Sensitivity, Specificity, and Efficiency in Detecting Opiates in Oral Fluid with the Cozart® Opiate Microplate EIA and GC–MS Following Controlled Codeine Administration

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Abstract

Oral fluid specimens (N = 1406) were collected from 19 subjects prior to and up to 72 h following controlled administration of oral codeine. Volunteers provided informed consent to participate in this National Institute on Drug Abuse Institutional Review Board-approved protocol. A modification of Cozart Microplate Opiate EIA Oral Fluid Kit (Opiate ELISA), employing codeine calibrators, was used for semiquantitative analysis of opiates, followed by gas chromatography–mass spectrometry (GC–MS) for the confirmation and quantitation of codeine, norcodeine, morphine, and normorphine in oral fluid. GC–MS limits of detection and quantitation were 2.5 pg/L for all analytes. The Substance Abuse and Mental Health Services Administration (SAMHSA) has proposed a 40-pg/L opiate screening and a 40-pg/L morphine or codeine confirmation cutoff for the detection of opiate use. Oral fluid opiate screening and confirmation cutoffs of 30 pg/L are in use in the U.K. Utilizing 2.5-, 20-, 30-, and 40-pg/L GC–MS cutoffs, 26%, 20%, 19%, and 18% of the oral fluid specimens were positive for codeine or one of its metabolites. Six Opiate ELISA/confirmation cutoff criteria (2.5/2.5, 10/2.5, 20/20, 30/20, 30/30, and 40/40 pg/L) were evaluated. Calculations for Opiate ELISA sensitivity, specificity, and efficiency were determined from the number of true-positive, true-negative, false-positive, and false-negative results at each screening/confirmation cutoff. Sensitivity, specificity, and efficiency for the lowest cutoff were 91.5%, 88.6%, and 89.3%. Application of the cutoff currently used in the U.K. yielded sensitivity, specificity, and efficiency results of 79.7%, 99.0%, and 95.1% when applying the SAMHSA criteria. These data indicate that the Opiate ELISA efficiently detects oral codeine use. In addition, the data, collected following controlled oral codeine administration, may aid in the interpretation of opiate oral fluid test results and in the selection of appropriate oral fluid screening and confirmation cutoffs.

Introduction

Currently, there is strong interest in monitoring drug use through oral fluid testing in driving under the influence, drug treatment, criminal justice, and workplace drug-testing programs. Oral fluid, as a matrix for biological testing of opiates, has advantages over urine testing including the ease and non-invasiveness of specimen collection and a reduction in the potential for specimen adulteration and substitution (1–4). Oral fluid testing is a greater analytical challenge because drug concentrations are generally lower than in urine, leading to shorter detection time windows (2,5).

Codeine is commonly used as an analgesic and antitussive and can be taken alone or in combination with other substances such as acetaminophen, caffeine, and acetylsalicylic acid, with total daily codeine doses ranging from 60 to 240 mg (6). Although used for pain relief and as a cough suppressant, codeine also is abused for its euphoric and depressant effects (7–9). The detection of codeine is included in military, workplace, and drug-testing programs because of its abuse and performance-impairing effects.

Microtiter plate enzyme immunoassays have been used to screen blood, urine, serum, oral fluid, sweat, and hair for a variety of drugs of abuse (10–14). Improved performance characteristics, increased throughput, smaller sample volumes, and decreased costs have increased their utility for qualitative and semiquantitative analysis (14,15). Microplate immunoassays are the primary replacement for radioimmunoassays of drugs in whole blood, utilized in many medical examiner and driving under the influence laboratories. Neogen Opiates Group (Lexington, KY) microtiter plate assay results for 127 postmortem blood specimens were compared with gas chromatography–mass spectrometry (GC–MS) results at two cutoffs (20 and 50 µg/L) (16). Sensitivity, specificity, and efficiency results utilizing the 20- and 50-µg/L cutoffs were 95.2%, 92.2%, and 95.1% when applying the SAMHSA criteria. These data indicate that the Opiate ELISA efficiently detects oral codeine use. In addition, the data, collected following controlled oral codeine administration, may aid in the interpretation of opiate oral fluid test results and in the selection of appropriate oral fluid screening and confirmation cutoffs.
Limited, controlled drug administration data in oral fluid are available for the interpretation of oral fluid test results. One of the underdetermined issues for oral fluid testing is the selection of appropriate screening and confirmation cutoffs. Recently, the Substance Abuse and Mental Health Services Administration (SAMHSA) proposed an oral fluid opiate screening cutoff of 40 µg/L and confirmation cutoffs of 40 µg/L for codeine and/or morphine. Oral fluid testing in the U.K. has utilized a 30-µg/L Opiate ELISA cutoff and a GC–MS cutoff of 30 µg/L for morphine, codeine, dihydrocodeine, 6-acetylmorphine, and/or heroin.

