Selection of an Immunoassay Screening Cutoff Concentration for Opioids in Oral Fluid

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Abstract

A retrospective analysis of data from oral fluid specimens was conducted in order to identify a relevant cutoff concentration for opiates and/or synthetic opiates in oral fluid. Previously proposed regulations from the Substance Abuse and Mental Health Services Administration (SAMHSA) have recommended 40 µg/L as a cutoff concentration. In this study, data from oral fluid specimens collected using the Quantisal™ device and screened with enzyme linked immunosorbent assays (ELISA) for both opiates and oxycodone were retrospectively assessed for screen positives > 20 µg/L and those between negative and 20 µg/L. Specimens identified at these concentrations were then analyzed using liquid chromatography with tandem mass spectral detection using a fully validated procedure. Overall, 156 positive specimens were identified using 40 µg/L; 191 specimens using 20 µg/L; and 241 specimens between negative and 20 µg/L. Specifically, the number of 6-acetylmorphine (6-AM) positives increased from 10 to 16; morphine from 4 to 9; codeine from 11 to 19; oxycodone from 56 to 74; hydrocodone from 73 to 119; and hydromorphone from 2 to 4 when specimens with enzyme inhibition between negative and 20 µg/L were analyzed. For workplace testing where only codeine, morphine, and 6-AM are considered, the use of a lowered cutoff concentration produced significant increases in the positive rate.

Introduction

Opiates and semi-synthetic opiates are widely prescribed for the management of mild to moderate pain [e.g., codeine (COD), hydrocodone (HYC)] and severe and chronic pain [e.g., morphine (MOR), oxycodone (OXYC)] and are present in illegal drugs (e.g., heroin). In 2004, the Substance Abuse and Mental Health Services Administration (SAMHSA) proposed the addition of oral fluid as a matrix for workplace drug-testing programs. The suggested cutoff concentration for opiates (COD, MOR) was 40 µg/L, and if separately screened, a cutoff of 4 µg/L proposed for 6-acetylmorphine (6-AM) (1). Oral fluid is increasing in popularity as drug testing matrix because of its ease of collection, difficulty of adulteration, and improving technology allowing for expanded drug test profiles.

One of the main issues with proposing cutoff concentrations for workplace programs in new matrices is a lack of controlled dosing studies because these are difficult to implement. A retrospective examination of previously collected specimens may provide some insight into the concentration of opiates present in oral fluid specimens from authentic users. The research described in this paper uses the Quantisal oral fluid collection device, which collects a known amount of neat oral fluid. The efficiency of recovery of the opioids from the collection pad into the transportation buffer was determined, in order to ensure confidence in the quantitative value, even though this has been reported previously by other research groups (2).

Several publications describe the analysis of opiates and opioids in oral fluid using liquid chromatography with tandem mass spectrometry (LC–MS–MS) with different instruments and ionization techniques, but always with good precision and performance (3–5). In 2007, Cone et al. (6) reported extensively on the prevalence and disposition of opioid treatment drugs in oral fluid. Dihydrocodeine was included in their paper because it is prescribed in the U.K. From over 5000 specimens positive for opioids, MOR (79.7%), COD (66.6%), and 6-AM (61.9%) were most frequently reported, often in combination. The detection rate of 6-AM when MOR was present was 77.5%, and both heroin and 6-acetylcodine (6-AC) were reportedly frequently detected (6). The screening cutoff concentration used was approximately 30 µg/L for opiates; confirmation was approximately 30 µg/L for COD and MOR; 15 µg/L for dihydrocodeine; and 3 µg/L for 6-AM, 6-AC, and heroin.

Because opioids are readily detectable in oral fluid, testing saliva is an effective means to determine their use, and oral fluid appears to have an advantage over urine for the detection of heroin intake.

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Materials and Methods

Oral fluid collection devices
Quantitative devices for the collection of oral fluid specimens were obtained from Immunalysis (Pomona, CA). The devices contain a collection pad with a volume adequacy indicator, which turns blue when 1 mL of oral fluid (±10%) has been collected. The pad is then placed into transport buffer (3 mL), allowing a total specimen volume available for analysis of 4 mL (3 mL buffer + 1 mL oral fluid). The buffer ensures stability of the drugs in the collection system during transportation to a testing facility.

