

## Abstract:

The performance characteristics of a method for detecting barbiturates and the time course of the elimination of barbiturates in oral fluid specimens were examined and compared with matching urine specimens. Oral fluid was obtained using the Intercept™ DOA Oral Specimen Collection device. To analyze specimens, a competitive EIA is used.

Matching Specimens collected simultaneously from 65 barbiturate non-users and several individuals who were prescribed phenobarbital or butalbital were first tested using an immunoassay cutoff of 20 ng/mL in oral fluids and 300 ng/mL in urine. Using a second aliquot, barbiturate confirmation in both oral fluid and urine was performed by GC/MS. The scope of the GC/MS analyses included amobarbital, butalbital, butabarbital, secobarbital and phenobarbital.

The immunoassay was tested for precision, stability, and the effects of potential cross-reactants and interferences. The total precision for 20 days of testing calculated using the NCCLS EP5-T2 protocol yielded CV's less than 10%. Refer to Cross-Reactivity and Interference tables for information.

The results yielded 92.2% agreement between oral fluid and urine and 97.6% agreement between oral fluid and GC/MS, suggesting that oral fluid may be a reliable matrix for barbiturate detection. The butalbital dose was a single 50 mg tablet and the phenobarbital was 120 mg tablet twice a day. The single dose butalbital subjects peaked between 0.5 and 2 hours after the dose at concentrations ranging from 67 to 268 ng/mL. Butalbital was detected for at least 48 hours after the dose.

## Background:

Barbiturates can be detected in saliva following administration due to a pH-dependent exchange between the blood system and salivary glands. Detection times for barbiturates are dependent upon the specific barbiturate ingested, with saliva detection times closely mimicking the detection times in blood.<sup>11</sup> The length of time following drug use for which a positive result may occur in saliva is dependent upon several factors including the frequency and amount of drug. In urine, detection times can range from 24 hours for the short-acting barbiturates to 3 weeks for the long-acting barbiturates, whereas, in saliva, detection times range from 3–50 hours.<sup>12,13</sup> Barbiturate metabolism depends upon the specific compound, with phenobarbital and barbital undergoing negligible metabolism while others such as secobarbital being extensively metabolized by oxidation. Only 1-5% of the dose is excreted unchanged in the urine.<sup>20</sup>

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 barbiturates are present in oral fluid following human use is well documented.<sup>11,21</sup>

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 Barbiturates. A dose response study was completed with single doses of butalbital over a time period of 48 hours.

## Oral Fluid Collection



The Intercept™ DOA 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 absorptive 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 barbiturates in the OTI Barbiturates Intercept™ MICRO-PLATE EIA manufactured by OraSure Technologies, Bethlehem, PA and GC/MS confirmation.

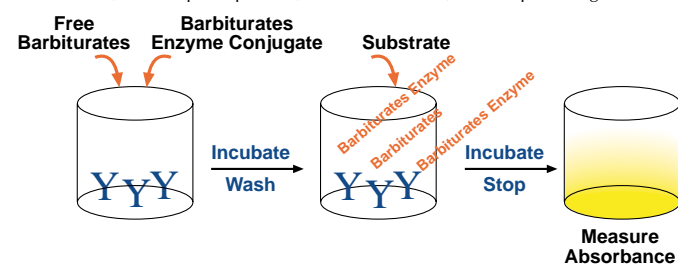


1. Peel open pad package far enough to allow easy removal of the Collection Pad.
2. Place pad between lower cheek and gum and gently rub back and forth until moist.
3. Keep the pad in place for 2 minutes (maximum 5 minutes) while timing.
4. Open vial in upright position.
5. Insert pad into the blue liquid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

## Assay Format and Procedure

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

1. Hold Collection Vial upright with the tip pointed up. Move the pad away from the vial tip by gently tapping the vial.
2. Break the pointed tip of the vial off with your thumb, place a tube over the vial, and invert the tube and vial.
3. Centrifuge at 6000-8000 x g for 15 minutes.
4. Add 50 µL of sample or calibrator to each well. Test all samples in duplicate.
5. Add 50 µL of Enzyme Conjugate to each test well and incubate for 30 minutes at room temperature (15-27°C) in the dark.
6. Wash the plate using a suitable plate washer; wash each well 6 times with 300 mL of distilled water.
7. Add 100 µL of Substrate Reagent to each well and incubate for 30 minutes at room temperature (15-27°C) in the dark.
8. 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.