The goals of this controlled drug administration study were to evaluate the sensitivity, specificity, and efficiency of the Opiate ELISA, as compared with GC–MS for detecting codeine use and to evaluate potential opiate oral fluid cutoffs for screening and confirmation assays.

Materials and Methods

Participants

Twelve men and seven women (14 African American, 3 Caucasian, and 2 Hispanic) participated in a controlled codeine administration protocol approved by the National Institute on Drug Abuse Institutional Review Board. Volunteers provided informed consent, remained on a closed clinical research ward under continuous medical surveillance for 10 weeks and reported a history of opiate use that was verified by a positive urine opiate test.

Drug administration

Codeine sulfate, obtained from Roxane Laboratories (Columbus, OH), was administered orally in capsules. After an initial washout period of three weeks to permit elimination of previously self-administered opiates, subjects received three low doses of oral codeine sulfate (60 mg/70 kg) within seven days and, following a three-week interval, three high doses (120 mg/70 kg) within the same time frame. The dosage regimen and route of administration were selected to minimize adverse drug effects.

Oral fluid collection

Oral fluid specimens (N = 1406) were collected prior to and up to 72 h after codeine administration using three different methods. Specimens were collected following citric acid candy stimulation and expectoration (N = 1077), and smaller subsets were collected with Salivet® neutral cotton swabs (N = 147) or Salivet citric acid-treated cotton swabs (N = 182). Oral fluid specimens collected with Salivet were extracted from the swabs by centrifugation and stored in polypropylene tubes at −20°C until Opiate ELISA and GC–MS analysis.

Cozart Microplate Opiate EIA Oral Fluid Kit

The Opiate ELISA is a competitive enzyme immunoassay for the detection of opiates in human oral fluid, developed by Cozart Bioscience Ltd. (Oxfordshire, U.K.) (18). The assay was performed according to the manufacturer's protocol, with the exception that the commercially available opiate kit was modified to optimize detection of codeine by substituting five codeine calibrators (0, 1, 5, 10, and 50 µg/L) for the morphine calibrators. Codeine calibrators were employed because codeine has a greater cross-reactivity with these opiate antibodies than morphine, and semiquantitative results were required to evaluate performance at different opiate cutoffs. Quality control samples were prepared by adding codeine to drug-free human oral fluid diluted 1:3 with 2 mol/L sodium acetate, the buffer from the Cozart fluid collection system.

Each microtiter plate was calibrated in duplicate, yielding semiquantitative results between 1 and 50 µg/L. Results greater than the upper limit of linearity were not extrapolated beyond the curve limits and were reported as greater than the highest calibrator. Concentrations of oral fluid specimens were determined in singlicate, and duplicate quality control samples were included to monitor assay performance. Between-run precision for the low and high quality control samples was calculated from absorbances across the 19 Opiate ELISA plates (N = 37). Inters assay precision for the low control was 21.3% with a mean absorbance of 0.179 ± 0.038 and 27.2% for the high control with a mean of 0.125 ± 0.034.

Experimental

Chemicals and reagents

Chemicals were obtained from the following sources: codeine-P04, [2H3]-codeine hydrochloride-2H2O, norcodeine hydrochloride-3H2O, morphine sulfate, [2H3]-morphine hydrochloride-3H2O, normorphine hydrochloride-H2O from Sigma Chemicals (St Louis, MO) and N,O-bis(trimethyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) with 1% tert-butyldimethylchlorosilane (TBDMS) from Pierce Chemical (Rockford, IL). Deuterated codeine was used as the internal standard for codeine and norcodeine, and deuterated morphine was used as the internal standard for morphine and normorphine. Solid-phase extraction (SPE) columns (Clean Screen ZSDAU020) and filtration columns (RFV02FP4) were obtained from United Chemical Technologies (Bristol, PA). Solvents were HPLC grade and purchased from the following sources: methylene chloride, 2-propanol, and...
acetonitrile from Mallinckroft Baker Inc. (Phillipsburg, NJ) and methanol from Fisher Scientific (Fair Lawn, NJ). Sodium acetate, acetic acid, ammonium hydroxide, and hydrochloric acid were ACS-reagent grade and obtained from Mallinckroft Baker Inc.

