Simultaneous Determination of Acetylcodeine, Monoacetylmorphine, and Other Opiates in Urine by GC–MS

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Abstract

In addition to 6-monoacetylmorphine (6-MAM), acetylcodeine (AC) has been suggested as a marker for the use of illicit heroin. We report a sensitive opiate gas chromatographic–mass spectrometric assay that detects AC, diacetylmorphine, and the propionylated derivatives of codeine, morphine, 6-MAM, and norcodeine. The analytes were extracted by solid phase with recoveries from 62 to 98%. The limits of detection (LOD) and quantitation (LOQ) for AC was 0.5 and 1.0 pg/L. The LOD of the other analytes was 2.0 pg/L and the LOQs ranged from 2 to 10 pg/L. The assay was linear for each analyte from the LOQ to 200 pg/L or 400 pg/L (morphine and codeine) with r ≥ 0.996, except for diacetylmorphine which was linear to 100 pg/L with r = 0.994. The within-run and between-run precision were below 10% CV for all analytes. There was no significant hydrolysis of AC to codeine in urine (pH 4.7 and 8.0) after 23 weeks of refrigeration or freezing. After storage at room temperature in urine of pH 8.0, AC was completely hydrolyzed after 5 weeks, but at pH 4.7, 58% of the AC remained after 15 weeks of storage at room temperature. The sensitivity of this assay was adequate to detect AC in the urine of heroin abusers. In preliminary studies, AC was detected in 6 of 69 opiate positive urines. Concentrations ranging from 1 to 48 pg/L were observed. These concentrations were found to be low when compared with the concentrations of 6-MAM, codeine, and morphine also detected in the urines.

Introduction

Forensic urine drug testing for the abuse of opiates involves the detection of morphine or codeine or both. Usually, only total morphine or codeine is determined, which makes it difficult to determine the source of the opiates detected in urine. There are five factors that may complicate the interpretation of the results of opiate analyses: 1. codeine contamination of heroin; 2. the presence of morphine and codeine in poppy seeds; 3. the metabolic conversion of codeine to morphine; 4. genetic differences that result in the variable metabolism of codeine; and 5. the different rates of urinary excretion of codeine and morphine (1,2). The presence of 6-monoacetylmorphine (6-MAM) in urine is regarded as a specific marker for the illicit use of heroin (3–5). Diacetylmorphine (DIAM) is hydrolyzed to 6-MAM by serum or liver esterases or spontaneous hydrolysis. 6-MAM is then further hydrolyzed to morphine. Unfortunately, the detection and confirmation of 6-MAM is not as routine as assays for codeine and morphine. Cone et al. (3) reported the results of a clinical study in which they found that 6-MAM had a short detection time of 2–8 h in urine (t1/2 = 0.6 h). Also, the concentrations of 6-MAM in urine are often very low compared with morphine or codeine, so detection is difficult.

Acetylcodeine (AC), which is an impurity of manufacture found in heroin, has also been mentioned as a specific marker for the illicit use of heroin (6,7). AC is present in heroin in varying amounts depending on the source and the extent to which morphine is purified from opium. Soine (6) reports that the AC concentration is usually 2 to 20% relative to DIAM and may be as high as 45%. Although AC has been mentioned as an additional marker of heroin use, there have been no reports in the literature on the detection or quantitation of AC in biological fluids. In order to determine if AC would be a suitable indicator of illicit heroin use, we describe a sensitive gas chromatographic–mass spectrometric (GC–MS) procedure for the detection and quantitation of AC in urine along with DIAM, 6-MAM, codeine, morphine, and norcodeine.

Materials and Methods

Chemicals

Acetylcodeine was purchased from Alltech (State College, PA). Codeine, 3H2-codeine, morphine, 3H3-morphine, 6-MAM, 3H6-6-MAM, norcodeine, and DIAM were purchased from Radian (Austin, TX). All solvents and reagents were analytical or HPLC grade. Methanol, methylene chloride, acetonitrile, concentrated ammonium hydroxide, and sodium carbonate...
were obtained from EM Science (Gibbstown, NJ). Sodium phosphate dibasic, propionic anhydride, β-glucuronidase (from Helix pomatia, Type H-2), and sodium azide were purchased from Sigma Chemical (St. Louis, MO). Sodium phosphate monobasic, glacial acetic acid, sodium bicarbonate, isopropanol, toluene, hexane, and isoamyl alcohol were purchased from Fisher Scientific (Fair Lawn, NJ). Pyridine was obtained from Aldrich Chemical (Milwaukee, WI).