Standards and reagents
An Opiates Direct ELISA kit (Catalog # 207) and an OXYC Direct ELISA kit (Catalog # 221) were obtained from Immunalysis and used for screening the oral fluid. For the confirmatory procedure, deuterated internal standards, MOR-d₃, COD-d₃, 6-AM-d₄, and OXYC-d₆, as well as unlabeled standards for MOR, COD, 6-AM, 6-AC, OXYC, oxymorphone (OXYM), hydromorphone (HYM), and HVC were purchased from Cerilliant (Round Rock, TX). Solid-phase extraction columns (ClinII, 691-0353T) were obtained from SPEWare, (Baldwin Park, CA). All solvents were HPLC grade, and all chemicals were ACS grade.

Calibrators
For the chromatographic calibration standards, a working solution for the deuterated internal standards and unlabeled drug standards were prepared in acetonitrile. All working solutions were stored at -20°C. For each batch, seven calibration standards were prepared in synthetic oral fluid (1 mL) then transportation buffer from the Quantisal collection device was added (3 mL). A synthetic oral fluid matrix, which matched the immunoassay responses of three human negative oral fluid specimens, was prepared as follows: 25 mM phosphate buffered saline (pH 7.0), 30 mM sodium bicarbonate, 0.1% albumin, amylase, and 0.1% Proclin 300 as a preservative. Synthetic oral fluid was used as opposed to authentic drug free saliva primarily because of the amount required in order to carry out all the experiments. The effect of real oral fluid on the drugs compared to the effect in synthetic material is minimized during to 1+3 dilution with transportation buffer. Drug concentrations of 4, 10, 20, 40, 80, and 160 µg/L of neat oral fluid equivalents were prepared.

Screening assay
Enzyme linked immunoabsorbent assay (ELISA) technology is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen in proportion to their concentration in the reaction well. The oral fluid specimens were screened at a concentration of 40 µg/L using MOR as the calibration standard for the opiate kit; OXYC for the OXYC specific kit. A standard curve consisting of a drug-free positive oral fluid specimen, and drug-free oral fluid specimens spiked at 50% and 200% of the recommended cutoff concentrations was analyzed with every batch. The sample volume was pipetted directly from the collection device into the microplate (sample size: 10 µL). Specimens screening positively using ELISA were carried forward to confirmation using the described procedure.

Over 5000 oral fluid samples previously analyzed gave a positive rate of approximately 2% for opioids. A retrospective analysis of the ELISA screening data associated with the same batches was carried out to assess those positive above the low control (20 µg/L) but not higher than the cutoff concentration (40 µg/L), as well as those showing some activity between the negative and low control.

Sample preparation for chromatographic analysis
An aliquot (1 mL) from the Quantisal collection device, equivalent to 0.25 mL of neat oral fluid was removed, and internal standard was added (100 µL of 250 µg/L solution). Potassium phosphate buffer (0.1 M, pH 6.0, 1 mL) was added to each calibrator, control or oral fluid specimen. Solid-phase mixed mode (cation exchange: hydrophobic) extraction columns were placed into a positive pressure manifold. Each column was conditioned with methanol (2 mL) and 0.1 M phosphate buffer (pH 6.0, 2 mL). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (2 mL), acetic acid (pH 4.0, 2 mL), methanol (1 mL), and ethyl acetate (1 mL). The columns were allowed to dry under nitrogen pressure (5 min). The drugs were finally eluted using freshly prepared ethyl acetate/ammonium hydroxide (98:2, v/v, 2 mL). The elution solvent was vortex mixed before adding to the solid-phase column so the ammonium hydroxide did not settle. The extracts were evaporated to dryness under nitrogen at 40°C and reconstituted in 20 mM ammonium formate pH 4.5/methanol (70:30, v/v, 50 µL).

