Paradoxical Results in Urine Drug Testing for 6-Acetylmorphine and Total Opiates: Implications for Best Analytical Strategy

Olof Beck1,* and Michael Büttcher2,†

1Department of Medicine, Division of Clinical Pharmacology, Karolinska University Hospital, SE-17176 Stockholm, Sweden and 2Arztpraxis f. Medizinische Mikrobiologie, Labordiagnostik and Hygiene, Dessau, Germany

Abstract

A major task in urine drug testing is to detect heroin intake. The most common way of doing this is by using morphine as the analytical target in opiate immunoassay screening. However, this strategy sometimes leads to false-positive results because morphine is not a metabolite unique to heroin. The objective of this study was to evaluate the usefulness of the unique heroin metabolite 6-acetylmorphine (6-AM) as the primary analytical target in combination with morphine in the screening assay. A total number of 3521 randomly collected urine samples from 707 patients undergoing heroin substitution treatment were investigated for 6-AM and opiates by CEDIA (cloned enzyme donor immunoassay) and for opiates by DRI immunoassays and by gas chromatography-mass spectrometry (free 6-AM, free morphine, total morphine, and total codeine). The rate of positive outcome in the screening for 6-AM was 9.1% (cutoff 10 pg/L), and for opiates, it was 22.6% (cutoff 300 pg/L), which is in accordance with a known shorter detection time for 6-AM following heroin intake. However, by comparing 6-AM and opiate screening results at different cutoff levels, it was observed that 7–8% of the samples and 12.5% of the patients with detectable 6-AM had an unexpected low content of free and total morphine in the urine. This study confirms earlier observations that certain individuals may escape detection in urine drug testing when morphine is being utilized for the detection of heroin intake. The underlying mechanism for this may be a metabolic defect and/or interaction. It is concluded that 6-AM is a valuable target analyte in the screening of drugs of abuse in urine and may be used in combination with opiate screening in clinical testing.

Introduction

Detection of heroin use is one of the major tasks in urine drug testing for opiates. The established way of doing this is by using the predominant urinary metabolite, morphine, as the principal analytical target both in the screening and confirmation, but this may sometimes be complicated, as the presence of morphine in urine is not a unique indicator of heroin intake (1). For example, intake of codeine, ethylmorphine, pholcodine, poppy seeds, and morphine itself may also be causing its presence in urine (2). In addition, other opiates can cause a positive response in the opiate immunoassay (e.g., dihydrocodeine, hydrocodone, hydromorphone, and oxycodone). In the clinical setting, this analytical sensitivity towards other possibly ingested substances may be of significant value (and not be a problem) as analytical data from the confirmation assay can be used for interpretation of the most likely source of the detected substances. In a therapeutic setting, ingestion of these substances, including poppy seeds, may not be tolerated, and in other applications, this may not be a possible or advisable approach (3).

One way to increase the level of confidence in detecting heroin intake is to focus on the unique intermediate metabolite 6-acetylmorphine (6-AM) (2). This was initially done by choosing 6-AM as an analytical target in the confirmation of samples that screened positive for opiates. However, the practice of screening with immunoassay for total opiates at a 2000 pg/L cutoff and confirming 6-AM at 10 pg/L introduced for workplace drug testing has been questioned as the optimal approach, based on observations of resulting false-negative results in physiological samples (4,5). Recently, immunochemical screening has become possible for 6-AM, which makes 6-AM a possible primary target in both screening and confirmation. However, it is well known that 6-AM levels in urine are lower for morphine, and that the detection time, therefore, is considerably shorter (6). In addition, the first evaluation of the 6-AM cloned enzyme donor immunoassay (CEDIA) in routine use reported a suspected interfering cross-reactivity from other opiate-related compounds (7). So far, 6-AM has not been commonly used as a primary screening target for heroin testing. Published observations suggest that 6-AM may offer increased sensitivity over morphine in certain individuals that display unexpected low levels of urinary morphine after heroin intake.
(8,9). Interestingly, methadone-treated patients, who were twins, showed a metabolic pattern of low excretion of total morphine and codeine together with 6-AM (8). Subsequently, von Euler and co-workers reported a similar phenomenon by observing that 7.6% of urines containing 6-AM (> 30 µg/L of 6-AM) had unexpected low levels (< 300 µg/L) of morphine-3-glucuronide (9). More recently, Fortner and co-workers (10) reported five individuals who were negative for opiates in urine using a 300 µg/L cutoff, although 6-AM was present in the range of 10–144 µg/L.

