

Abstract

The performance characteristics of an immunoassay method for detecting benzodiazepines in oral fluid specimens were examined and compared with urine specimens. Oral fluid was obtained using a simple device that collects approximately 0.4 mL of oral fluid and dilutes it with 0.8 mL of diluent. When specimen or standard is added to an EIA well containing an oral fluid specimen positive for benzodiazepines, there is a competition between the drug and the enzyme-labeled hapten to bind the antibody fixed onto the EIA well. The EIA wells are then washed, substrate is added, and color is produced.

Matching oral fluid and urine specimens were collected from 144 benzodiazepines non-users and users from a drug treatment center and were first tested using an immunoassay cutoff of 1 ng/mL in oral fluids and 300 ng/mL in urine. Using a second aliquot, benzodiazepines confirmation in urine was performed by GC/MS and in oral fluids by GC/MS/MS. The combined immunoassay and GC/MS/MS procedures were completed with less than 600 µL of oral fluid.

The immunoassay was tested for precision, stability, and the effects of potential cross-reactants and interferents. The total precision for 20 days of testing calculated using the NCCLS EP5-T2 protocol yielded CV's less than 15%. The assay is specific for nordiazepam but also has good cross-reactivity to a wide range of other benzodiazepines. The assay exhibited no cross-reactivity to compounds such as acetylsalicylic acid, benzoylecgonine, caffeine, cotinine, d-amphetamine, ibuprofen, morphine, naproxen, penicillin, pseudoephedrine, and Δ⁹-THC. The following adulterants did not interfere with the assay: sugar water, toothpaste, cranberry juice, baking soda, cola, cough syrup, antiseptic, and orange juice.

The results yielded 84.7% agreement between oral fluid and urine EIA and 87.9% agreement between oral fluid EIA and GC/MS, suggesting that oral fluid may be a reliable matrix for benzodiazepines detection.

Key Words: Benzodiazepines, Oral fluid, Saliva

Study Design Section

- The benzodiazepine study included 144 matched oral fluid and urine samples from patients. There were no restrictions on food or beverage throughout the study, except 10 minutes prior to collection.
- All assays were screened by immunoassay at 1 ng/mL for oral fluid using nordiazepam as the calibrator and 300 ng/mL in urine. Oral fluid was confirmed by GC/MS/MS at 0.5 ng/mL for nordiazepam, oxazepam, chlordiazepoxide, diazepam and at 5 ng/mL for alprazolam and triazolam. Urine was confirmed by GC/MS at 300 ng/mL for nordiazepam, oxazepam, hydroxy-alprazolam and hydroxy-triazolam.

Background

Benzodiazepines can be detected in saliva following administration due to a pH-dependent exchange between the blood system and salivary glands. Detection times for benzodiazepines are dependent upon the specific benzodiazepine ingested with saliva detection times closely mimicking the detection times in blood at concentrations 1 to 10% of those found in blood.⁽¹⁾ The length of time following drug use for which a positive result may occur in saliva is dependent upon several factors including the frequency of use, the amount of drug used, and the protein binding of the benzodiazepine to plasma proteins. Only the free or unbound portion of the drug circulating in the plasma crosses from the blood to the saliva. In urine, detection times can range from 24 hours for a single dose of one of the low-dose benzodiazepines to 1 week for the chronic administration of the high dose benzodiazepines, whereas, in saliva, detection times have been reported up to 50 hours.^(2,3) The benzodiazepines are a very diverse group of compounds. The 1,4 benzodiazepines including diazepam, nordiazepam, temazepam, and oxazepam share a common metabolic path and are generally administered in doses of 10 mg and higher. The nitrobenzodiazepines (flunitrazepam, clonazepam, and nitrazepam) and the triazolobenzodiazepines (alprazolam, triazolam, and estazolam) are typically prescribed in doses as low as 0.25 mg. Most benzodiazepines undergo extensive metabolism including conjugation to glucuronides prior to excretion in the urine.⁽²⁾

Saliva is a complex mixture of parotid, submandibular, sublingual, and minor salivary gland secretions mixed with mucin, bacteria, leukocytes, sloughed epithelial cells, and gingival crevicular fluid. The fact that benzodiazepines are present in oral fluid following human use is well documented.^(1,2)

Our research has been focused on the development and qualification of an oral fluid collection device combined with an immunoassay screening system for several of the benzodiazepines.

