In Vitro Conversion of Morphine to 6-Acetylmorphine in Urine Samples during Enzymatic Hydrolysis

To the Editor:

Heroin is an illegal drug and monitoring heroin use is important in rehabilitation, pain management services, and workplace employment testing. In humans, heroin is rapidly converted to 6-acetylmorphine (6AM),¹ which is quickly hydrolyzed to morphine. Morphine glucuronide is the major metabolite of heroin in urine (1). However, 6AM is a unique metabolite of heroin and is considered the definitive indicator of heroin intake. 6AM in urine is measured by GC-MS, LC-MS, immunoassay, or thin-layer chromatography methods. For measuring opioids in urine, hydrolysis using β-glucuronidase is frequently employed to improve consistency because of large variable glucuronide conjugation rates between and within individuals. Acetate buffer is the primary choice for preparing enzymatic hydrolysis solution due to the favorable pH (4.5–5.0). In our routine LC-MS analysis (2, 3), a number of low-concentration 6AM results were observed in patient urine samples with high concentrations of morphine. A Medical Review Officers’ advisory cites possible in vitro synthesis of 6AM in high-morphine samples during derivatization in GC-MS analyses (4). To the best of our knowledge, there are no reports on in vitro conversion of morphine to 6AM in LC-MS analyses. We hypothesized that the acetate buffer used for enzymatic hydrolysis may act as an acetylating agent to chemically convert morphine to 6AM.

In the first study, morphine and the major morphine metabolite, morphine-3-glucuronide (M3G), were spiked into morphine and 6AM-negative urine samples (n = 3) to create 2 sets of samples: 5 concentrations of morphine samples (10 000, 50 000, 100 000, 150 000, and 200 000 μg/L) in 1 set and 5 concentrations of M3G samples (16 250, 81 250, 162 500, 243 750, and 325 000 μg/L) in the other set. Enzymatic hydrolysis was performed at 60 °C using a β-glucuronidase from Patella vulgata in 1.0 mol/L sodium acetate buffer (pH 4.5). Aliquots were taken at 0, 2, 4, 6, and 18 h. In a similar experiment, the samples spiked with morphine (100 000, 150 000, and 200 000 μg/L) (n = 3) and M3G (162 500, 243 750, and 325 000 μg/L) (n = 3) were incubated for 0 and 12 h under the same conditions. In the second study, patient urine samples (n = 4) with increased concentrations (97 000–110 000 μg/L) of morphine were incubated at 60 °C using β-glucuronidase in acetate buffer for 0 and 18 h. In the third study, 2 samples spiked with 200 000 μg/L morphine, 2 samples spiked with 325 000 μg/L M3G, and the 4 patient urine samples used in the second study were incubated at 60 °C using β-glucuronidase in 1.0 mol/L citrate buffer (pH 4.5) for 0 and 18 h. In the fourth study, 3 concentrations of spiked morphine samples (100 000, 150 000, and 200 000 μg/L) (n = 3) and 3 separate concentrations of spiked M3G samples (162 500, 243 750, and 325 000 μg/L) (n = 3) were incubated in the acetate buffer without β-glucuronidase for 0 and 18 h. Examining patient urine samples in this study after clinical testing was approved by the Cleveland Clinic Institutional Review Board. In the LC-MS method, the analytical measurement ranges for morphine and 6AM were 5–5365 μg/L and 5–4800 μg/L, respectively. The total imprecision was 3%–9% for morphine and 4%–13% for 6AM.

After incubation for 18 h using β-glucuronidase in acetate buffer, all urine samples with increased morphine (>100 000 μg/L) and 78% of the samples with increased M3G (>162 500 μg/L) formed measurable 6AM (≥5 μg/L). One of the 9 samples with >100 000 μg/L morphine and 2 of the 9 samples with >162 500 μg/L M3G incubated for 12 h using β-glucuronidase in acetate buffer formed measurable 6AM (Fig. 1). All samples with <100 000 μg/L morphine or <162 500 μg/L M3G, and all samples incubated for <12 h using β-glucuronidase in acetate buffer, did not form measurable 6AM. All 4 patient urine samples with increased morphine formed 6AM (5–20 μg/L) when incubated for 18 h using β-glucuronidase in acetate buffer. No samples incubated using citrate buffer formed measurable 6AM, whereas 67% of the samples incubated for 18 h in the acetate buffer without β-glucuronidase formed measurable 6AM (5–8 μg/L).

Our results show that false-positive identification of heroin is possible when using acetate buffer to hydrolyze urine samples with extremely high morphine (>100 000 μg/L). After 18 h of incubation, 16 of the 18 samples with extremely high morphine or M3G formed 6AM (>5 μg/L), and 2 samples formed 6AM >10 μg/L (the common cutoff concentration for 6AM in workplace programs to identify heroin). A retrospective analysis of 2 years’ worth of patient data in our laboratory identified 1116 morphine-positive samples, among which approximately 2% had morphine concentrations >100 000 μg/L. Caution should be taken in result interpretation. An alternate buffer with no acetate, for example citrate buffer, should be considered for this application.

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¹ Nonstandard abbreviations: 6AM, 6-acetylmorphine; M3G, morphine-3-glucuronide.
requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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References


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Letters to the Editor

To the Editor:

In the December 2013 issue of Clinical Chemistry, Huestis and colleagues published an article on cannabinoids in exhaled breath following smoked cannabis (1). This intriguing study prompted a valuable conversation regarding current legislation and future possibilities for detecting cannabis-induced impairment. We therefore commend the authors for their initial efforts to assess a breath test for cannabinoid detection. There are, however, several aspects of this study that deserve further discussion.

First, the possibility of laboratory interference must be considered. Although all study participants tested negative 1 h before smoking, there were positive tests that were not well explained. Two participants (I and K) had a detectable 9-tetrahydrocannabinol (THC) concentration on admission. In this scenario, the positivity of THC can be explained by 2 possibilities: those participants smoked marijuana after admission, which is unlikely, or the assay also detected 11-nor-9-carboxy-THC (THCCOOH), which is often increased in chronic users. Subsequently, by the 1-h assessment, the THCCOOH concentration may

Fig. 1. Formation of 6AM from morphine and M3G after 12 and 18 h of incubation in 1.0 mol/L acetate buffer. 6AM concentrations below 5 µg/L (15 nmol/L) are considered undetectable.