Laboratory Analysis of Remotely Collected Oral Fluid Specimens for Opiates by Immunoassay

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Abstract

The performance characteristics of a method for detecting opiates (morphine, codeine, heroin, and 6-acetylmorphine [6-AM]) in oral fluid specimens were examined and compared with methods for urine specimens. The oral fluid was easily obtained using a simple device that collects between 1 and 1.5 mL of fluid for laboratory analysis. Simultaneously collected specimens from 60 known opiate abusers from a drug-treatment center were first tested using an immunoassay cutoff of 10 ng/mL in oral fluids and 2000 ng/mL in urine. Using a second aliquot, opiate confirmation in urine was performed by gas chromatography–mass spectrometry (GC–MS) and in oral fluids by GC–MS–MS. The combined immunoassay and GC–MS–MS procedures were completed with less than 250 µL of oral fluid. Opiates identified in oral fluid specimens from heroin users included morphine, codeine, heroin, and 6-AM. The immunoassay was tested for precision, stability, and the effects of potential cross-reactants. The results yielded 93.6% agreement between oral fluid and urine, suggesting that oral fluid may be a reliable matrix for opiate detection.

Introduction

The use of saliva or oral fluid specimens for drug analysis has been of great interest for years (1). Because humans produce up to 1.5 L of saliva each day, the opportunity exists to create simple collection procedures. Recently, even the word "saliva" has been abandoned for the term "oral fluid" to better describe the combination of glandular and cellular debris present in the oral cavity. Oral fluids are the product of multiple glands in the mouth (2) and components of blood that transfer into the oral cavity introducing additional proteins such as antibodies. In just the last few years, the first commercial tests using oral fluid specimens for the detection of circulating antibodies to HIV and for drug metabolites such as cotinine and benzoylecgonine have been introduced (3).

As with a number of drugs of abuse, opiates may be deposited into the oral cavity from two sources. The first possibility is that they would remain in the oral cavity after insufflation or inhalation of heroin (or the smoking of opium), and the second is that opiates and their metabolites would transfer into the oral cavity from the plasma. Indeed, previous studies have shown that these agents will appear in the oral cavity regardless of the route of administration (4,5). Following heroin administration, target analytes are heroin, morphine, codeine, and 6-monoacetylmorphine (6-AM). Although these compounds have been found in blood, urine, and hair, limited data on the presence and concentration of all these materials in oral fluids are available.

This paper presents information on the use of immunoassay and gas chromatography–tandem mass spectrometry (GC–MS–MS) for the detection and confirmation of opiates in oral fluid specimens. These specimens were obtained using a collection device consisting of a pad on the end of a stick. This pad was originally designed for the collection of mucosal transudate to test for HIV antibodies. Mucosal transudate is a combination of oral fluid plus components of serum. The pad also has been shown to be efficient in absorbing a number of drugs of abuse. The pad contains dried salts that increase osmotic pressure, facilitating increased collection efficiency. Following collection, the pad is stored in a vial containing preservative fluid and shipped to a laboratory for analysis (Figure 1). The collection pad absorbs approximately 400 µL of oral fluid. This is diluted 1:3 in the shipping container (preservative fluid), after which the entire solution is submitted to analysts.

Materials and Methods

Chemicals and reagents

Intercept™ Oral Specimen Collection Devices were obtained from OraSure Technologies, Inc. (Bethlehem, PA). Immunoassay kits (product no. 1150I) were also provided by OraSure Technologies, Inc. All other chemicals were obtained

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from the Sigma-Aldrich Chemical Company (St. Louis, MO). Specific materials used for GC–MS–MS confirmation testing are listed in that section. Urine specimens were analyzed using commercial kits of ONLINE® reagents from Roche Diagnostics (Indianapolis, IN) according to package insert instructions.