The objective of the present investigation was to provide further analytical data on the use of 6-AM as a screening target as compared to total opiates for the detection of heroin intake in an addiction patient population. The study compared the use of three different cutoff levels for the CEDIA opiate test with the CEDIA 6-AM test at two cutoff levels using samples from patients undergoing heroin substitution treatment.

**Material and Methods**

**Clinical urine samples**

A total of 3521 randomly selected urine specimens were obtained from 707 out-patients (486 men and 221 women; age range 18–55 years) undergoing heroin substitution treatment with d,l-methadone (581 patients), l-methadone (“Polamidon”; 67 patients), or buprenorphine (“Subutex”; 59 patients). The urine specimens were collected during a period of 11 consecutive weeks. The urine sampling frequency of each patient varied from weekly to once during the study period (Figure 1) and was decided by the patient’s physician on clinical grounds. Screening results for other drugs of abuse in the study samples revealed 22% positive samples for benzodiazepines, 9% for cocaine (from 3521 samples tested), and 1.9% for amphetamines (from 2772 samples tested).

**Immunochemical assays**

Urine samples were screened routinely for benzodiazepines (with enzymatic “online” hydrolysis), benzylecgonine, methadone metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), amphetamines, 6-AM, and opiates with CEDIA (Microgenics, Passau, Germany). In addition, the DRI opiates assay (Microgenics) was conducted on selected samples. Assays were performed on a Hitachi 911 (Roche, Mannheim, Germany), according to the manufacturers instructions with the following specifications: amphetamines (cutoff 500 µg/L) calibration points 0, 500, 1000, and 3000 µg/L; benzodiazepines (cutoff 100 µg/L) calibration points 0, 100, 300, 800, 1600, and 2400 µg/L; benzylecgonine (cutoff 100 µg/L) calibration points 0, 100, 300, 1000, and 2000 µg/L; EDDP (cutoff 100 µg/L) calibration points 0, 100, 500, and 2000 µg/L; 6-AM (cutoff 5 and 10 µg/L) calibration points 0, 5, 10, and 20 µg/L; opiates-CEDIA (cutoff 100, 300, and 2000 µg/L) calibration points 0, 100, 300, 800, and 2000 µg/L; and opiates-DRI (cutoff 100 µg/L) calibration points 0, 100, 300, 800, and 1466 µg/L. Calibration points not supplied were prepared from Microgenics calibrators by the Hitachi 911 by simply pipetting modified sample volumes. Quality control was conducted using the CEDIA Specialty Control Set and CEDIA 6-AM Control Kit (Microgenics) at least once within a series. The between-day coefficient of variation (CV) at the 225 µg/L opiate control level was 5.3% ($\bar{x} = 135$) for the CEDIA and 6.1% ($\bar{x} = 37$) for the DRI application, and the 6-AM control at 7.5 µg/L gave a CV of 9.2% ($\bar{x} = 52$).

For cross-reactivity studies of the CEDIA 6-AM assay, prepared solutions of 25, 12.5, 6.25, and 3.13 mg/L morphine in saline were tested. For cross-reactivity studies of 6-AM in the CEDIA and DRI opiates assays, prepared solutions of 150, 75, 37.5, 18.75, and 9.38 µg/L 6-AM in saline were tested. Cross-reactivity values were calculated by linear regression analysis.

Sample integrity was monitored by measuring the pH, sample check, and creatinine (reagents from Microgenics) on every sample according to protocols from the manufacturer.