Instrumentation

The GC was a Hewlett Packard (Palo Alto, CA) 5890 with a split/splitless injection port and a 7673 autosampler. The mass selective detector (MSD) was a Hewlett Packard model 5791A with HP Chemstation software to control the operation of the GC and MSD. The GC oven temperature program was as follows: initial temperature, 170°C; initial time, 1.0 min; rate, 10°C/min; final temperature, 280°C; and final time, 3.0 min. The injection port temperature was 260°C, and the transfer line temperature was 290°C. The column was an HP-1 capillary column (12 m x 0.2 mm, 0.33-μm film thickness).

The MSD was operated in the selected ion monitoring (SIM) mode using the following ions: 341, 282, and 229 for AC; 355, 282, and 229 for codeine; 358 and 285 for 2H3-codeine; 327, 369, and 268 for diacetylmorphine; 383, 324, and 268 for 6-MAM; 333 and 389 for 2H6-6-MAM; 341, 268, and 397 for morphine; 344 and 271 for 2H3-morphine; 223, 224, and 397 for norcodeine; and 299 and 242 for hydrocodone. Dwell times were set at 40 ms. Calibration was based on a single-point calibration using peak-area ratios of the first ion listed for each analyte. The calibrator was 25 μg/L for AC, 6-MAM, and DIAM and 50 μg/L for codeine, morphine, and norcodeine. 6-MAM, codeine, and morphine were quantitated using their respective deuterated internal standards. In addition, AC and diacetylmorphine quantitation was based on 2H6-6-MAM and norcodeine was based on 2H3-codeine as internal standards. In a separate method that did not require derivatization, quantitation of AC and codeine were based on calibration curves using hydrocodone as an internal standard.

Sample preparation

Twenty microliters of 2H6-6-MAM and 50 μL of 2H3-codeine and 50 μL of 2H3-morphine (all solutions were 10 mg/L) were added to 5.0 mL of calibrators, controls, and samples. The urine was buffered to pH 6.0 with 2 mL of 0.1M phosphate buffer. The samples were vortex mixed for 10 s and centrifuged for 5 min. The urine was then extracted with ZSDAU020 Clean Screen extraction columns (United Chemical Technologies, Bristol, PA). Briefly, the procedure consisted of conditioning the columns with methanol (3 mL), deionized water (3 mL), and phosphate buffer (1 mL). The samples were added to the columns, followed by deionized water (2 mL), 2 mL 0.1M acetic acid buffer (pH 4.5), and methanol (3 mL). The columns were then reconstituted with 50 μL of toluene/hexane/isoamyl alcohol (78:20:2). Two microliters of the reconstituted sample were injected onto the GC-MS.

There were a few modifications to the described procedure. For the stability study only, 40 μL of a 10-mg/L solution of hydrocodone was added to each calibrator, control, and sample as the internal standard. The samples were not derivatized, so the eluent was 50 μL of the toluene/hexane/isoamyl alcohol solvent. For the analysis of total morphine and codeine the samples were hydrolyzed with β-glucuronidase before extraction by solid phase. One milliliter of 0.2M acetic acid buffer (pH 4.5) and 40 μL of enzyme (4000 units) were added to each sample and heated overnight (18 h) at 55°C.

Validation protocol

The extraction recovery was determined by adding the analytes to drug-free urine at a low and high concentration (n = 3). After extraction, the above deuterated standards were then added as external standards. Peak-area ratios were then compared with unextracted standards of equal concentrations.
The within-run precision of the method was determined by analysis of a low and a high concentration of each analyte on the same day (n = 10). The between-run precision was determined by the analysis of the same concentrations once daily on 10 separate days. The lower limit of detection (LOD) for each analyte was the lowest concentration, obtained by serial dilution, with a signal-to-noise ratio of 3 or greater, and the ion ratios within ±20% of the calibrator ion ratios. The lower limit of quantitation (LOQ) was the lowest concentration yielding a result within ±20% of the target concentration and with a coefficient of variation (CV) of<br>
had hydrolyzed to codeine after 5 weeks at pH 8 (Figure 2). After 23 weeks of storage in a refrigerator at pH 8, 90% of the AC still remained unhydrolyzed in the urine, and codeine was not detected in the frozen urine. It was determined that the preservative, sodium azide, had no effect on the stability of AC in urine because hydrolysis did not occur at a faster rate in unpreserved urine stored at room temperature at either pH.