Materials and Methods

Specimen collection

Oral fluid specimens were collected using the Intercept collection device provided by OraSure Technologies, Inc. The Intercept device consists of a cellulose pad containing buffered salts on a plastic stick. The pad is placed in the mouth for between 2 and 5 min. The saturated pad and stick are then placed into the vial provided. Half of the stick is snapped off, and the tube is capped with the pad inside. When collection is complete, the specimen is sent to a laboratory for analysis. After arrival at the laboratory, the plastic nipple at the end of the tube is removed and the tube is centrifuged at 600–800 × *g* for 15 min. Generally, 1.5 mL of oral fluid is collected in this manner. Oral fluid and urine samples were analyzed at Lab*One*, Inc. (Lenexa, KS).

Immunoassay

Immunoassay kits were provided by OraSure Technologies, Inc. The opiate micro-plate assay is a competitive, solid-phase, enzyme immunoassay. In this assay, free drug in the specimen, calibrator, or control competes with morphine-labeled horseradish peroxidase to bind to antibodies immobilized on the bottom of the microtiter well. Kit calibrators and controls were prepared using a defined, artificial, saliva matrix that mimics human specimens. The initial immunological step takes 30 min at room temperature (20–27°C) followed by a wash step (6 times 300 µL dH_2O). Substrate (100 µL 3,3',5,5'-tetramethylbenzidine) is then added to each well. Color is allowed to develop for 30 min, after which 100 µL of 2N sulfuric acid is added. The absorbance is then measured at 450 nm and referenced at 630 nm. The absorbance measured is inversely proportional to the amount of opiate present in the specimen, calibrator, or control. The volume of specimen used for the analysis is 25 µL. Morphine calibrators containing 0, 5, 10, and 20 ng/mL morphine using morphine sulfate are prepared and included in every run. The immunoassay is complete in 60 min with the immunological and color development steps each requiring 30 min.

The LOD (limit of detection) for the immunoassay is determined by obtaining the mean absorbance value from multiple replicates of the negative calibrator. The absorbance value minus three standard deviations (extrapolated from the curve) represents the sensitivity of the assay.

The stability of opiates in oral fluid specimens is determined by "spiking" the collection device or the diluent in the Intercept shipping vial with morphine and 6-AM at various concentrations and storing them at –80, 4, 25, and 37°C for up to 90 days. The stability is determined by calculating the percent displacement demonstrated at each time point. This value was calculated with the following equation:

$$\% \text{ Displacement} = \left[\frac{\text{Absorbance 0 ng/mL standard} - \text{Absorbance of standards}}{\text{Absorbance 0 ng/mL}}\right] \times 100$$

In a second study, the stability of morphine and 6-AM was evaluated after storage of spiked oral fluid at 4°C and 37°C for 14 days. Kit precision was determined by testing the immunoassay calibrators (0, 5, 10, and 20 ng/mL). For intra-assay precision, each standard was analyzed 20 times. For interassay precision, 20 samples at each calibrator concentration were analyzed daily for 5 days.

The assay also was tested for cross-reactivity of structurally related compounds as well as common materials. For structurally related compounds, drug was spiked into the assay matrix at a number of concentrations and tested in the assay as an unknown. The percent cross-reactivity was defined by the apparent morphine concentration divided by the spiked concentration times 100. Non-structurally related compounds were tested up to 10,000 ng/mL.

In addition, because the ingestion of poppy seeds has been found to result in urines positive for morphine, the effects of poppy seeds were also evaluated. Five volunteer subjects (two males, three females) consumed between 5.2 and 40 g of commercially available, uncooked poppy seeds. Oral fluid and urine specimens were collected at time points up to 24 h. All urine and oral fluid specimens were analyzed by immunoassay.

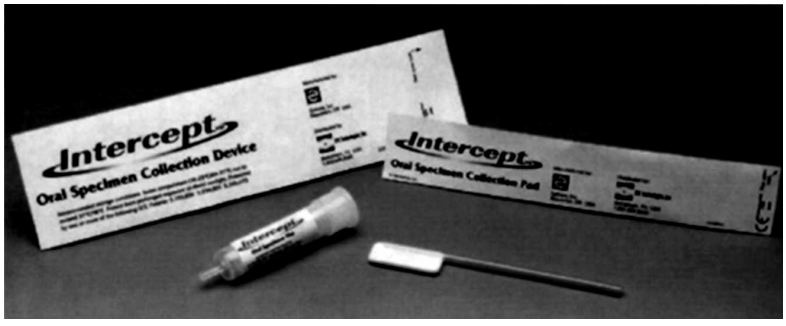

Figure 1. How Intercept works: an overview. Step 1, instruct donor to place the collection pad between lower cheek and gums; step 2, leave collection pad in between cheek and gum for a full 2 min; step 3, place collection pad in container and close; and step 4, ship specimen to laboratory.