**Gas chromatography–mass spectrometry (GC–MS) methods**

The following procedure (derivatization and chromatographic system taken from Paul and co-workers (4)) was used for quantification of free opiates (6-AM, morphine, and codeine): 3 mL of urine was spiked with the corresponding deuterated internal standard: 30 ng 6-AM-d$_3$, 300 ng morphine-d$_3$, and 300 ng codeine-d$_3$ (all from Promochenn, Wesel, Germany). Sample pH was adjusted to between 8.5 and 9.5 with 0.1M NaOH. Subsequently, solid-phase extraction was performed on SPEC-dau 3-mL columns (Varian, Darmstadt, Germany), which had been conditioned with 0.2 mL methanol. The sample was applied onto the cartridge and pulled through by slowly applying gentle vacuum (~20 kPa). Cartridges were washed by sequential addition of 0.5 mL deionized water, 0.5 mL 0.1M acetic acid, and 0.5 mL 50% methanol (v/v). Cartridges were then dried under a vacuum for 5 min. The analytes were eluted slowly with 0.8 mL freshly prepared ethyl acetate/methanol/ammonia (40:10:1, by volume) into a glass vial. The eluate was immediately evaporated to dryness under nitrogen in a heated metal block kept at 65°C. After cooling, the extract was dissolved in 75 µL.
pentafluoropropionic anhydride (Supelco, Taufkirchen, Germany) and derivatized at 65°C for 20 min in the heating block. The excess reagent was evaporated as described earlier, and the residue was then dissolved in 70 µL of ethyl acetate. One microliter was injected (splitless, injector temperature 280°C) into the GC-MS (Shimadzu, Duisburg, Germany) with an AOC20i autosampler (Shimadzu). GC-MS was operated in electron impact mode with helium as the carrier gas. Chromatographic separation was achieved on a DB5-MS fused-silica column (J&W Scientific, Promochem, Wesel, Germany), 15-m × 0.25-mm i.d., 0.25-µm film thickness. The oven was initially held at 70°C for 2 min, ramped to 200°C/min to 160°C. The temperature was further increased at 10°C/min to 250°C and then heated at 200°C/min to 300°C and held 1 min. The transfer line was maintained at 300°C. Data were acquired in the selected-ion monitoring mode. The pentafluoropropionyl (PFP) derivatives were monitored at m/z 414 (target ion), 577, 430, and 266 for morphine (dipPF); 417 (target ion), 580, 445, and 285 for codeine (monopPF); 285 (target ion), 448, and 286 for codeine-d3; 414 (target ion), 473, and 361 for 6-AM (monopPF); and 417 (target ion), 476, and 364 for 6-AM-d3. Retention times were 12.6 min for codeine-d3; 269 (target ion), 473, and 361 for 6-AM (monoPFP); and 417 (target ion), 285, 448, and 286 for codeine-d3; 414 (target ion), 577, 430, and 266 for morphine (dipPFP); 417 (target ion), 473, and 361 for 6-AM (monoPFP); and 414 (target ion), 580, 445, and 285 for codeine-d3; 414 (target ion), 473, and 361 for 6-AM (monopPF); and 417 (target ion), 476, and 364 for 6-AM-d3. Retention times were 12.6 min for morphine, 13.0 min for codeine, and 13.5 min for 6-AM.

Linear nine-point calibration was done from 1 mL each fortified drug-free pooled urine, covering the range 1.95 to 500 µg/L for 6-AM, morphine, and codeine (Promochem) with 10 ng 6-AM-d3, 100 ng morphine-d3, and 100 ng codeine-d3 as internal standard. The limit of detection (LOD) was calculated according to German standard DIN 32645 (11,12) using software B.E.N. version 2.03 [Arvecon GmbH, Walldorf, Germany (2003)] and resulted in 0.5 µg/L for 6-AM, 0.8 µg/L for morphine, and 1.0 µg/L for codeine when 3 mL of sample was prepared as described previously. The intra- and interassay CV for morphine was ≤ 5% at the 50 and 100 µg/L level and for 6-AM, < 14% at the 4 µg/L level. Extraction efficiency was investigated using spiked drug-free pooled urine samples at concentrations of 100 µg/L for morphine and codeine and 10 µg/L for 6-AM by adding the corresponding internal standards after solid-phase extraction. Recovery was found to be 84% for morphine, 85% for codeine, and 90% for 6-AM with interassay CVs below 5% (n = 5).

Total morphine and codeine was analyzed according to Lin and co-workers (13) using GC-MS and hydrochloric acid hydrolysis. The LOD was about 10 µg/L for both compounds using a 3 mL sample volume, and the interassay CVs were 8% for morphine and 7% for codeine.

### Results

#### Opiates and 6-AM screening results at different cutoffs

Using the standard cutoff at 300 µg/L for opiates, 23% of the samples and 43% of the patients were detected positive during the study period (Table I). The number of positive samples increased to 24.0% (6% increase) when using 100 µg/L as a cutoff and decreased to 18.4% (19% decrease) when using 2000 µg/L as cutoff. For 6-AM, the rate of positive results were about half of the values for opiates (Table I). The use of 5 µg/L as the cutoff value for 6-AM instead of 10 µg/L resulted in a 14% increase in positive outcome (Table I).

