Forensic Science International 252 (2015) 150–156

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

# Possible mechanism for inhibition of morphine formation from 6-acetylmorphine after intake of street heroin

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ARTICLE INFO

*Article history:*
Received 4 December 2014
Received in revised form 14 April 2015
Accepted 3 May 2015
Available online 12 May 2015

*Keywords:*
Heroin
6-acetylmorphine
Morphine
LC–MS/MS
Metabolism
Inhibition

ABSTRACT

Heroin is de-acetylated in the body to morphine in two steps. The intermediate 6-acetylmorphine (6-AM) is formed rapidly and is considered important for the pharmacological effect of heroin. In urine drug testing, an atypical pattern of morphine and 6-AM is known to occur in low frequency. The aim of this study was to investigate this atypical pattern in more detail and to identify responsible substances for a possible inhibition of the conversion from 6-AM to morphine. Urine samples were selected from a routine flow of samples sent for drug testing. Out of 695 samples containing morphine and 6-acetylmorphine, 11.5% had the atypical pattern of a 6-AM to morphine ratio above 0.26 as derived from a bimodal frequency distribution. An *in vitro* study of the conversion of 6-acetylmorphine to morphine in human liver homogenates demonstrated that a number of known carboxylesterase inhibitors were able to inhibit the reaction mimicking the situation *in vivo*. Compound **3** (3,6-Dimethoxy-4-acetoxy-5-[2-(N-methylacetamido)ethyl]phenanthrene) a substance formed from thebaine during the production of heroin was found to be a strong inhibitor. Liquid chromatography–mass spectrometry was used to identify possible inhibitors present *in vivo*. This part of the investigation demonstrated that several components may contribute to the effect. It is concluded that inhibition of liver carboxylesterase activity is a possible mechanism causing the atypical pattern and that one candidate compound is the result of the heroin production process. An inhibition of 6-AM metabolism is likely to increase the pharmacological effect of heroin and may be related to a higher risk of lethal toxicity.


## 1. Introduction

Heroin (diacetylmorphine) is converted to morphine *in vivo* by de-acetylation in two hydrolytic steps. It is generally assumed that the intermediate 6-acetylmorphine (6-AM) is rapidly formed by chemical and/or enzymatic hydrolysis and contributes to the pharmacological effect of heroin, and that 6-AM is further metabolized to morphine by carboxylesterases [1] (Fig. 1). The esterase enzymes are subjected to polymorphic variability, indicating inter-individual differences in the metabolic formation of morphine from 6-AM [2].

Urine drug testing is common for detecting a possible heroin intake. Because of the rapid conversion of heroin to morphine, drug testing is directed towards detection of morphine in urine. Since the presence of morphine in urine could have several causes, the focus may be on detecting 6-AM in the confirmation method after a morphine positive screening result. 6-AM presence in urine has been advocated as the safest and most sensitive criteria for heroin intake [3–5]. Since 6-AM concentrations usually are low in urine, the possibility to include 6-AM routinely in this investigation has not been possible until more recently [6]. It is expected that morphine is accompanied with low amounts of 6-AM, and it is therefore surprising that several authors have observed that in some individuals, 6-AM is accompanied with very low levels of morphine, i.e. showing an atypical metabolic pattern in the urine samples [5,7–11] (Fig. 1). Interestingly, one study showed that the very same individual can have this atypical metabolic pattern one time and a more normal pattern another time [8], indicating that genetic factors alone could not explain this phenomenon. Notably, the mechanism behind this atypical pattern of heroin metabolism is still not known. A number of studies after administration of pure heroin have not displayed this atypical pattern in any of the subjects [12–16]. Street heroin is an impure product that is produced from raw opium extracts by acetylation and is diluted by

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http://dx.doi.org/10.1016/j.forsciint.2015.05.002
0379-0738/

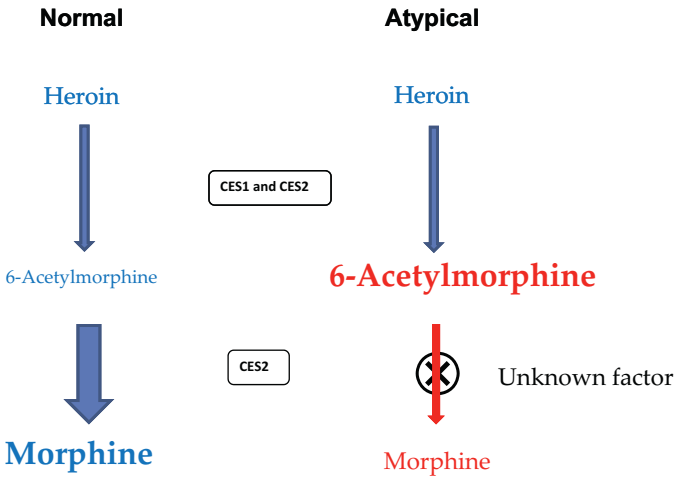


**Fig. 1.** Simplified presentation of heroin metabolism showing the normal and atypical metabolic pattern. The first step from heroin to 6-acetylmorphine (6-AM) can be enzymatic and chemical. The second conversion of 6-AM to morphine is catalyzed by carboxylesteraseses. In the subjects showing an atypical pattern of heroin metabolism, an unknown factor is inhibiting the second enzymatic conversion from 6-AM to morphine.