GC–MS–MS: analysis of oral fluids

GC–MS–MS confirmation was performed using a Varian Saturn instrument (Walnut Creek, CA). Calibrators of codeine, morphine, heroin, and 6-AM were prepared at 0, 2, 5, 10, 20, 40, 80, and 150 ng/mL in a defined, artificial, saliva matrix that mimics human oral fluid. Corresponding deuterated internal standards at concentrations of 20 ng/mL were added to each calibrator, control, or specimen. A total of 200 µL of

each oral fluid specimen was used for the extraction. To each tube, 1.8 mL of 0.1M phosphate buffer (pH 6) was added. The tubes were then vortex mixed and placed on a RapidTrace™ Workstation for extraction. The RapidTrace procedure (6) was as follows: (i) conditioning a solid-phase column with 1.3 mL of methanol, 1.3 mL of deionized water, and 1.3 mL of 0.1M pH 6 phosphate buffer; (ii) loading the sample onto the column at a rate of 1 mL/min; (iii) rinsing the column with 1.3 mL deionized water, 1.3 mL 0.1M acetic acid, and 1.7 mL methanol; (iv) drying the column for 1 min with nitrogen and eluting the opiates with 1.3 mL 78:20:2 methylene chloride/isopropanol/ammonium hydroxide at a rate of 1 mL/min; (v) removing the tubes from the RapidTrace workstation and transferring the extracts to screw-top tubes; (vi) evaporating to dryness at 45°C; and (vii) derivatizing the opiates with 25 µL of BSTFA/1%TMCS at 70°C for 30 min (7). The extracted specimens were analyzed directly by GC–MS–MS using a Varian (Walnut Creek, CA) 3800 GC with a 15-m × 0.18-mm DB-1, 0.25-µm, capillary column, splitless, injection port, 280°C, program; 100°C for 1.2 min, 20°C/min to 290°C. The detector was a Varian Saturn 2000 ion trap that was set at 220°C with its transfer line at 290°C and manifold at 40°C. First, full-scan analysis was performed over a mass range of 210–450 amu with the MS operated in the electron-impact mode and a filament emission current of 35 µA. The following parent ions were selected for each compound to form product ions: *m/z* 371, codeine; *m/z* 374, codeine-d_3; *m/z* 429, morphine; *m/z* 432, morphine-d_3; *m/z* 399, 6-AM; *m/z* 402, 6-AM-d_3; *m/z* 329, heroin; and *m/z* 334, heroin-d_9. The following product ions were used for quantitation: *m/z* 234, codeine; *m/z* 237, codeine-d_3; *m/z* 412, morphine; *m/z* 415, morphine-d_3; *m/z* 287, 6-AM; *m/z* 290, 6-AM-d_3; *m/z* 268, heroin; and *m/z* 272, heroin-d_9. To be considered positive, both the parent and product ions needed to be present in a peak within 0.1% of the retention time of the standards and the parent ion at approximately 10% the area of the product ion. This method of identification was also used in determining the limit of quantitation (LOQ). The LOQ for codeine was 2 ng/mL. The LOQ for morphine, heroin, and 6-AM was 5 ng/mL. For some clinical samples, results below the LOQ are reported later in this report. These values should be considered approximations within 50% of the stated value.

Urine samples positive by immunoassay were confirmed by GC–MS for morphine, codeine, and 6-AM. The LOQ for GC–MS was 200 ng/mL morphine/codeine and 2.5 ng/mL for 6-AM. For urine, the LOQ was determined as the lowest concentration that demonstrated ± 20% the target values on both quantitative and ion ratios.