**Correspondence of opiate and 6-AM screening results**

Of the 370 6-AM-positive samples, 79% had a 6-AM response above 10 and an opiate response above 2000 µg/L (Table II). Of the 51 samples with a 6-AM response between 5 and 10 µg/L, the proportion that had an opiate response above 2000 µg/L was 88%. The majority (73%) of 6-AM-positive samples had levels exceeding 20 µg/L (Table I). Of the samples containing detectable 6-AM, 7% to 8% (depending on 6-AM cutoff limit) had a response for opiates below the 300 cutoff limit, and 3% to 4% has a response below 100 µg/L (Table II).

The agreement of results for opiate and 6-AM assays at different cutoff levels are summarized in Figure 2. Between 10% to 16% of the samples had deviant results between the two parameters, indicating that they reveal somewhat complementary information.

#### Characterization of 6-AM-positive samples with low opiate content

Twenty-nine samples containing detectable 6-AM but low levels of opiates (CEDIA opiates < 2000 µg/L) were further characterized using GC–MS and opiate DRI methods (Table III). These 29 samples came from 24 individuals, of
which 10 were females and 22 were receiving d,l-methadone. Four samples with results below, but very close to, the 2000 pg/L limit, were not included. The 29 samples from 24 individuals represented 12.5% of all 6-AM-positive patients and 3.4% of the total number of patients. The GC-MS results confirmed the presence of 6-AM in all cases as well as the low levels of both free and total morphine and codeine. The quantitative responses from the immunoassays were in agreement with each other and with GC-MS data. The concentration of creatinine tended to be low in this subgroup of samples. The median creatinine concentration was 74 mg/dL, and 90% of the results were below the median concentration (133 mg/dL) of the total 3521 samples in the study. One sample (#10) had undetectable creatinine and EDDP levels but contained a high level of methadone, indicating sample adulteration.