In 100 opiate positive urine specimens obtained from criminal justice clients, 69 confirmed positive for morphine and codeine. Of these 69 samples, 6 (8.7%) were found to contain AC in concentrations ranging from 1 to 48 µg/L with a mean of 16.2 µg/L. The GC–MS chromatogram of the free fraction of the urine extract for Sample 2 is presented in Figure 3. 6-MAM was detected in 13 (18.8%) of the specimens. 6-MAM was present in all the specimens that contained AC in concentrations ranging from 106 to 1470 µg/L. DIAM was not detected in any of the specimens. Quantitative results for the specimens containing AC are listed in Table IV. The AC concentration was low compared with the 6-MAM concentration in each specimen. The AC concentrations averaged 4.1% (range, 0.7–12.9%) of the 6-MAM concentrations for the six specimens.

Discussion

Because AC is only present in heroin at a concentration 2–20% of the DIAM dose, we speculated that the concentration would be lower than that of 6-MAM, and we would, therefore, need a sensitive assay for the detection of AC. The assay was designed specifically for the optimization of AC sensitivity. We were able to achieve an LOD of 0.5 µg/L and an LOQ of 1.0 µg/L. This assay is one of the most sensitive assays reported in the literature using an MSD for the routine detection and quantitation of morphine and codeine. Few assays detect opiates as low as 2 µg/L and quantitate as low as 10 µg/L. In an attempt to improve the sensitivity of the assay, several parameters were investigated: extraction recovery of solid-phase versus liquid–liquid extraction, derivatizing reagents, and reconstitution solvents.

Most of the methods employing liquid–liquid extraction of opiates from urine use 4:1 or 9:1 mixtures of methylene chloride or chloroform with an alcohol, usually isopropanol (8). The mixtures, that are 4:1 have a higher recovery than the 9:1 mixtures because of the increased polarity. Solid-phase extraction was determined to give a higher recovery of AC and a cleaner extract than liquid–liquid extraction with various solvent systems. The recovery of AC from solid-phase extraction is 6 to 23% higher than 4:1 mixtures of methylene chloride/ethanol, methylene chloride/isobutanol, methylene chloride/isopropanol, chloroform/ethanol, and chloroform/isopropanol.

Propionylated derivatives of the opiates were used because they were more stable and displayed fewer coelution problems. The propionylated derivatives were found to be stable for at least 72 h whereas the silyl derivatives were only stable for 24 h. Also, there was an interference peak that coeluted with the 6-MAM silyl derivative preventing quantitation. AC does not hydrolyze to codeine during derivatization even at 70°C for 20 min. No propionylated codeine was detected in the AC standard. The mild derivatizing conditions of 40°C for 1 h were chosen to eliminate interferences from the enols of hydromorphone, hydrocodone, oxymorphone, and oxydone. Also, less DIAM is hydrolyzed to 6-MAM. The conditions were sufficient to completely derivatize morphine so that only dipropionylated morphine was detected.

The underivatized assay was initially developed for the stability study in which only AC and codeine were determined. It was thought that an assay without a derivatization step would be more sensitive for AC, but we found derivatization with propionic anhydride had no negative effects on the sensitivity for AC. Both assays have an LOD of 0.5 µg/L and an LOQ of 1.0 µg/L. Derivatization greatly improves the chromatography of codeine and 6-MAM, and without it, morphine cannot be detected. In addition, without derivatization, specimens containing codeine cannot be analyzed by GC–MS after derivatized samples, especially silyl derivatives, without first changing the injection port liner.

### Table III. The Within-Run and Between-Run Precision for the Analysis of the Propionylated Derivatives of the Opiates (n = 10)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (µg/L)</th>
<th>Within-run %CV</th>
<th>Between-run %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC*</td>
<td>10</td>
<td>3.4</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.7</td>
<td>7.5</td>
</tr>
<tr>
<td>Codeine</td>
<td>10</td>
<td>1.5</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td>DIAM</td>
<td>10</td>
<td>3.4</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4.4</td>
<td>6.0</td>
</tr>
<tr>
<td>6-MAM</td>
<td>10</td>
<td>3.8</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Morphine</td>
<td>10</td>
<td>4.0</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Norcodeine</td>
<td>10</td>
<td>8.5</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.6</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*Abbreviations: AC, acetylmorphine; DIAM, diacetylmorphine; 6-MAM, 6-monoacetylmorphine; CV, coefficient of variation.