**Extraction**

Samples were analyzed for codeine and three metabolites using SPE and GC-MS according to a previously published procedure (19-22). Drug-free oral fluid was spiked with codeine, norcodeine, morphine, and normorphine at concentrations ranging from 2.5 to 500 µg/L. For each analytical run, two calibration curves were generated, 2.5-50 µg/L and 50-500 µg/L. Splitting the curves improved sensitivity and linearity for all analytes of interest (r > 0.98) and allowed us to achieve limits of detection and quantitation (LOQ) of 2.5 µg/L for all analytes. Quality control samples utilized stock solutions from a different source and were spiked to achieve limits of detection and quantitation (LOQ) of 500 pg/L. Splitting the curves improved sensitivity and calibration curves were generated, 2.5-50 lag/L and 50-500 lag/L. For each analytical run, two quality control samples utilized stock solutions from a different source and were spiked to achieve limits of detection and quantitation (LOQ) of 2.5 µg/L for all analytes. Quality control samples required to be within +20% of theoretical values. Ion ratios of quality control samples and participant specimens were required to be within ±20% of those observed for the 10 and 100-ng calibrators’ ion ratios for the low and high calibration curves, respectively. Briefly, 1 mL of oral fluid and 100 µL of (1 µg/mL) internal standard solution (COD-d3, MOR-d3) were extracted by SPE (Clean Screen ZSDAU020) and derivatized with MTBSTFA containing 1% TBDMCS and BSTFA containing 1% TMCS.

GC-MS analysis of the derivatized extract was performed on a Hewlett-Packard (Palo Alto, CA) 5890A GC interfaced with a Hewlett-Packard 5973 mass selective detector or a Hewlett-Packard 5972 mass selective detector utilizing electron impact operated in the splitless mode. Separation of analytes was achieved using an HP-1 fused-silica capillary column (12 m x 0.2-mm i.d., 0.33 µm film thickness). Three ions for each analyte were monitored (quantitative ion in parenthesis): [2H3]-codeine, m/z (374), 237, 181; codeine, m/z (371), 234, 178; norcodeine, m/z (429), 254, 292; [2H3]-morphine, m/z (417), 474, 281; morphine, m/z (414), 471, 278; and normorphine, m/z (472), 529, 350.

**Performance evaluation**

True-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) results were determined by comparing immunoassay with GC–MS results. A sample was considered positive if the analytical result was greater than or equal to the specified cutoff. Therefore, a sample was considered TP if there was a positive immunoassay result and codeine, norcodeine, morphine, and/or normorphine were positive by GC–MS. If both results were negative, the sample was a TN. A positive immunoassay result and a GC–MS result negative for all analytes was considered an FP. A positive immunoassay result and a GC–MS result positive for at least one of the opiate analytes was considered an FN. Sensitivity of the immunoassay at a specific cutoff was calculated as TP/(TP + FN) x 100 and specificity as TN/(TN + FP) x 100. Efficiency was calculated as (TP + TN)/total number x 100.

**Screening and confirmation cutoff concentrations**

Six Opiate ELISA/GC–MS cutoffs were evaluated. The lowest cutoffs, 2.5/2.5 and 10/2.5, were at the LOQ of the GC–MS assay. In addition to the SAMHSA (40/40) and U.K. (30/30) cutoffs, thresholds at 20/20 and 30/20 were assessed. Adhering to the criteria for the U.K. and SAMHSA cutoffs, only codeine and morphine were used in calculations to determine TP, TN, FP, and FN tests. However, for the other ELISA/confirmation cutoffs, codeine, norcodeine, morphine, and normorphine concentrations were evaluated.

**Results**

**Cross-reactivity**

We evaluated the cross-reactivity of the Opiate ELISA to determine which prescription or illicit opioids could produce positive immunoassay results. Table 1 lists the cross-reactivity of opioid and closely related compounds as compared with codeine activity. Even at elevated concentrations, norcodeine exhibited poor cross-reactivity. In contrast to norcodeine, there was good cross-reactivity and, hence, high sensitivity for hydrocodone, dihydrocodeine, and pholcodine.