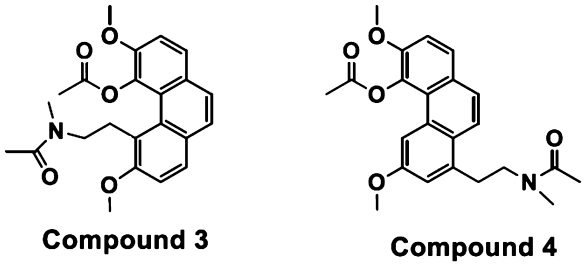


**Fig. 2.** Chemical structures of Compounds **3** and **4** formed from thebaine during the production of heroin.

several different chemicals, e.g. caffeine or lidocaine. These circumstances make a dose of street heroin undefined and variable regarding amount and purity of heroin and identity of other congener substances.

Lethal drug overdosing of heroin is a leading cause of death in the young age group (<35 years old) and several causes for overdosing have been proposed. Loss of tolerance and co-administration of alcohol and benzodiazepines have been demonstrated as a risk for overdosing [17–21]. If heroin had a direct effect on death, it could be expected that higher blood levels would occur in lethal cases as compared to survivors. However, this is not the case and this fact has puzzled the field [19,21,22]. It is therefore possible that additional factors beyond high serum levels of the parent compound of morphine/heroin could contribute to the risk of fatality. However, these possible factors or metabolites remain to be determined. Here we propose that inhibition of 6-AM metabolism could be one such factor.

The aim was to investigate the subgroup of cases further that present the atypical metabolic pattern of morphine and 6-AM in urine. Since genetic factors most likely cannot explain this phenomenon, we hypothesized that a drug–drug interaction or drug–metabolite interaction may explain the atypical pattern.

## 2. Materials and method

### 2.1. Chemicals

6-AM, 6-AM-$d_3$, acetylcodeine, buprenorphine-$d_4$, cocaine, codeine-$d_3$, heroin, 3,4-methylenedioxymethamphetamine-$d_5$ (MDMA-$d_5$), morphine, morphine-$d_3$ and morphine-3-glucuronide-$d_3$ (M3G-$d_3$) were obtained from LGC Standards (Teddington, United Kingdom). Acetyl salicylic acid (ASA), ammonium formate, lidocaine (Lid), loperamide (Lop), procaine (Proc), thebaine and uridine 5′-diphosphoglucuronic acid were obtained from Sigma–Aldrich (St Louis, MO, USA). Ethanol (95%) was obtained from Kemetyl AB (Haninge, Sweden). Compound **3** (3,6-Dimethoxy-4-acetoxy-5-[2-(N-methylacetamido)ethyl]phenanthrene) and Compound **4** (3,6-Dimethoxy-4-acetoxy-8-[2-(N-methylacetamido)ethyl]phenanthrene) see Fig. 2 were prepared by a contract laboratory (Xenochem AB, Stockholm, Sweden) according to published procedure (Allen et al., [33]). Acetonitrile (LiChrosolv isocratic grade for Liquid Chromatography), ammonia solution (25%) and formic acid (pro analysis quality) and were obtained from Merck KGaA (Darmstadt, Germany). Methanol (HiPerSolv CHROMANORM for HPLC gradient grade) was obtained from VWR International (Radnor, Pennsylvania, USA). Ultra-pure water was produced in-house by a Milli-Q Millipore Water system.

### 2.2. Urine samples

The urine samples included in this study were de-coded surplus samples collected during a 3 years period from the routine flow sent to the Pharmacological laboratory, Karolinska University Hospital for drug testing of opiates. The primary sample selection criterion was a positive screening result (>300 ng/ml) using CEDIA opiate reagent (Thermo Fisher Scientific, Waltham, MA, USA) and the second criterion was a positive confirmation result for 6-AM (>2 ng/ml) using an LC–MS/MS method with direct injection after five times dilution [23]. Both the screening and confirmation methods are routine methods which are validated according to the EMA guidelines. In the confirmation methods, free morphine, morphine-3-glucuronide and morphine-6-glucuronide were quantified and the summary value of corresponding morphine value was calculated as total morphine (MTOT). The total numbers of samples with such results was 695 and out of these, 250 were randomly selected and stored at −20 °C for further analytical investigation.

### 2.3. Study of 6-AM metabolism in vitro

Human liver tissue from 10 individuals was obtained from the human liver bank established at our department. Pieces of liver tissues (0.30–2.1 g) were put in a glass homogenizer tube and homogenized in 0.05 M Tris–HCl buffer (pH 7.5, 1 ml buffer per 0.2 g tissue) using a Teflon piston and manual rotation. The liver homogenates were stored at −80 °C until analysis. Protein concentration was determined according to the method described by Lowry et al. [24].

A volume of 6.4–14.8 μl human liver homogenate (0.385 mg protein/ml) and 2.5–4.1 μl of inhibitor solution to a final concentration of 6.1 μM, 30.5 μM or 61 μM for cocaine, caffeine, lidocaine, procaine, acetyl salicylic acid and a final concentration of 0.1‰, 1‰ and 10‰ for ethanol. Finally a volume of 177.1–185.5 μl of 0.05 M Tris–HCl buffer (pH 7.5) was mixed in a glass test-tube ending up with a total volume of 200 μl. This mixture was pre-incubated at 37 °C for 5 min; 4 μl of a 6AM solution (6.1 μM in acetonitrile) was added and the incubation continued for 15 min. The reaction was stopped with the addition of 200 μl ice-cold acetonitrile and by putting the test-tube on ice. A volume of 10 μl of the sample, and 10 μl of internal standard solution (codeine-$d_3$, 20 μg/ml) was mixed with 80 μl of 0.1% formic acid (mobile phase A) in an autosampler vial. The prepared samples were analyzed for