LC-MS-MS
A 1200 series LC pump coupled to a 6410 triple-quadrupole MS, operating in positive electrospray ionization mode (ESI) mode was used for analysis (Agilent Technologies, Santa Clara, CA). The LC column was also supplied by Agilent Technologies, and was a Zorbax Eclipse XDB C₈ (4.6 x 50 mm x 1.8 mm). The column temperature was held at 60°C, and the injection volume was 5 µL. The mobile phase consisted of 20 mM ammonium formate pH 6.4 (Solvent A) and methanol (Solvent B). Initially, the mobile phase composition was 85% A/15% B at a flow rate of 0.7 mL/min. Over 7 min, the percentage of methanol was increased to 90%; and returned to 15%B after 8min. The post-column stabilization time was 3min. The gas temperature was 350°C, the gas flow was 10 L/min, and the nebulizer pressure was 35 psi. Nitrogen was used as the collision gas and the capillary voltage was 4000V. Two transitions were selected and optimized for each drug (quantifying transition is underlined); 6-AC 342.3 > 225.2, 165.3; 6-AM-d₄ 331.3 > 165.3; 6-AM 328.3 > 165.3, 211.2; COD-d₃ 303.3 > 285.2; COD 300.3 > 181.1, 282.2; HYM 286.3 > 185.3, 157.4; MOR-d₃ 289.2 > 211.2; MOR 286.3 > 165.2, 155.2; OXYC-d₆ 322.2 > 247.5; OXYC 316.3 > 298.4, 241.2; OXYM 302.3 > 227.2, 198.3. The dwell time for all transitions was 50 ms, and optimal fragmentor and collision energy voltages were determined. The ratio of the qualifier transition to the quantifier transition was selected at the calibration point of 40 µg/L. The retention times (min) for the drugs were as fol-
described procedures in order to assess interference from drug-free volunteers, extracted and analyzed according to the traction or matrix, or potential ion suppression. Ion suppression is more prevalent in the operational mode of electrospray ionization (ESI) rather than atmospheric pressure chemical ionization. The protocol from Matuszewski (7) was used to assess matrix effects and process efficiency. In order to perform experiments according to these protocols, a non-extracted drug standard at a concentration of 10 µg/L was prepared as well as drug-free matrix extracts and negative controls (extracts containing only internal standard).

The recovery of the opioids from oral fluid was determined by first assessing the response of the extracted samples (n = 6) at a concentration of 40 µg/L [R_{RE}] Next, oral fluid was extracted and drug was added post-extraction at a concentration of 40 µg/L (n = 6) [R_{RES}]. The percentage recovery was then calculated from the equation (R_{ES}/R_{RES}) × 100.

The percentage reduction / improvement in response due to matrix effects (ion suppression / ion enhancement) was determined by assessing the peak-area response of a non-extracted neat drug standard (n = 6) at a concentration of 40 µg/L [R_{RES}]. The non-extracted solution was analyzed in the same reconstitution solvent as the extracted specimen. The % matrix effect was then calculated using the equation (R_{ES}/R_{RES}) × 100. A negative result indicated ion suppression, and a positive result indicated ion enhancement of the signal. The overall efficiency of the process was calculated as (R_{ES}/R_{RES}) × 100. Reduction of matrix effects is best achieved by utility of deuterated internal standards where possible, extensive matrix clean-up before injection, and optimal chromatographic and mass spectral conditions.

Exogenous interference. In order to assess potential problems arising from exogenous sources, commonly encountered drugs were added to the drug-free oral fluid specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed at a concentration of 10,000 µg/L: buprenorphine, norbuprenorphine, cocaine, benzoylcegonine, cocaethylene, norcocaine, tramadol, fentanyl, γ-hydroxybutyrate (GHB), tetrahydrocannabinol, 11-nor-9-carboxy-tetrahydrocannabinol, amphetamine, methamphetamine, nortriptyline, amitriptyline, methylenedioxyamphetamine (MDMA), methylenedioxymethamphetamine (MDEA), carisoprodol, methadone, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, nitrazepam, triazolam, secobarbital, pentobarbital, butalbital, and phenobarbital.

Stability

Finally the stability of the drugs in the extracts was assessed. The extracts remained in the vials inside the autosampler, which was maintained at 7°C. The extracts were re-analyzed after 24 h and after 48 h against a fresh calibration curve.