**Codeine and metabolites in oral fluid**

Following controlled administration of three 60-mg/70-kg and three 120-mg/70-kg oral codeine sulfate doses, codeine was the primary analyte and norcodeine the only metabolite detected in oral fluid with our GC–MS LOQ of 2.5 µg/L. Mor-

| Table I. Cross-Reactivity of the Cozart Microplate Opiate ELIA Oral Fluid Kit |
|---------------------------------|----------------|----------------|----------------|
| Compound                        | Concentration | Apparent Codeine Concentration | % Cross-Reactivity* |
| Codeine                         | 100,000 µg/L  | 12.6 µg/L                  | 0.00126          |
| Dextromethorphan                | 10,000 µg/L   | 2.3 µg/L                   | 0.0226           |
| Norcodeine                      | 100,000 µg/L  | 19.8 µg/L                  | 0.198            |
| Normorphine                     | 1000 µg/L     | 2.8 µg/L                   | 0.28             |
| Hydromorphone                   | 1000 µg/L     | 4.4 µg/L                   | 0.44             |
| Heroin                          | 1000 µg/L     | 8.1 µg/L                   | 0.81             |
| Morphine-3-glucoronide          | 1000 µg/L     | 2.7 µg/L                   | 2.7              |
| Nalorphine                      | 1000 µg/L     | 16.5 µg/L                  | 16.5             |
| Hydrocodone                     | 1000 µg/L     | 34.2 µg/L                  | 34.2             |
| Pholcodine                      | 1000 µg/L     | 51.0 µg/L                  | 51.0             |
| Pholcodine                      | 5000 µg/L     | 56.2 µg/L                  | 56.2             |
| Codeine                         | 1000 µg/L     | 69.9 µg/L                  | 69.9             |

* Cross-reactivity = (Apparent Concentration/Target Concentration) x 100.

* Codeine calibrators were used as the reference.
phine and normorphine were not detected in any oral fluid specimens. Norcodeine was never present without concurrent codeine and, on only one occasion, did the concentration of norcodeine exceed that of codeine. At the GC–MS LOQ, 26% (N = 365) of the specimens were positive for codeine and 13% (N = 183) for norcodeine. Codeine concentrations ranged from 2.5 to 3961 μg/L and norcodeine from 2.6 to 191 μg/L. In addition, 1041 specimens (74%) had codeine and norcodeine concentrations below the GC–MS LOQ and were defined as negative specimens.

Performance characteristics at different cutoffs

The designation of individual specimens as TP, TN, FP, and FN and the performance characteristics of the Opiate ELISA at different immunoassay and GC–MS cutoffs are summarized in Table II. As expected, the lowest cutoff (2.5/2.5) exhibited the greatest overall sensitivity (91.5%), yet produced the greatest number (119) of FP results. Specificity and efficiency were both greater than 88%, but this large number of FP tests would put an unnecessary burden on the confirmation section of a forensic laboratory or, if employed in a screening only treatment program, would produce too many unsupportable results. Raising the Opiate ELISA cutoff from 2.5 to 10 μg/L and maintaining the GC–MS cutoff of 2.5 μg/L yielded an 18% loss of assay sensitivity because of the large number (97) of FN specimens. However, this higher Opiate ELISA screening cutoff greatly improved specificity and decreased the number of FP results. A similar efficiency of 91% was found. The use of a 2.5-μg/L cutoff may not be practical for forensic applications because of analytical limitations of the confirmation assay. Performance characteristics at higher confirmation cutoffs of 20 μg/L or greater had efficiencies of better than 94%.

SAMHSA has proposed a 40-μg/L opiate screening cutoff with a confirmation cutoff of 40 μg/L morphine and/or codeine to detect opiate use in oral fluid for federally mandated drug testing programs. Comparing assay performance at these cutoffs with our lowest cutoff (2.5/2.5) identified 143 fewer TP specimens, but decreased the number of FP results by 109. Specificity and efficiency were high, 99.1% and 95.1%, respectively, but there was a 14.8% decrease in sensitivity to 76.7%. We anticipated that lowering the SAMHSA threshold cutoffs to those currently utilized in the U.K. (30/30) would improve performance characteristics. Although specificity and efficiency differed only fractionally, 17 additional TP specimens were identified with the 30/30 U.K. cutoff.