Figure 2. The agreement between CEDIA 6-AM and CEDIA opiate screening results at different applied cutoff levels.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Patient Number</th>
<th>Creatinine (mg/dL)</th>
<th>6-AM CEDIA (&gt; 5 pg/L)</th>
<th>Opiate CEDIA (pg/L)</th>
<th>Opiate DRI (pg/L)</th>
<th>Free 6-AM GC-MS (pg/L)</th>
<th>Free Morphine GC-MS (pg/L)</th>
<th>Total* Morphine GC-MS (pg/L)</th>
<th>Total* Codeine GC-MS (pg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>52</td>
<td>&gt; 20</td>
<td>80</td>
<td>69</td>
<td>54.5</td>
<td>4.9</td>
<td>86</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>66</td>
<td>19.8</td>
<td>41</td>
<td>42</td>
<td>18.5</td>
<td>1.5</td>
<td>62</td>
<td>57</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>91</td>
<td>&gt; 20</td>
<td>197</td>
<td>148</td>
<td>30.0</td>
<td>7.9</td>
<td>158</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>15</td>
<td>&gt; 20</td>
<td>85</td>
<td>54</td>
<td>26.0</td>
<td>2.9</td>
<td>64</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>351</td>
<td>&gt; 20</td>
<td>79</td>
<td>43</td>
<td>48.1</td>
<td>&lt; 0.8</td>
<td>83</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>14</td>
<td>&gt; 20</td>
<td>125</td>
<td>119</td>
<td>35.3</td>
<td>41.5</td>
<td>108</td>
<td>52</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>40</td>
<td>&gt; 20</td>
<td>212</td>
<td>212</td>
<td>34.5</td>
<td>43.6</td>
<td>151</td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>14</td>
<td>5.0</td>
<td>81</td>
<td>60</td>
<td>2.4</td>
<td>30.2</td>
<td>64</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>90</td>
<td>19.0</td>
<td>68</td>
<td>29</td>
<td>14.0</td>
<td>2.4</td>
<td>&lt; 30</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>&lt; 1</td>
<td>&gt; 20</td>
<td>217</td>
<td>203</td>
<td>8.9</td>
<td>125</td>
<td>157</td>
<td>54</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>106</td>
<td>&gt; 20</td>
<td>114</td>
<td>77</td>
<td>19.7</td>
<td>&lt; 0.8</td>
<td>&lt; 150</td>
<td>&lt; 150</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>63</td>
<td>7.5</td>
<td>56</td>
<td>40</td>
<td>5.7</td>
<td>2.6</td>
<td>54</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>5</td>
<td>&gt; 20</td>
<td>273</td>
<td>251</td>
<td>86.9</td>
<td>2.3</td>
<td>212</td>
<td>68</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>220</td>
<td>6.7</td>
<td>76</td>
<td>25</td>
<td>1.1</td>
<td>8.4</td>
<td>66</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>15</td>
<td>11</td>
<td>74</td>
<td>15.7</td>
<td>59</td>
<td>40</td>
<td>8.9</td>
<td>&lt; 0.8</td>
<td>60</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>16</td>
<td>12</td>
<td>100</td>
<td>16.9</td>
<td>99</td>
<td>79</td>
<td>10.2</td>
<td>2.4</td>
<td>92</td>
<td>60</td>
</tr>
<tr>
<td>17</td>
<td>13</td>
<td>108</td>
<td>&gt; 20</td>
<td>115</td>
<td>107</td>
<td>64.0</td>
<td>16.9</td>
<td>106</td>
<td>51</td>
</tr>
<tr>
<td>18</td>
<td>13</td>
<td>105</td>
<td>&gt; 20</td>
<td>147</td>
<td>117</td>
<td>72.3</td>
<td>36.4</td>
<td>117</td>
<td>52</td>
</tr>
<tr>
<td>19</td>
<td>14</td>
<td>114</td>
<td>19.0</td>
<td>182</td>
<td>172</td>
<td>19.6</td>
<td>9.7</td>
<td>108</td>
<td>58</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>47</td>
<td>&gt; 20</td>
<td>354</td>
<td>308</td>
<td>218.0</td>
<td>1.5</td>
<td>232</td>
<td>61</td>
</tr>
<tr>
<td>21</td>
<td>16</td>
<td>89</td>
<td>9.3</td>
<td>71</td>
<td>23</td>
<td>5.4</td>
<td>5.7</td>
<td>54</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>22</td>
<td>17</td>
<td>74</td>
<td>&gt; 20</td>
<td>69</td>
<td>70</td>
<td>18.7</td>
<td>2.0</td>
<td>76</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>23</td>
<td>18</td>
<td>90</td>
<td>8.7</td>
<td>110</td>
<td>93</td>
<td>9.3</td>
<td>&lt; 0.8</td>
<td>86</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>24</td>
<td>19</td>
<td>97</td>
<td>13.5</td>
<td>136</td>
<td>132</td>
<td>9.2</td>
<td>18.4</td>
<td>116</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>185</td>
<td>&gt; 20</td>
<td>109</td>
<td>70</td>
<td>29.6</td>
<td>21.4</td>
<td>97</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>26</td>
<td>21</td>
<td>14</td>
<td>&gt; 20</td>
<td>72</td>
<td>49</td>
<td>30.1</td>
<td>8.3</td>
<td>66</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>27</td>
<td>22</td>
<td>33</td>
<td>&gt; 20</td>
<td>80</td>
<td>55</td>
<td>34.4</td>
<td>&lt; 0.8</td>
<td>74</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>28</td>
<td>23</td>
<td>74</td>
<td>&gt; 20</td>
<td>169</td>
<td>151</td>
<td>98.8</td>
<td>21.2</td>
<td>150</td>
<td>57</td>
</tr>
<tr>
<td>29</td>
<td>24</td>
<td>132</td>
<td>&gt; 20</td>
<td>113</td>
<td>75</td>
<td>29.3</td>
<td>13.8</td>
<td>91</td>
<td>&lt; 30</td>
</tr>
</tbody>
</table>

* LOD for morphine and codeine depended on sample volume available for analysis.

Sample 10 (patient 6) was included despite the low creatinine concentration as it contained EDDP (153 pg/L), and typical urinary endogenous compounds were detected with GC-MS. In addition, sample check was valid.
Cross-reactivity

The cross-reactivity of free morphine in the 6-AM CEDIA assay was found to be 0.06%. The cross-reactivity of 6-AM in the opiate CEDIA assay was 88% and in the opiate DR assay was 91%.