### Table IV. Quantitative Results of Six Criminal Justice Urine Specimens Containing AC (µg/L)

<table>
<thead>
<tr>
<th>Case no.</th>
<th>AC*</th>
<th>6-MAM</th>
<th>Codeine</th>
<th>Norcodeine</th>
<th>Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-MAM</td>
<td>Free</td>
<td>Total</td>
<td>ND</td>
<td>3650</td>
</tr>
<tr>
<td>1</td>
<td>48</td>
<td>1470</td>
<td>307</td>
<td>540</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>124</td>
<td>137</td>
<td>400</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>1190</td>
<td>104</td>
<td>340</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>300</td>
<td>171</td>
<td>220</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>106</td>
<td>189</td>
<td>560</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>310</td>
<td>63</td>
<td>135</td>
<td>12</td>
</tr>
</tbody>
</table>

*Abbreviations: AC, acetylmorphine; 6-MAM, 6-monoacetylmorphine; ND, not detected.
During method development, we observed variable recoveries of the analytes with different reconstitution solvents, especially underivatized hydrocodone and codeine. Most assays reported in the literature use ethyl acetate, isopropanol, or chloroform to reconstitute samples before injection onto the GC–MS. We observed greatly reduced recoveries (with high CVs) for hydrocodone and codeine. Most assays for the reconstitution solvent greatly improving recovery to 97% for hydrocodone (compared with an unextracted standard). Recovery using other solvents was <50%. We believed that there were compounds in the urine that were coextracting with the analytes of interest preventing them from dissolving in the reconstitution solvent. Using a less polar reconstitution solvent, toluene/hexane/isoamyl alcohol, solved this problem.

AC was found to be more stable at pH 4.7 than pH 8.0 in urine. These results were in agreement with studies in the literature (4,5,9–13) that report compounds with acetyl groups like DIAM and 6-MAM are more stable at acidic pHs. Poochikian and Cradock (9) found that DIAM was most stable at pH 4.5. Bertol et al. (10) commented that AC is more stable than 6-MAM in illicit heroin samples, but it is not known if this is true of biological fluids as well. There has been a lot of variation in the literature on the stability of DIAM in urine. Rop et al. (11) reported that 6-MAM was completely hydrolyzed in urine (pH unknown) in seven days at room temperature, and 9% was hydrolyzed after 14 days at -20°C. Poochikian and Cradock (9) found a >50% loss after nine days of storage in an aqueous solution (pH 8.6) at room temperature. Paul et al. (5) reported a 44% loss of 6-MAM after 2 weeks in urine (pH 6.8), whereas Barrett et al. (12) found no hydrolysis at pHs 4.0 to 7.4 under the same conditions. Fuller and Anderson (4) found that it took 12 weeks for the complete hydrolysis of 6-MAM to morphine in urine at pH 8.0 at room temperature. The reason for the large variation in the hydrolysis rates may be the different ionic strengths of the buffers used in each study. Beaumont (13) observed a linear relationship between ionic strength (μ) and the stability of DIAM in an aqueous solution. Small increases in μ resulted in large decreases in the stability of DIAM. Maximum shelf-life of DIAM was observed when no buffer was present. Beaumont reported that μ will have a greater affect on the stability of compounds than the pH of the solution. In our stability study, both phosphate buffers, pH 4.7 and pH 8.0, had an ionic strength of 0.2, so that any difference in the rate of hydrolysis was due to the pH difference alone.

From the results of preliminary studies with actual opiate positive urines, it was determined that AC may be present in the urine of heroin abusers. The AC concentrations observed in these urines were low compared with the 6-MAM, morphine, and codeine concentrations. We assume most of the AC administered with DIAM had been hydrolyzed to codeine, but, because AC is stable in urine, the hydrolysis was in vivo and not from spontaneous hydrolysis after storage. All urines were stored at room temperature for less than one week and then frozen. The total codeine content (AC, norcodeine, and total codeine) was found to be 0.9 to 5.0% of the total morphine content (6-MAM and total morphine). This may be a low estimation of the actual codeine content because it is possible all the codeine-6-glucuronide was not hydrolyzed. As Romberg and Lee (14) report β-glucuronidase from Helix pomatia will only hydrolyze 52% of codeine-6-glucuronide even after 22 h. Therefore, the codeine content may actually be higher. We are currently investigating other sources of β-glucuronidase for an improved recovery of codeine.

Conclusion

AC is found in illicit heroin concentrations varying from 2 to 20% of the DIAM concentration and has been suggested as an additional marker for illicit heroin use. We validated a sensitive GC–MS method for the determination of AC and other opiates in urine. Although AC was found to be stable in urine under various conditions (e.g., preliminary studies of the urines of heroin abusers), it was found in fewer specimens and at lower concentrations than 6-MAM.

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References


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