## Cross Reactivity of the OTI Barbiturates Intercept™ MICRO-PLATE EIA

Compound (structurally related)	Cross Reactivity (%)
Allobarbital	23.6
Amobarbital	43.3
Aprobarbital	28.9
Barbital	9.1
Butabarbital	184.7
Butalbital	109.4
Hexobarbital	0.1
Mephobarbital	0.1
Methohexital	0.01
Pentobarbital	68.0
Phenobarbital	50.0
Talbutal	170.4

The OTI Barbiturates Intercept™ MICRO-PLATE EIA has a high percent cross-reactivity to several common barbiturates. The percent cross reactivity was calculated based on a 20 ng/mL secobarbital Cutoff.

## Precision of the OTI Barbiturates Intercept™ MICRO-PLATE EIA

Secobarbital (ng/mL)	Intra-Assay % CV (n=64)	Inter-Assay % CV (n=2, 20 days)
0	4.1	8.5
10	4.4	8.9
20	3.8	8.9
30	7.1	8.9
40	4.9	9.4

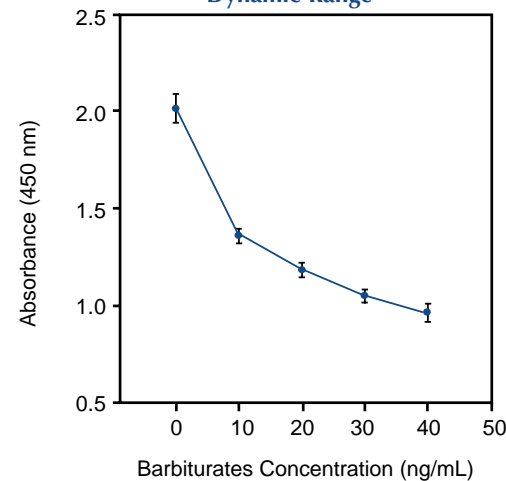
The intra-assay precision was determined by analyzing each calibrator at n=16 per micro-plate and running four micro-plates within one day. The inter-assay precision was determined by analyzing 2 samples of each calibrator twice per day for 20 days. The assay precision allows discrimination ±50% around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

## Effects of Common Adulterants on the OTI Barbiturates Intercept™ MICRO-PLATE EIA

Secobarbital (ng/mL)	Sugar	Toothpaste	Cranberry Juice	Orange Juice	Cola	Cough Syrup	Antiseptic
0	N	N	N	N	N	N	N
10	N	N	N	N	N	N	N
30	P	P	P	N	P	N	P
40	P	P	P	P	P	N	P

Each adulterant was consumed/used by a total of five volunteers. Oral fluid samples were collected from each volunteer using the Intercept, Drugs of Abuse Collection Device after a five-minute waiting period (current procedure is to wait 10-minutes) following the consumption/use of each material. This study shows that after a five-minute waiting period, all the common adulterants listed above except for cough syrup and orange juice should be cleared from the oral cavity and will not interfere with the assay. After a ten-minute waiting period, both the orange juice and the cough syrup had positive results with a drug concentration of ≥40 ng/mL.

## OTI Barbiturates Intercept™ EIA Dynamic Range



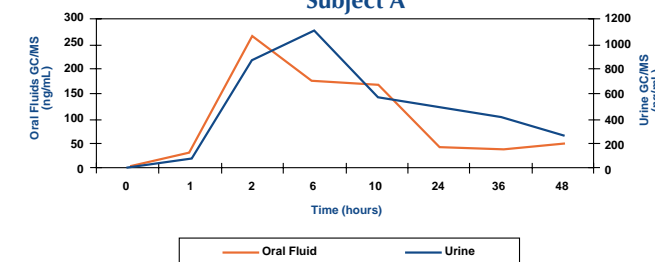
(0, 10, 20, (Cutoff Calibrator), 30, 40 ng/mL: each data point represents the mean of 16 runs)

Figure 1

## Study Design

- The Barbiturate study included 5 patients from which paired oral fluid/urine specimens were collected over a 48-hour time period after a single 50 mg dose of butalbital for migraine headaches. There were no restrictions on food or beverage throughout the study, except 10 minutes prior to collection. Data from two patients shown in Figure 2.
- A summary of butalbital concentrations in oral fluid and urine at 0, 1, 2, 6, 10, 24, 36, and 48 hours are shown in Figure 2.
- All assays were screened by immunoassay and GC/MS using a secobarbital cutoff of 20 ng/mL for oral fluid and 300 ng/mL in urine.