Results and Discussion

Method validation

The LC–MS–MS procedure developed for the opioids was validated according to accepted protocols. Four internal standards, MOR-d_3, COD-d_3, 6-AM-d_3, and OXYC-d_6 were employed. Deuterated COD was used as the internal standard for HYC; deuterated MOR for HYM; 6-AM was used as the internal standard for 6-AC; and deuterated OXYC for the OXYM quantitation.

Linearity and sensitivity

The limit of quantitation was 4 µg/L for all drugs and linearity was obtained with an average correlation coefficient for
all the drugs of > 0.99 over the range 4–160 µg/L of oral fluid. The mean correlation of the calibration curves was $r^2 > 0.99$ for all compounds.

**Recovery, accuracy, and precision**

The recovery, accuracy, and precision of the assay at two concentrations, both interday and intraday, are shown in Table I. The precision did not exceed 6.1% for inter- or intraday studies at either concentration, and the accuracy of the assay for all drugs ranged from 96% to 110% of the target concentration. The lowest recovery from the collection pad using the Quantisal device was 74.7% (HYM).

**Selectivity**

**Ion suppression.** The oral fluid is diluted during collection, deuterated internal standards are added, and specific solid-phase procedures are employed. Overall these steps in the procedure contributed to minimal ion suppression. The percentage matrix effect and percentage process efficiency were determined for each drug and are given respectively: HYM –13%, 76%; MOR 1%, 96%; OXYM 1%, 92%; 6-AM 7%, 104%; COD –4%, 94%; HYC –10%, 83%; OXYC –11%, 93%; and 6-AC –1%, 99%. All matrix effects were less than 14% for all drugs and process efficiencies higher than 76%, showing very limited ion suppression in the assay.

**Interference.** Oral fluid specimens collected from drug-free individuals showed no interference, which was not unexpected, because it is unlikely these drugs are similar to endogenous substances in oral fluid. For exogenous interferences, commonly encountered drugs of abuse were studied as described in the Materials and Methods section. No chromatographic interference was observed in the channels of the transitions.

**Stability**

The stability of the drugs in the extracts was assessed, and were stable for at least two days when kept inside the autosampler, which was maintained at 7°C. There was less than a 5% difference in the quantitation of the extracts after 48 h.

**Retrospective analysis**

Over the past several months, over 5000 oral fluid specimens have been

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### Table I. Recovery of Opiates from Oral Fluid Collection Pad at a Concentration of 40 µg/L—Accuracy and Imprecision of the Assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>Recovery (%) (n = 5)</th>
<th>Accuracy (%) 30 µg/L</th>
<th>Precision CV (%) 30 µg/L</th>
<th>Accuracy (%) 60 µg/L</th>
<th>Precision CV (%) 60 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocodone</td>
<td>84.8</td>
<td>101</td>
<td>98.6</td>
<td>3.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Hydromorphone</td>
<td>74.7</td>
<td>100</td>
<td>101</td>
<td>5.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Codeine</td>
<td>83.4</td>
<td>96.1</td>
<td>98.0</td>
<td>5.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Morphine</td>
<td>84.2</td>
<td>110</td>
<td>106</td>
<td>2.2</td>
<td>2.9</td>
</tr>
<tr>
<td>6-Acetylmorphone</td>
<td>82.7</td>
<td>105</td>
<td>102</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>6-Acetylcodine</td>
<td>101</td>
<td>99.4</td>
<td>100</td>
<td>2.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>95.8</td>
<td>99.4</td>
<td>102</td>
<td>3.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Oxymorphone</td>
<td>79.1</td>
<td>106</td>
<td>103</td>
<td>3.6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

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### Table II. Oral Fluid Specimens Identified as Containing Opioids Depending on Screening Cutoff Concentration

<table>
<thead>
<tr>
<th>Opiate positives (&gt; 40 µg/L)</th>
<th>HYC</th>
<th>OXYC</th>
<th>COD</th>
<th>6-AM</th>
<th>MOR</th>
<th>HYM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screen values &gt; 20 and &lt; 40 µg/L</td>
<td>73</td>
<td>56</td>
<td>11</td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>156</td>
</tr>
<tr>
<td>% Increase</td>
<td>27%</td>
<td>16%</td>
<td>36%</td>
<td>0%</td>
<td>50%</td>
<td>0%</td>
<td>22%</td>
</tr>
</tbody>
</table>