Clinical accuracy

The clinical accuracy of the immunoassay was assessed by collecting paired oral fluid and urine specimens from 60 self-admitting opiate abusers in drug rehabilitation. Specimens were collected up to three times per week for up to eight weeks from each subject. In addition, 30 negative specimens were

Table I. Intra-assay Precision Determined by Testing Each Standard 20 Times*

Calibrator	Mean (absorbance)	Standard deviation (absorbance)	Intra-assay CV%	Interassay CV%
0 ng/mL	2.150	0.160	3.5	7.5
5 ng/mL	0.582	0.052	6.4	8.9
10 ng/mL	0.349	0.033	6.6	9.5
20 ng/mL	0.191	0.017	6.9	8.7

* For interassay precision, 20 samples at each concentration were analyzed daily for 5 days.

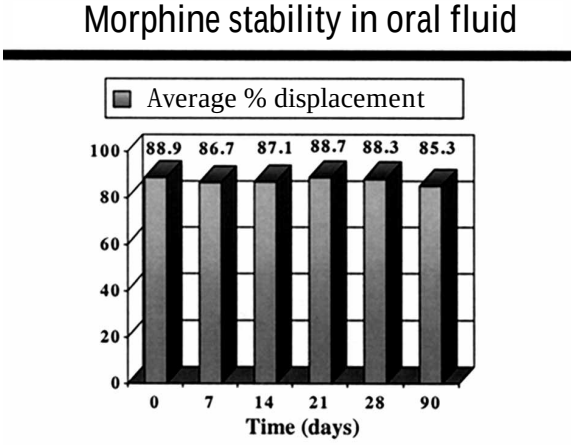

Figure 2. The stability of 10 ng/mL morphine on the Intercept collection pad was tested at –80, 4, 25, and 37°C. No degradation of morphine was seen in the immunoassay as demonstrated by the stability of the assay curves. The plot shows the average percent displacement at all temperatures over a 90-day period.

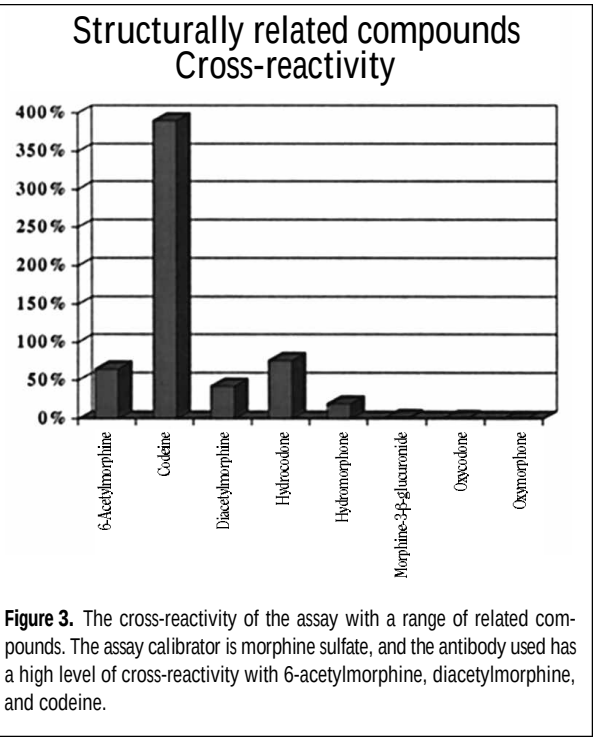

Figure 3. The cross-reactivity of the assay with a range of related compounds. The assay calibrator is morphine sulfate, and the antibody used has a high level of cross-reactivity with 6-acetylmorphine, diacetylmorphine, and codeine.

collected and tested by immunoassay. Collection was open to either males or females who were 18 years or older and signed an informed consent document.

As part of the collection procedure, volunteers completed questionnaires that included general demographic information and current drug-abuse habits. If accepted into the study, two oral fluid specimens were collected bilaterally—one from each side of the mouth—in addition to a urine sample. Oral fluid specimens were frozen, and urine samples were stored at 4°C until shipped for analysis.

