

Flurazepam	800	12.5
2-OH-ethyl- flurazepam	50	13.3
Flunitrazepam	25	31
-desmethyl-Flunit- razepam	00	12.5
Halazepam	50	13.3
Lorazepam	725	13.8
Lorazepam Gluc.	800	5.5
Lormetazepam	550	18
Medazepam	98	25
Midazolam	100	9
Nitrazepam	00	33
Nordiazepam	7	150
Oxazepam Gluc.	900	11
Prazepam	200	8.3
Temazepam	20	83
Triazolam	50	10.5
á-OH Triazolam	5000	<2

6. Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 10,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (2 ng/ml). Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Ascorbic acid, Atropine, Benzoyllecgonine, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, 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, Phensuximide, PEMA, Primidone, Phencyclidine, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, THC-COOH

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BENZODIAZEPINES DIRECT ELISA KIT

Catalog No. BQ 214-096 (96 tests)

INTENDED TO USE

The Benzodiazepines Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

SUMMARY AND EXPLANATION

The Benzodiazepines Direct ELISA Kit is a sensitive in-vitro test to detect the presence of Benzodiazepines in samples such as whole blood, serum, plasma and urine. Benzodiazepines - are a class of widely prescribed central nervous system depressant drugs with sedative, muscle relaxant and anti-convulsant activities. Chronic use does result in moderate dependence and tolerance to the drug. The use of alcohol in conjunction with the benzodiazepines has been shown to have a greater suppressive effect to the central nervous system than that attributable to either chemical alone. Benzodiazepines are usually administered orally and are absorbed rapidly. The metabolism of Benzodiazepines is mainly in the liver and excreted in the urine as a variety of structurally related metabolites. Metabolic similarities include removal of substituents from the B ring of the 1,4 benzodiazepines and alpha hydroxylation of the triazolobenzodiazepines, hydroxylation of the 3 position carbon of the B ring and conjugation of hydroxylated metabolites followed by urinary excretion as glucuronides.(6)

PRINCIPLES OF THE TEST

The Benzodiazepines Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled Benzodiazepine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 2 ng/ml. The Benzodiazepines Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs an Oxazepam directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This results in extremely small sample size reducing matrix effects and interference with binding proteins(s) or other Macromolecules.

MATERIALS PROVIDED	96 TESTS
Microwells coated polyclonal anti-Oxazepam	12x8x1
Benzo- Conjugate	12.5 ml
Immunoanalysis Positive Reference Standard	1 ml
Negative Standard	1 ml
Stop Solution	12.5 ml
TMB Substrate	15 ml

MATERIALS NOT PROVIDED

1. 12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).
2. Manual or electronic micropipets (single channel / multichannel) or automated pipetting stations.
3. Refrigerator (for kit storage).
4. Interval Timer.
5. Wash bottle or Plate Washer.
6. Microplate reader capable of reading at 450 nm. And 650 nm.

WARNING AND PRECAUTIONS

1. This kit is designed for Research Use Only. There should be no eating or drinking within work area. Always wear gloves and a protective lab coat.
2. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
3. Do not add sodium azide to samples as preservative Don't use external controls containing sodium azide.
4. Use disposable pipette tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue. Do not pour chromogenic substrate back into container after use.
5. Do not freeze reagents. Do not mix reagents from different kit lot numbers.
6. Keep reagents out of direct sunlight.
7. Handle stop reagent with care, since it is corrosive. Bring all reagents to room temperature.
8. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
9. Ensure the bag containing the micro-plate strips and desiccant is well sealed if only a partial plate is used.
10. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4 C.
11. A drop of greater than 50% in the A0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.
12. The Benzodiazepines Direct ELISA Kit is to be used with human samples, such as urine, whole blood, serum and plasma. Have not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.

SPECIMEN HANDLING

Urine samples should be stored at 2 - 4 C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

ASSAY PROCEDURE.

All reagents must be brought to room temperature (20-25 C) before use. The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:10 for a Oxazepam cutoff of 200 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25 C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

Oxazepam	Absorbance ng/ml
0	3.043
25	0.750
50	0.548
100	0.388

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

Results

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is **POSITIVE** for Benzodiazepines. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called **NEGATIVE** for Benzodiazepines . Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

PERFORMANCE CHARACTERISTICS**1. Accuracy**

50 whole blood samples and 35 urine samples collected from presumed non-users were tested in the Benzodiazepines Direct ELISA Kit . One hundred percent of these normal samples measured negative at 100 ng/ml of oxazepam . Thirty whole blood samples which were previously confirmed positive for Benzodiazepines by GC-MS, 28 of the samples were found to be positive i.e. above the cut-off of 100 ng/ml of oxazepam equivalents the remaining 2 samples would have screened positive at an oxazepam cutoff of 50 ng/ml.

2. Precision

The precision of the BENZODIAZEPINES Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

3. Intra-assay Precision

Intra-assay precision was determined with reference controls. A 0,25,50 and 100 ng/ml Oxazepam standard was assayed five times in the same assay. The results are tabulated in Table.

Oxazepam (ng/ml)	Mean Abs	S.D.	C.V.%
0	3.061	0.163	5.3
25	0.997	0.091	9.1
50	0.665	0.047	6.6
100	0.458	0.051	11.2

4. Sensitivity

Assay sensitivity based on the minimum oxazepam concentration required to produce a four standard deviation from assay Ao is 2 ng/ml.

5. Specificity

The specificity of the Benzodiazepines ELISA for was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table.

Cross-reactivities related drugs		
Compound	Approx. ng/ml equivalent to 100 ng Oxazepam	Cross-reactivities
Alprazolam	85	118
â-OH Alprazolam	150	66
Glucuronide	1200	8.3
Bromazepam	525	19
Chlordiazepoxide	600	17
Clorazepate	480	21
Clonazepam	1200	8.3
7-amino-clonazepam	>5000	<2
Demoxepam	285	35
Diazepam	10	91
Estazolam	90	111