Discussion

The results of this study confirm previous reports (8,9) about the existence of individuals displaying paradoxical results regarding 6-AM and morphine in urine drug testing. This reinforces the value of using 6-AM as the primary analytical target when heroin intake is the reason for testing. As was already pointed out by Glass and co-workers (8) the common practice of screening urine for opiates based on morphine detection has the consequence that individuals displaying this atypical pattern would escape detection; however, the prevalence of this phenomenon has not been well described. Apart from the two individuals reported by Glass and co-workers, another five individuals displaying this atypical pattern were recently reported by Fortner and co-workers (10). Von Euler and co-workers reported further cases, but the selection of cases and the analytical approach was somewhat different from standard practice, as morphine-3-glucuronide (M3G) was the analytical target in the confirmation by liquid chromatography-MS (9). The selected cases were positive by immunoassay screening at a 300 μg/L cutoff. Out of a total of 1923 positive samples from immunoassay screening, 423 contained 6-AM (> 30 μg/L) and, out of those, 7.6% could be considered atypical based on an M3G concentration below 300 μg/L. It is probable that further cases with an atypical pattern remained undetected. The 32 atypical samples were obtained from a total of 13 individuals. It was noted in the discussion part of the report (9) that the atypical pattern was not always observed in the same individual, but no details were given.

The current study is novel as it reports the atypical pattern where the selection of samples is based on 6-AM screening. Because 6-AM is the unique metabolite of heroin, this gives an estimate of the prevalence of the observed atypical pattern of about 7% to 8%, based on samples, and 12.5%, based on individuals. These numbers are in accordance with the report of von Euler and co-workers (9). The fact that a significant number of heroin users can escape detection when using commercial opiate screening at 2000 and even 300 μg/L cutoff levels is intriguing, as it is generally assumed in urine drug testing that opiate screening safely detects heroin intake for several days. In addition, because of its shorter detection time, 6-AM is not to be expected together with low total opiate (morphine) concentrations. Therefore, the observed presence of 6-AM at low total opiate (morphine) concentrations may indicate that a somewhat longer detection time than usually assumed can occur in certain individuals.

A number of studies on 6-AM exist where the atypical pattern has not been observed. In the controlled clinical trials of heroin administration (6,14–17), a total number of only 24 different subjects have been studied. The rather limited number of subjects may be one reason why the atypical pattern was not observed in these studies. In the study by Staub and co-workers, which focused on the occurrence of acetylcocaine in urine from illicit and medical heroin users, individuals displaying the atypical pattern is evident from the data but not commented on by the authors (18). The study by Spanbauer and co-workers (19) is, however, more contradictory as no case of the atypical pattern was observed when 27 outpatients undergoing heroin substitution treatment were studied (3 samples/week, in a total of 1377 samples). In 55 samples detected by 6-AM screening (> 10 μg/L), not one example of atypical low content of total morphine (< 2000 μg/L) was found. Further studies are needed to explain the reason for this apparent discrepancy.

Several mechanisms for producing the atypical pattern have been put forward (8,9). One reason could simply be that the timing of sampling is close to administration, and that the difference in pharmacokinetics of morphine and 6-AM may lead to the existence of a time window where 6-AM and not morphine is being excreted in urine. Although this is possible from a theoretical point of view and has been mentioned by Paul and co-workers (4), we think this is more unlikely based on published plasma and urine pharmacokinetic investigations (6,14–18). For example, in the urine pharmacokinetic study of heroin by Cone and co-workers (6), no sample with the predominance of

![Figure 3. Schematic presentation of the heroin metabolism. The first conversion of heroin can be enzymatic and chemical 1. The second conversion of 6-AM to morphine involves esterases, which are subjected to polymorphic variability 2. The demethylation of morphine to normorphine is a minor pathway 3. The phase II conversion to form glucuronide and sulfate conjugates are subjected to polymorphic variability 4. In addition, interactions are possible for all these metabolic conversions.](https://academic.oup.com/jat/article-abstract/30/2/73/725988)
6-AM could be observed. Furthermore, the plasma pharmacokinetic study of Girardin and co-workers (15) would suggest that such a window would only be minutes wide and occur within an hour after administration. Therefore, this pharmacokinetic explanation would require that a substantial number of individuals routinely are being subjected to sampling during a narrow window shortly after administration and while still being in the acute phase of intoxication, which is unlikely to occur at such a high frequency. Another aspect that relates to the timing of sampling is the general assumption that the 6-AM detection window after intake is short (6,5,18) and related to a more recent (< 24 h) intake. It might be possible that a prolonged excretion of 6-AM compared to morphine may underlie the observed cases of the atypical pattern. This should be taken into consideration when assuming that 6-AM must reflect a very recent intake (e.g., in clinical situations or in cases of suspected driving impairment) (20).

