine Solution: The Stock Benzodiazepine Solution is supplied by the manufacturer in a ready to use liquid form. Store refrigerated. The Stock Benzodiazepine Solution is stable for 30 days (or until the expiration date marked on the vial ) when refrigerated at 2-8  $^{\circ}$ C.

**Note: The Stock Benzodiazepine Solution is the radioactive component of the procedure. The Solution, along with anything contacted by the solution, must be handled properly, and must be disposed of as a radioactive waste.**

- 9.3 Benzodiazepine Antibody: The Benzodiazepine Antibody is a sheep anti-benzodiazepine serum, supplied by the manufacturer in a ready to use liquid form. The Benzodiazepine Antibody is stable when refrigerated at 2-8  $^{\circ}$ C.
- 9.4 Second Antibody: The Second Antibody is complexed with PEG, and is supplied by the manufacturer in a ready to use liquid. The Second Antibody is stable for 30 days after opening when it is refrigerated at 2-8  $^{\circ}$ C.
- 9.5 Benzodiazepine-Free Urine:

## 10 Standards:

- 10.1 Primary Oxazepam Standard, 1000  $\mu$ gm/mL: Cerilliant Catalog Number O-XXX, or equivalent.
- 10.2 Benzodiazepine Calibration Standards, 0, 25, 50, 100, and 250 ng/mL oxazepam : The Benzodiazepine Calibration Standards are supplied by the manufacturer in a ready to use liquid form. The Calibration Standards are supplied as a set of 6 vials. The vials are labeled "A" through "F", with "A" being the 0 ng/mL and "F" being the 1,000 ng/mL Standard. The calibration standards are stable for 30 days (or until the expiration date marked on the vial) after opening, when refrigerated at 2-8  $^{\circ}$ C. If necessary, the Calibration Standards may be stored for up to 6 months by freezing -20  $^{\circ}$ C.
- 10.3 Benzodiazepine Control, 150 ng/mL oxazepam: Add 650  $\mu$ L of the Benzodiazepines-Free Urine (Section 9.5) to a clean screw-top sample vial. Add 450  $\mu$ L of the 1,000 ng/mL Benzodiazepine Calibration Standard (Section 10.1) to the vial. Mix the contents by inverting the vial 15 times. The Control is stable for 30 days (or until the expiration date marked on the vial ) after opening, when refrigerated at 2-8  $^{\circ}$ C. If necessary, the Control may be stored for up to 6 months by freezing -20  $^{\circ}$ C.
- 10.4 Benzodiazepine Positive Standard, 100 ng/mL oxazepam: The Benzodiazepine Positive Standard is supplied by the manufacturer in a ready to use liquid form. The Positive Standard is stable for 30 days (or until the expiration date marked on the vial ) after opening, when refrigerated at 2-8  $^{\circ}$ C.
- 10.5 Benzodiazepine Negative Standard, 0 ng/mL oxazepam: The Benzodiazepine Negative Standard is supplied by the manufacturer in a ready to use liquid form. The Negative Standard is stable for 30 days (or until the expiration date marked on the vial ) after opening, when refrigerated at 2-8  $^{\circ}$ C.



## 11 Procedure

**Note: All of the Reagents and Standards must be at room temperature (15-28 °C) before use.**

**Note: Be careful to pipette the samples, standards, and reagents directly to the bottom of the tubes.**

### 11.1 On Receipt of the Immunalysis Kit

11.1.1 Record the date of receipt of the kit on a new log sheet.

11.1.2 Label a new polypropylene scintillation vial for each of the Calibration Standards (Section 10.2).

11.1.3 Pipette 25  $\mu$ L of each of the Benzodiazepine Calibration Standards (Section 10.1) into the respective vials.

11.1.4 Add 200  $\mu$ L of the Stock Benzodiazepine Solution (Section 9.2) to each of the vials.

**Note: The pipette tip must be discarded of in the container designated for solid radioactive wastes.**

11.1.5 Add 100  $\mu$ L of the Benzodiazepine Antibody (Section 9.3) to each of the vials.

11.1.6 Add 200  $\mu$ L of the Second Antibody (Section 9.4) to each of the vials.

11.1.7 Vortex all of the vials.

11.1.8 Incubate the vials for one hour at room temperature (15-28 °C).

11.1.9 Centrifuge the vials for 20 minutes.

11.1.10 Thoroughly decant the liquid from all of the vials, taking care to retain all of the precipitate. Allow the vials to stand inverted for 10 minutes to fully drain the supernatant. Strike the vials gently on absorbent paper to shake off the last of the residual moisture. Wipe the rim with the paper to ensure the removal of any remaining liquid.