Upon receipt at the laboratory, the oral fluid and urine specimens were processed and analyzed by immunoassay. All urines were analyzed using Roche ONLINE® immunoassay reagents. Positive urine specimens were also analyzed by GC–MS for morphine, codeine, and 6-AM, and all oral fluid specimens were analyzed for codeine, morphine, 6-AM, and heroin by GC–MS–MS.

Results

The limit of detection (LOD) for the immunoassay kit was calculated as the absorbance of the negative calibrator minus three standard deviations extrapolated from a standard curve. Using this method, the LOD (or analytical sensitivity) of the immunoassay was 0.2 ng/mL.

The intra- and interassay precision were measured between 0 and 20 ng/mL. Table I shows the summary of the results. The intra-assay precision was measured for 20 replicates at 0, 5, 10, and 20 ng/mL, whereas the interassay precision was measured for 20 replicates/day across 5 days. The highest CV for precision was 9.5% at the 10 ng/mL standard for intra-assay. On average, the precision of the assay for all data points was 7.2% CV.

The stability of morphine, both on the collection pad and in the preservative fluid, was measured over 90 days at –80, 4, 25, and 37°C. For each test day, the unknowns were compared to a standard curve from a kit stored at 4°C. Using this method, the unknowns had to be within the expected CV for absorbance values as determined by the reliability test. Figure 2 shows the plot of the average displacement for all temperatures and all drugs over the 90-day period. Morphine was stable at all temperatures tested, both on the pad and in the preservative fluid. In addition, samples spiked with morphine or 6-AM tested after storage at 4°C and 37°C were stable for one week as determined by GC–MS.

Cross-reactivity studies revealed that the immunoassay cross-reacts with a variety of opiates. Figure 3 presents the relative cross-reactivities of several important compounds. It is evident that the kit is highly cross-reactive to codeine. It is also sensitive to 6-AM, diacetylmorphine, hydrocodone, and, to a lesser extent, hydromorphone.

Table II lists non-structurally related compounds that were tested for cross-reactivity. These compounds were tested at 10,000 ng/mL in the assay's control matrix. None produced a positive response in the assay above the cutoff calibrator containing 10 ng/mL morphine.

To test the influence of poppy seeds on oral fluid specimens, matching oral fluid and urine specimens were collected from each subject over 24 h after consuming between 5.2 and 40 g of seeds. It was found that these specimens produced a positive response in both oral fluid and urine specimens by im-

Table II. The Cross-Reactivity of Non-Structurally Related Compounds Determined by Spiking Drug Up To 10,000 ng/mL in Control Matrix Buffer*

Acetylsalicylic Acid	L-Ephedrine
Alprazolam	L-Methamphetamine
Amobarbital	Lidocaine
Ampicillin	Medazepam
β-Phenethylamine	Methadone
Benzoylecgonine	Metoprolol
Butabarbital	Naproxen
Butalbital	Niacinamide
Caffeine	Norchlordiazepoxide
Chlordiazepoxide	Nordiazepam
Chlorpromazine	PCP
Clonazepam	Penicillin
Clorazepate	Pentobarbital
Cocaethylene	Phenobarbital
Cocaine	Phenylephrine
Cotinine	Phenylpropanolamine
D-Amphetamine	Procainamide
D-Methamphetamine	Procaine
Fenoprofen	Pseudoephedrine
Gemfibrozil	Quinidine
Gentisic Acid	Temazepam
Glipizide	Δ^9-THC
Ibuprofen	Theophylline
Imipramine	Zomepirac

* None of the compounds shown in Table II showed a response greater than the cutoff calibrator of 10 ng/mL morphine.