Oral Fluid Collection

The Intercept® Oral Specimen Collection Device was developed for the purpose of collecting oral fluid for diagnostic testing. The collection device consists of a treated, absorbent, cotton-fiber pad affixed to a nylon stick (Collection Pad) and a preservative solution in a plastic container (Specimen Vial). The collection pad is impregnated with a mixture of common salts and gelatin which creates a hypertonic environment and an increased



osmotic pressure wherever it contacts oral mucosal cells. The pad is placed in contact with the gingival mucosa (between the lower gum and cheek) which enhances the flow of mucosal transudate across the mucosal surfaces onto the absorbent cotton fibers of the pad. Following the collection period, the collection pad is placed into a vial containing a preservative solution which serves to inhibit the growth of oral micro-organisms recovered on the collection pad. The vial is sealed with a plastic cap and transported to a laboratory for processing and testing. Following processing, a fluid containing a mixture of saliva components and the preservative solution is recovered which is suitable for testing for the presence of benzodiazepines in the OTI Benzodiazepines Intercept® MICRO-PLATE EIA manufactured by OraSure Technologies, Bethlehem, PA and GC/MS/MS confirmation.

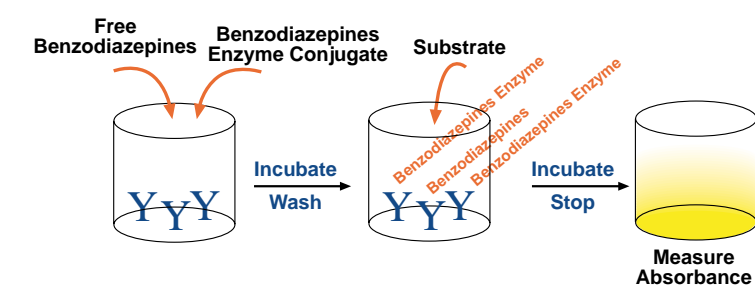


- Peel open pad package far enough to allow easy removal of the Collection Pad.
- Place pad between lower cheek and gum and gently rub back and forth until moist.
- Keep the pad in place for 2 minutes (maximum 5 minutes) while timing.
- Open vial in upright position.
- Insert pad into the blue liquid at the bottom of the vial.
- Break the pad handle by snapping it against the side of vial.
- Replace the cap with a snap.
- Place seal over top of vial and send sample to a laboratory for processing and testing.

Assay Format and Procedure

The OTI Benzodiazepines Intercept® MICRO-PLATE Enzyme Immunoassay (EIA) is a competitive immunoassay for the qualitative detection of benzodiazepines in oral fluid (see diagram below).

- Hold Collection Vial upright with the tip pointed up. Move the pad away from the vial tip by gently tapping the vial.
- Break the pointed tip of the vial off with your thumb, place a tube over the vial, and invert the tube and vial.
- Centrifuge at 600-800 x g for 15 minutes.
- Add 25 µL of sample or calibrator to each well. Test all samples in duplicate.
- Add 100 µL of Enzyme Conjugate to each test well and incubate for 30 minutes at room temperature (15-27°C) in the dark.
- Wash the plate using a suitable plate washer; wash each well 6 times with 300 µL of distilled water.
- Add 100 µL of Substrate Reagent to each well and incubate for 30 minutes at room temperature (15-27°C) in the dark.
- Add 100 µL of Stopping Reagent to each well and then measure the absorbance (A) at 450 nm. If the A is < cutoff, the sample is positive; if the A is > cutoff, the sample is negative.



The intra-assay precision was determined by analyzing each calibrator at n=16 per micro-plate and running four micro-plates within one day. The inter-assay precision was determined by analyzing 2 samples of each calibrator twice per day for 20 days. The assay precision allows discrimination ±50% around the 1 ng/mL cutoff level.