**Note: All of the supernatant liquid must be disposed of in the shielded bottle designated for the disposal of liquid radioactive wastes. The absorbent paper must be disposed of in the shielded box designated for the disposal of solid radioactive wastes.**

11.1.11 Using the gamma counter, count the standards for 1.5 minutes. Record the count for each of the vials.

**Note: Once counted, the liquid free tubes must be disposed of in the shielded box designated for the disposal of solid radioactive wastes.**

11.1.12 Plot and perform a linear regression of the resulting data.

11.1.13 Attach the results of the regression analysis to the log sheet, and file the log sheet in the binder for RIA logs.

### 11.2 Analytical Procedure

11.2.1 Label three new polypropylene scintillation vials "Positive", "Negative", and "Control". Label an additional vial for each of the samples.

11.2.2 Pipette 25  $\mu$ L of the Benzodiazepine Negative Standard (Section 10.5) into the vial labeled "Negative".

11.2.3 Pipette 25  $\mu$ L of the Benzodiazepine Positive Standard (Section 10.4) into the vial labeled "Positive".

11.2.4 Pipette 25  $\mu$ L of the Benzodiazepine Control (Section 10.3) into the vial labeled "Control".



- 11.2.5 Pipette 25  $\mu\text{L}$  of each sample into its correspondingly labeled vial.
- 11.2.6 Add 200  $\mu\text{L}$  of the Stock Benzodiazepine Solution (Section 9.2) to each of the vials.

**Note: The pipette tip must be discarded of in the container designated for solid radioactive wastes.**

- 11.2.7 Add 100  $\mu\text{L}$  of the Benzodiazepine Antibody (Section 9.3) to each of the vials.
- 11.2.8 Add 200  $\mu\text{L}$  of the Second Antibody (Section 9.4) to each of the vials.
- 11.2.9 Vortex all of the vials.
- 11.2.10 Incubate the vials for one hour at room temperature (15-28  $^{\circ}\text{C}$ ).
- 11.2.11 Centrifuge the vials for 20 minutes.
- 11.2.12 Thoroughly decant the liquid from all of the vials, taking care to retain all of the precipitate. Allow the vials to stand inverted for 10 minutes to fully drain the supernatant. Strike the vials gently on absorbent paper to shake off the last of the residual moisture. Wipe the rim with the paper to ensure the removal of any remaining liquid.

**Note: All of the supernatant liquid must be disposed of in the shielded bottle designated for the disposal of liquid radioactive wastes. The absorbent paper must be disposed of in the shielded box designated for the disposal of solid radioactive wastes.**

- 11.2.13 Using the gamma counter, count the standards and samples for 1.5 minutes. Record the count for each of the vials.

**Note: Once counted, the liquid free tubes must be disposed of in the shielded box designated for the disposal of solid radioactive wastes.**

## 12 Quality Control

- 12.1 The regression analysis for the calibration curve created in Section 11.1 (On Receipt of the Immunalysis Kit) should exhibit a correlation coefficient of 0.99 or greater. If the fit is less than 0.99, the calibration curve should be re-run.
- 12.2 Samples should be divided into batches of not more than 10 samples. A Negative, Positive, and Control should be run with each batch of samples.
- 12.3 The counts obtained for the Control should be compared to the calibration curve created in Section 11.1. If the concentration calculated for the control is more than 10% from the expected value, the batch of samples should be re-run.
- 12.4 The air displacement pipettes must be calibrated and maintained according to the SASL procedure entitled "Air Displacement Pipettes".
- 12.5 The procedure calls for centrifuging at  $3000\times g$  for 15 minutes. Lower accelerations are satisfactory only if the centrifugation time is increased appropriately, for example, centrifuging for 30 minutes at  $1500\times g$ . A high-speed, refrigerated centrifuge is desirable but not essential. Use the equation in Section 13.2 to calculate the acceleration of the centrifuge.
- 12.6 Do not use the standards or reagents beyond their expiration dates.

**Note: All of the parts must be disposed of in the shielded bottle designated for the disposal of liquid radioactive wastes. The absorbent paper must be disposed of in the shielded box designated for the disposal of solid radioactive wastes.**



### 13 Calculations

13.1 Compare the counts for the “Sample” to those for the “Positive”.

13.1.1 If the counts for the “Sample” are greater than the counts for the “Positive”, the result is negative (i.e. within the precision of the procedure, the sample contains less than 100 ng/mL benzodiazepine (as oxazepam)).

13.1.2 If the counts for the “Sample” are lower than the counts for the “Positive”, the result is positive, and the sample contains more than 100 ng/mL benzodiazepine (as THC oxazepam), within the precision of the procedure.

13.2 Relating the acceleration to the speed of a centrifuge:

$$F = 28.38 \times \left( \frac{S}{1000} \right)^2 \times r$$

where:

F = Force (g)

S = Speed (rpm)

r = Radius of the rotor (inches)

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#### Authorizing Signatures:

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Revision Log

<b>Revision Number</b>	<b>Section(s) Revised</b>	<b>Effective Date</b>	<b>Revision</b>
1.00	All	10/28/03	Finished version to go to reviewers.
1.50	All	1/18/04	Edited for use with the new vendor (Immunoanalysis).