### Butalbital Oral Fluid GC/MS vs. Urine GC/MS Subject A



### Butalbital Oral Fluid GC/MS vs. Urine GC/MS Subject B

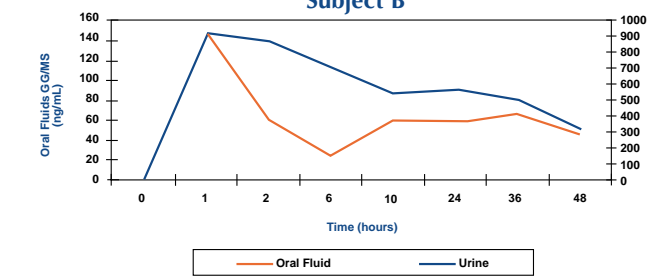


Figure 2

## Oral Fluid GC/MS Method

1. Add d-5 butalbital and d-5 phenobarbital internal standard to 500 µL of oral fluid and then dilute to 4 mL with 0.1M acetate buffer (pH 7) and mix.
2. This solution is then extracted on a RapidTrace under the following conditions using Varian Bond Elute Certify II 200mg 3mL columns:

Condition	2 mL Methanol
Condition	2 mL 0.1M acetate buffer (pH 7)
Load	4 mL Sample
Rinse	2 mL Water
Rinse	2 mL 0.1M acetate buffer (pH 7)
Dry	4 minutes
Rinse	2 mL Hexane/Ethyl acetate (95/5)
Purge	2 mL Hexane/Ethyl acetate (70/30)
Collect	2 mL Hexane/Ethyl acetate (70/30)
Rinse	2 mL Methanol
Rinse	2 mL Methanol
Purge	2 mL Water

3. The solvent is dried under nitrogen at 55°C.
4. 25 µL BSTFA+1%TMCS is added to the dried down extracts. The mixture is then heated at 70°C for 30 minutes.
5. 2 µL is injected into the Hewlett Packard 5970 GC/MSD 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
Flow rate	1mL/min.
Injector	250°C
Detector	275°C

## Accuracy

The clinical accuracy of the OTI Barbiturates Intercept™ MICRO-PLATE assay was determined from specimens collected from self-reported barbiturates users and non-users. The cutoff for EIA and GC/MS was 20 ng/mL for oral fluid specimens and 300 ng/mL for urine specimens.

A total of 128 oral fluid and urine specimens pairs were collected. All oral fluid and urine samples were tested by EIA. Of the 128 samples collected, 127 oral fluid samples were confirmed by GC/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 at LabOne (Lenexa, Kansas).

### GC/MS of Intercept™ Specimens (20 ng/mL cutoff)

		+	-
OTI Intercept™ EIA (20 ng/mL cutoff)	+	57	1
	-	2	67
		% Agreement = 97.6%	

### Urine EIA (300 ng/mL cutoff)

		+	-
OTI Intercept™ EIA (20 ng/mL cutoff)	+	51	8
	-	2	67
		% Agreement = 92.2%	

## Conclusion

- Butalbital concentrations for each subject peaked between 0.5 and 2 hours and were still detectable after 48 hours in oral fluid after a single dose of 50 mg. Also Butalbital concentrations for urine peaked between 0.5 and 6 hours and could be detected for up to 48 hours after the single dose.
- The OTI Barbiturates Intercept™ MICRO-PLATE EIA has a broad cross-reactivity profile which includes Allobarbital, Amobarbital, Aprobarbital, Barbital, Butabarbital, Butalbital, Pentobarbital, Phenobarbital, and Talbutal.
- After a ten-minute waiting period all adulterants screened were cleared from the oral cavity and did not interfere with samples containing drug concentrations greater than 40 ng/mL.
- Twenty patient samples from known barbiturates users were collected and screened in the OTI Barbiturates Intercept™ MICRO-PLATE EIA. Concentrations of samples containing drug ranged from 90 ng/mL to 2910 ng/mL.
- Oral fluid screening has shown acceptable agreement with urine screening.

## REFERENCES

1. Kidwell, D., Holland J., and Athanasis, S., "Testing for Drugs of Abuse in Saliva and Sweat," *Journal of Chromatography*, 1998; 713: 111-135.
2. Schramm, W., Smith, R.H., and Craig, P.A., "Drugs of Abuse in Saliva: A Review," *Journal of Analytical Toxicology*, 1992; 16:1-9.
3. Cone, E.J., "Saliva Testing for Drugs of Abuse," Presented at the NY Acad. Sciences meeting Oct. 22-25, 1992.