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For the past several months, over 5000 oral fluid specimens have been
received into our facility. On average, approximately 2% confirm positively for opioids, with more than 150 oral fluid specimens positive for COD, MOR, 6-AM, 6-AC, HYC, HYM, OXYC, and/or OXYM identified during the various projects. A retrospective analysis of the ELISA screening data associated with the same batches was carried out to assess those positive above the low control (20 µg/L) but not higher than the cutoff concentration (40 µg/L), as well as those showing some activity between the negative and low control. It is difficult to quantitate the exact screening value for those below 20 µg/L. Oral fluid specimens identified were then subjected to confirmation using the described procedure, using 4 µg/L as the limit of quantitation. The results are shown in Figure 1 and Table II; no specimens contained OXYM or 6-AC. Overall, when assessing those specimens screening above 20 µg/L but below the cutoff, the greatest increase in positivity rate was for MOR (50% increase: 4 samples to 6) followed by COD (36% increase: 11 samples to 15) and HYC (27.4% increase: 73 specimens to 93).

### Workplace opiates

For the drug-free workplace program, only COD, MOR, and 6-AM are likely to be analyzed. Lowering the screening cutoff to 20 µg/L resulted in the identification of four additional positive samples for COD (36% increase); two additional positives for MOR (50% increase); and no increase for 6-AM. However, when samples just below the low control were analyzed by LC–MS–MS, six more specimens positive for 6-AM were identified; one with a concentration of 14 µg/L and MOR at 11 µg/L (Figure 2). This specimen screened very closely to the low control absorbance value equivalent of 20 µg/L indicating that the screening level for 6-AM should be lower than 20 µg/L and the proposed concentration of 4 µg/L may be appropriate.

Although it is obvious that any reduction in a screening or confirmation cutoff concentration will result in an increased number of positive specimens, if workplace rules are to be introduced, a reduction in the number of false-negative results should be considered. Simply dropping the screening cutoff to 20 µg/L for COD and MOR, retaining 4 µg/L for 6-AM, and reducing the confirmation level would result in an increase of over 30% in the identification of true positive oral fluid specimens.

### Limitations of the study

There were two major limitations to this retrospective analysis. Firstly, it was difficult to assess the true concentration of any screening result below the low positive control, because any specimen showing enzyme activity was subjected to confirmation; therefore, a suggested concentration below 20 µg/L does not yet have sufficient data to support the recommendation, based on this retrospective analysis. Future projects should include calibration standards in the assay at lower concentrations so that specific recommendations can be supported.

A second major limitation was the lack of a separate, specific screening assay for 6-AM at the cutoff of 4 µg/L, and this is absolutely essential for workplace testing if heroin users are to be identified. Oral fluid is an excellent matrix for the expression of 6-AM and should be utilized in a workplace system for this analyte. Even though the opiate kit (targeted to MOR) showed a cross-reactivity of 83% towards 6-AM, not all oral fluid samples have a high level of MOR associated with a measurable concentration of 6-AM, so a separate assay is necessary to avoid false-negative results.

Lowering the cutoff is also a concern in the issue of poppy seed intake causing a positive result. In 2001, Niedbala et al. (8) reported oral fluid opiate results from individuals who had ingested between 5.2 and 40 g of uncooked poppy seeds, concluding that the saliva would have been positive for only 15 min at an adjusted cutoff concentration of 10 µg/L. Later in 2003, Rohrig and Moore (9) reported that measurable concentrations of MOR were detectable in oral fluid for approximately 1 h after the ingestion of 9–20 g of poppy seeds. These small studies ought to be repeated and expanded if lowering of cutoff concentrations is to be considered.

### Conclusions

The retrospective analysis of oral fluid specimens containing opioids is described, with specific emphasis on screening cutoff concentration. The extraction and LC–MS–MS procedures are reproducible, robust, and precise. An in-
crease of > 30% was observed in true opiate positives when using a screening cutoff concentration of 20 µg/L compared to the current recommendation of 40 µg/L; for 6-AM an even lower concentration is already recommended.

References


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