The explanation most favored by Glass and co-workers (8) was a metabolic defect and was mainly based on the repeated observation of the atypical pattern in the male twins during a period of several months. This explanation is somewhat contradicted by the observation of von Euler and co-workers (9), that an individual showing the atypical pattern also can produce a normal pattern. However, this cannot be regarded as a conclusive observation at present, and more data should be collected on this matter. An enzyme-metabolic explanation involving esterase activity can be hypothesized from the fact that 6-AM can be produced from heroin by chemical hydrolysis (21,22), but morphine must be formed enzymatically. Polymorphism in the enzymes involved in these transformations was recently reported with significant variability observed in both European and African populations (23). Although the relation of these forms to enzyme functional activity remains to be investigated, a metabolic defect underlying the observed phenomenon must be seriously considered at this point. A schematic presentation of the pathways of heroin metabolism is shown in Figure 3.

A third possible reason for the atypical urine excretion pattern after heroin intake is an interaction from another co-administered substance. Von Euler and co-workers favored this explanation and focused their interest on the formation of glucuronide conjugates. Interactions and polymorphism underlying such a mechanism are known (9). However, based on current results, such an explanation can be ruled out because the determination of both free and total opiates demonstrated a lack of morphine in any form and not specifically conjugated derivatives. In addition, recent studies on glucuronide conjugation do not give support to such a hypothesis, as genetic variability of the glucuronyltransferase involved in morphine conjugation had no correlation with the serum ratio between free and glucuronidated morphine (24,25). Yet another possibility for interaction, which should be considered, is with ethanol. It is well known that ethanol is commonly taken together with heroin, and that this constitutes a major risk factor for fatality (26-28). In addition, a pharmacokinetic interaction of heroin and ethanol is supported from the altered morphine/6-AM ratio observed in a forensic investigation (29). The possibility that an interaction with ethanol accounts for the atypical pattern requires further investigation and may be related to the documented higher risk for fatality.

If 6-AM screening is becoming more generally used, it is important to gain knowledge about possible pitfalls and drawbacks. One potential analytical pitfall is related to the detected cross-reactivity with free morphine. The cross-reactivity of free morphine in the CEDIA 6-AM assay may contribute to a significant response only at high concentrations (> 2000 µg/L) and is seen as an offset between immunoassay and GC–MS response. However, it has been observed by us that urine from patients receiving continuous administration of morphine may contain enough free morphine to produce a positive screening result for 6-AM. In such cases, the confirmation assay must be validated for artificial formation of 6-AM during the analytical procedure (e.g., acylation derivatization agents may contain traces of acetyl moieties) (30). No other substances are known to cross-react in the CEDIA 6-AM assay at present. A recently published study using 6-AM CEDIA screening in a criminal justice drug-testing program reported a 98% confirmation rate and pointed out the potential interference from morphine, oxycodone, and pentazocine (31). The high cross-reactivity of 6-AM in the CEDIA and DRI opiate assays is interesting to note because this alone can cause a positive response in the opiate screening.

One further aspect of this investigation is the issue of the best strategy when performing heroin drug testing in urine. It is obvious that the practice of screening for opiates at 2000 µg/L and confirmation of 6-AM at 10 µg/L used in workplace drug testing suffers from producing false-negative results and, consequently, limited sensitivity for detecting heroin intake. The study by Paul and co-workers (4) demonstrated the inability to detect true-positive samples when screening for opiates at 2000 µg/L. Our data demonstrates that this is not fully prevented by lowering the cutoff to 300 µg/L. The study by Holler and co-workers (5) demonstrated that a substantial number (19.5%) of false-negative results are produced in the standard procedure and suggested screening for 6-AM instead in both military and civilian drug-testing programs. The advantage of using 6-AM as the target compound has been suggested for fatal intoxications (32,33). However, as 6-AM can be considered the most optimal target compound in forensic and workplace drug testing, the combination of 6-AM and opiate screening at a low cutoff limit is the optimal strategy in clinical testing. The continuing use of opiate screening in clinical testing is warranted by the longer detection time after intake and the range of substances covered.

References


Manuscript received August 11, 2005.