Nordiazepam (ng/mL)	Intra-Assay % CV (n=64)	Inter-Assay % CV (n=2, 20 days)
0	5.1	7.6
0.5	6.1	10.4
1	6.5	11
1.5	4.9	11.4

OTI BENZODIAZEPINES INTERCEPT® EIA DYNAMIC RANGE

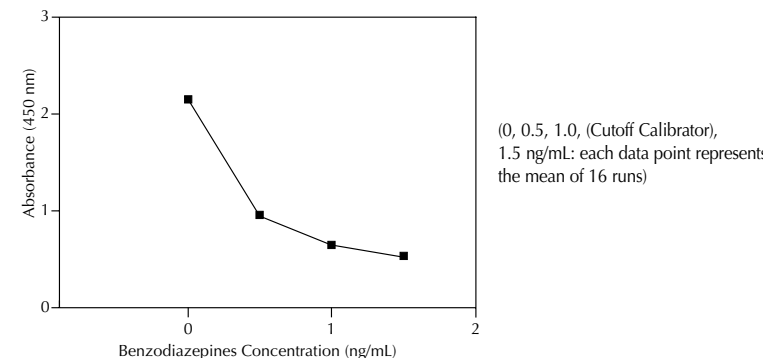


Figure 1

Cross Reactivity and Precision of the OTI Benzodiazepines Intercept® MICRO-PLATE EIA

Compound (structurally related)	Cross-Reactivity (%)
Alpha-Hydroxyalprazolam	9.9
Alpha-Hydroxytriazolam	15
Alprazolam	150
7-aminoflunitrazepam	6.4
Bromazepam	2.3
Clorazepate	70
Desalkylflurazepam	17
Diazepam	135
Estazolam	130
Flurazepam	49
2-Hydroxyethylflurazepam	8.3
Lorazepam	0.19
Medazepam	17
Midazolam	49
Nitrazepam	39
Norchlordiazepoxide	3.1
Oxazepam	7.1
Prazepam	110
Temazepam	55
Triazolam	26

The OTI Benzodiazepines Intercept® MICRO-PLATE EIA has a high percent cross-reactivity to several common benzodiazepines. The percent cross-reactivity was calculated based on a 1 ng/mL nordiazepam cutoff.

Effects of Common Adulterants on the OTI Benzodiazepines Intercept® MICRO-PLATE EIA

Nordiazepam 1 (ng/mL)	Sugar Water	Toothpaste	Cranberry Juice	Baking Soda	Cola	Cough Syrup	Antiseptic	Orange Juice
0	N	N	N	N	N	N	N	N
0.5	N	N	N	N	N	N	N	N
1	P	P	P	N	P	P	P	P
2	P	P	P	P	P	P	P	P

Each adulterant was spiked onto 2 devices per drug level. Devices were processed according to PI and were tested by EIA. Results were compared to a water control. None of the substances were found to interfere in the assay.

Sample	pH Range	pH Mean
Blank (random samples)	6.52 - 7.88	7.18
Orange Juice	6.58 - 7.15	6.90
TUMS®	7.25 - 7.89	7.69

Forty random oral fluid samples and oral fluid samples collected from ten volunteers five-minutes after consuming Orange Juice or TUMS® were collected using the Intercept® Drugs of Abuse Collection Device. The pH was measured for each sample. The results indicate that oral fluid samples under normal conditions, acidic conditions, and basic conditions have a narrow pH range of 6.5-7.9. Oral fluid samples with a pH of 6.0 to 7.0 will cause a depression in the assay but will not cause a positive result in the absence of drug. Oral fluid samples with a pH > 7.0 work acceptably in the assay.