We evaluated two additional cutoffs with concentrations below the U.K. and SAMHSA thresholds. When comparing assay performance at 20/20 and 30/20, as expected, we found that the higher Opiate ELISA screening cutoff increased specificity and decreased sensitivity. In contrast, sensitivity increased when the screening cutoff was maintained and the confirmation threshold increased, as illustrated with the 30/20 and 30/30 cutoffs. The best assay efficiency (95.6%) of the six evaluated was obtained with the 20/20 cutoff. Sensitivity was greater than 80% and specificity greater than 98%. Assay performance characteristics for the four higher cutoffs were comparable with total differences of 36 TP results and 5 FP results between the 20/20 and 40/40 cutoffs. All four threshold concentrations are within the analytical capabilities of the Opiate ELISA and routine GC–MS analysis, however the 20/20 cutoff showed improved performance characteristics over both SAMHSA and U.K. cutoffs.

### Table II. Sensitivity, Specificity, and Efficiency of the Cozart Microplate EIA Opiate Oral Fluid Kit for Detection of Codeine and Metabolites in Oral Fluid at Different Immunoassay/GC–MS Cutoffs (N = 1406)

<table>
<thead>
<tr>
<th>Cutoff (μg/L)</th>
<th>2.5/2.5*</th>
<th>10/2.5*</th>
<th>20/20*</th>
<th>30/20*</th>
<th>U.K.*</th>
<th>SAMHSA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>334</td>
<td>268</td>
<td>227</td>
<td>210</td>
<td>208</td>
<td>191</td>
</tr>
<tr>
<td>FP</td>
<td>119</td>
<td>21</td>
<td>15</td>
<td>9</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>TN</td>
<td>922</td>
<td>1020</td>
<td>1107</td>
<td>1113</td>
<td>1124</td>
<td>1137</td>
</tr>
<tr>
<td>FN</td>
<td>31</td>
<td>97</td>
<td>47</td>
<td>64</td>
<td>53</td>
<td>58</td>
</tr>
<tr>
<td>Sensitivity* (%)</td>
<td>91.5</td>
<td>73.4</td>
<td>82.9</td>
<td>76.6</td>
<td>79.7</td>
<td>76.7</td>
</tr>
<tr>
<td>Specificity* (%)</td>
<td>88.6</td>
<td>98.0</td>
<td>98.7</td>
<td>99.2</td>
<td>99.0</td>
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<td>Efficiency* (%)</td>
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<td>91.6</td>
<td>95.6</td>
<td>94.8</td>
<td>95.4</td>
<td>95.1</td>
</tr>
</tbody>
</table>

* Cozart Microplate Opiate EIA Oral Fluid Kit screening cutoff/morphine, normorphine, codeine, and norcodeine confirmation cutoff by GC–MS.

### Discussion

The testing for opiate use and abuse historically has included detection and confirmation of codeine and morphine. The inclusion of norcodeine in the GC–MS confirmation analysis had no effect on assay performance characteristics because norcodeine was never present without concurrent codeine. In addition, norcodeine has low cross-reactivity with Opiate ELISA antibodies. Following our controlled codeine administration, norcodeine was detected at a concentration range of 2.6–191 μg/L, concentrations well below those needed to generate a positive Opiate ELISA result. Compounds with higher cross-reactivity (hydrocodone, dihydrocodeine, and pholcodine) were not present in our controlled study and did not contribute to sensitivity, specificity, and efficiency results. However, if present in routine oral fluid specimens, these compounds most likely would produce positive Opiate ELISA tests that would not be confirmed if only codeine and morphine were targeted analytes.

These oral fluid screening and confirmation data following controlled codeine drug administration will aid in the interpretation of oral fluid drug tests. In addition, they permit quantitative evaluation of potential immunoassay and confirmation cutoffs. The selection of appropriate screening and confirmation cutoffs will be important in establishing oral fluid as an alternative matrix for drug testing.
alternative matrix for drug testing. This controlled codeine administration study demonstrates that the Cozart Opiate ELISA provides a suitable screening procedure for detecting codeine exposure, with supporting evidence that a 20/20 cutoff improves performance characteristics.

References