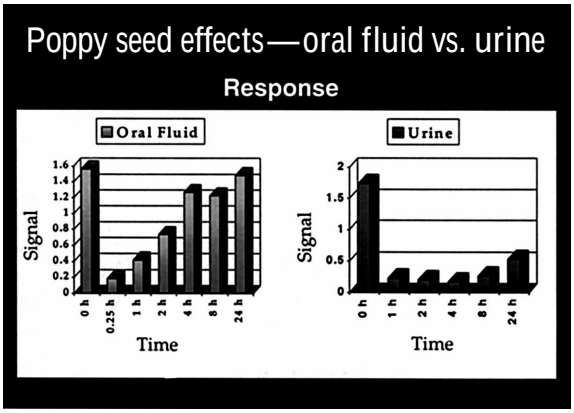

Figure 4. This figure shows the immunoassay response for one individual after consumption of 40 g of commercially available poppy seeds. The poppy seeds were consumed within 15 min after which matching oral fluids and urine samples were collected for 24 h. The immunoassay is inversely proportional to the amount of opiate in the specimen. Therefore, the lower the signal, the higher the concentration of opiate. The oral fluid and urine specimens both showed an immediate response in the immunoassay. Using administrative cutoffs of 10 ng/mL for oral fluid and 300 ng/mL for urine, the oral fluid would have been positive for only 15 min while the urine was at or below the urine cutoff for 4 h.

munoassay. However, the time course in each fluid was different for one of the subjects as illustrated in Figure 4. These results show that poppy seeds were detectable in oral fluids in as little as 15 min. The corresponding urine samples showed a response by 1 h. Using administrative cutoffs of 10 ng/mL for oral fluid and 300 ng/mL for urine, the oral fluid would have been positive for only 15 min, and the urine was at or below the urine cutoff for 4 h.

A series of experiments was conducted to demonstrate how to differentiate poppy-seed influence from true drug abuse. In the first experiment, various amounts of poppy seeds were soaked in the oral fluid collector diluent for 24 h at room temperature. As expected, GC–MS–MS analysis of these specimens for morphine, codeine, and 6-AM only identified the presence of morphine and a small amount of codeine. (Figure 4).

In a follow-up experiment, oral fluid specimens were collected from subjects attending a drug rehabilitation center and tested by GC–MS–MS. These specimens were analyzed for morphine, codeine, heroin, and 6-AM (Table III). The key finding here was a significant concentration of 6-AM in these specimens. Concentrations as high as 248 ng/mL were found. However, one of the specimens that was positive for morphine by immunoassay but relatively low in concentration did not contain any opiate upon confirmation by GC–MS–MS. Finally, one of the specimens contained 33 ng/mL heroin, suggesting that oral fluid specimens could be used to detect very recent use.

Clinical Accuracy

In total, 296 samples were collected from all 60 volunteers participating in the study. For the purposes of ROC analysis of oral fluids, any sample was considered a true positive sample if it contained any combination of morphine, 6-AM, and codeine greater than 10 ng/mL. Using this criterion, the optimal cutoff was determined to be 10 ng/mL morphine for the immunoassay. Further, it was found that the agreement between oral fluids and urine using immunoassay cutoffs of 10 ng/mL and 2000 ng/mL, respectively, was 93.6%. Examination of alternative ROC-based cutoffs at 5.0 ng/mL or 20 ng/mL resulted in 91.2 and 93.0% agreement with the urine immunoassay, respectively.

Discussion

The deposition of drugs into the oral cavity may occur from remnants of oral/nasal administration or redistribution from the circulation. The ease of distribution into the oral cavity appears dependent on characteristics of the compound and its metabolites, that is, pK_a and hydrophobicity or hydrophilicity (1,4,5,8,9). For opioids, their levels in oral fluids appear to mimic blood levels suggesting that comparable results between these matrices may be achieved (10–14).

Assuming the feasibility of opioid distribution into oral fluids, the next challenge is to develop practical methods for collection of the oral fluid sample, screen for opiate presence or absence by immunoassay, and confirm by GC–MS. These components are considered essential because they are part of currently accepted practices for blood or urine analysis.

In this report, samples were collected using a commercial device (Intercept) that absorbs approximately 400 µL of oral fluid. The sample pad was placed into a storage tube containing a preservative fluid that dilutes the initial sample 1:3. Once received by a laboratory, the sample was centrifuged and the fluid was analyzed by enzyme immunoassay. Any presumptive positives were further analyzed using GC–MS–MS to confirm the presence of one or more opioids.