Oral Fluid GC/MS/MS Methods

- Add d-5 nordiazepam internal standard to 500 µL of oral fluid sample and then dilute to 3.5 mLs with 0.1M acetate buffer (pH 5) and mix.
- This solution is then extracted on a RapidTrace workstation under the following conditions using Varian Bond Elute Certify 130mg 3mL columns:⁽⁴⁾

Condition	1.3 mL Methanol
Condition	1.3 mL Water
Condition	1.3 mL 0.1M (pH 6) phosphate buffer
Load	4 mL Sample
Rinse	1.3 mL Water
Rinse	1.3 mL 20% Acetonitrile in 0.1M (pH 6 phosphate buffer)
Dry	2 minutes
Rinse	1.3 mL Hexane
Purge	2 mL Ethyl Acetate
Collect	1.3 mL Ethyl Acetate
Collect	1.3 mL Ethyl Acetate
Rinse	2 mL Methanol
Rinse	2 mL Methanol
Purge	2 mL Water
- The solvent is dried under nitrogen at 55°C.
- 25 µL BSTFA+1% TCMS is added to the dried down extracts and heated at 70°C for 20 minutes. After allowing the derivatized extract to cool, 25 µL acetonitrile was added, and then the mixture was transferred to an autosampler vial.
- 2 µL is injected into the Varian 1200 GC/MS/MS and analyzed under the following conditions:

Column:	Chrompack CP-SIL 5 CB Low Bleed, 15 meter, 0.25 mm
Oven	100°C for 1 min., 20°C/min. up to 300°C, hold 3 minutes
Flow rate	1 mL/min.
Injector	280°C
Transfer line	280°C
Source	200°C
Multiplier	1800 mv
Collision gas	2 mtorr argon

Time On	Q1	Q2	Collision Energy
6.0	341	269	-20 volts
	341	290	-20 volts
	345	273	-20 volts
9.5	429	267	-23 volts
	429	341	-23 volts
10.0	256	165	-20 volts
	256	206	-20 volts
10.3	282	218	-22 volts
	282	247	-22 volts
12.0	279	224	-20 volts
	279	223	-20 volts
12.9	313	242	-20 volts
	313	277	-20 volts

Analyte	Description	Quant Ions
Nordiazepam d-5	Internal Std	273
Nordiazepam	Std	269, 290
Oxazepam	Std	267, 341
Diazepam	Std	165, 206
Chlordiazepoxide	Std	218, 247
Alprazolam	Std	224, 223
Triazolam	Std	242, 277

Accuracy

The clinical accuracy of the OTI Benzodiazepines Intercept® MICRO-PLATE assay was determined from specimens collected from self-reported benzodiazepines users and non-users. The oral fluid cutoffs for EIA and GC/MS/MS were 1 ng/mL and 0.5 ng/mL respectively. The cutoffs for urine were 300 ng/mL for both EIA and GC/MS.

A total of 144 oral fluid and urine specimen pairs were collected. All oral fluid and urine samples were tested by EIA. Of the 144 samples collected, all 144 oral fluid samples plus an additional 5 oral fluid samples to which there were not matching urine samples were confirmed by GC/MS/MS at OraSure Technologies, Inc. (Bethlehem, Pennsylvania). All urine samples that were positive by EIA and approximately 10% of the EIA negatives were confirmed by GC/MS by LabOne (Lenexa, Kansas).

GC/MS/MS of Intercept® Specimens (0.5 ng/mL cutoff)

OTI Intercept® EIA (1 ng/mL cutoff)	+	-
+	28	10
-	8	103

% Agreement = 87.9%

Urine EIA (300 ng/mL cutoff)

OTI Intercept® EIA (1 ng/mL cutoff)	+	-
+	23	12
-	10	99

% Agreement = 84.7%

Conclusion

- The OTI Benzodiazepines Intercept® MICRO-PLATE EIA has a broad cross-reactivity profile which includes alprazolam, triazolam, estazolam, midazolam, temazepam, oxazepam, nitrazepam, flurazepam, chlordiazepoxide, diazepam as well as many of the major metabolites of these compounds.
- All adulterants screened did not interfere with samples containing drug concentrations greater than 2 ng/mL.
- Of the 36 oral fluid patient samples that were positive for benzodiazepines by GC/MS/MS, concentrations ranged from 0.5 ng/mL to 49 ng/mL. The number of false positives would have been reduced if the GC/MS/MS scope better matched the cross-reactivity of the screening assay.
- Oral fluid screening has shown acceptable agreement with urine screening.

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