The Intercept collector was found to collect oral fluids satisfactorily. Stability was 90 days for morphine and 1 week for 6-AM. This time period would be adequate for shipment back to a laboratory for analysis and is comparable to other opiate

Table III. Eleven Oral Fluid Specimens Collected from Subjects Participating in a Drug-Rehabilitation Program*

Oral Fluid GC–MS–MS Analysis of Specimens Collected from Heroin Addicts						
	Opiates (ng/mL oral fluid)					
Subject	Codeine (ng/mL)	Morphine (ng/mL)	6-AM (ng/mL)	Heroin (ng/mL)	Codeine/morphine (ratio)	6-AM/morphine (ratio)
1	84	333	119	0	0.25	0.36
2	143	246	166	0	0.58	0.67
3	4	77	11	0	0.05	0.14
4	0	14	5	0		0.36
5	2	98	28	0	0.02	0.29
6	0	14	0	0		
7	164	472	304	0	0.35	0.64
8	39	123	91	0	0.32	0.74
9	188	345	149	0	0.54	0.43
10	130	419	382	33	0.31	0.91
11	89	233	248	0	0.38	1.06

* These specimens were analyzed for morphine, codeine, 6-AM, and heroin by GC–MS–MS; 6-AM was present in all but one of the specimens tested.

stability studies (13,15). Testing of the oral fluid by immunoassay was straightforward because the sample was in buffer when centrifuged off the pad; thus, mucous-like material did not impair test equipment or pipettors.

The EIA was also tested for robustness with a variety of nonclinical parameters. The assay was precise, contained stable reagents, and utilized antibodies that cross-reacted with a number of opiates. As contained in other reports, the most important cross-reactants, morphine, codeine, and 6-AM, were detected (7,15).

Finally, for the oral fluid collected, confirmation analysis by GC–MS–MS was achievable to very low levels of all compounds of interest. The LOQs were 2.0 ng/mL for codeine and 5.0 ng/mL for morphine, 6-AM, and heroin. Derivatization using readily available BSTFA/1%TMCS was performed routinely with a minimum of 200 µL of oral fluid specimen. Thus, replicate immunoassay and/or GC–MS–MS analyses were possible.

Clinical evaluation of parallel oral fluid/urine samples suggested a cutoff of 10 ng/mL as optimal according to ROC analysis. Out of 302 specimens tested, 48 were positive in the initial immunoassay screen. Confirmation by GC–MS–MS found 92% positive for one or more opiates using a confirmation cutoff of 10 ng/mL. In comparison to urine, using an immunoassay cutoff of 2000 ng/mL, the agreement between EIAs was 93.6%

Thus, the data suggest not only good agreement between immunoassay and confirmation for either urine or oral fluids, but also for positive rates between fluids. The agreement between urine and oral fluid immunoassay indicates that opiates are readily detectable in either fluid in the population tested.

Finally, it is incumbent on any test to perform well even in the presence of potential interferants and adulterants, and ideally to be unaffected by ubiquitous medicines or foods. It has long been established that certain food stuffs and household chemicals can invalidate some urine immunoassays (16,17). In this report, no exhaustive studies were conducted to examine all potential interferants. Rather, attention was given to the common problem of urinalysis detecting opiates in poppy seeds (18,19). Well-documented and differentiated from abuse by the presence of 6-AM in urine, poppy seeds are easily explained. However, this subject has not been studied in oral fluids. Here we report that poppy-seed ingestion also may be differentiated in oral fluids by the absence of 6-AM. Samples analyzed after ingestion of seeds contained no 6-AM, whereas samples from opiate abusers contained high levels of 6-AM and in one case heroin. Levels of 6-AM at the concentrations found in Table III are easily confirmed and appear almost equal to morphine.

Conclusions

The detection of opiates in oral fluid specimens provides comparable results to urine on the populations tested. Given the ease of collection of oral fluid specimens, these protocols now offer a viable alternative to urine testing.

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