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

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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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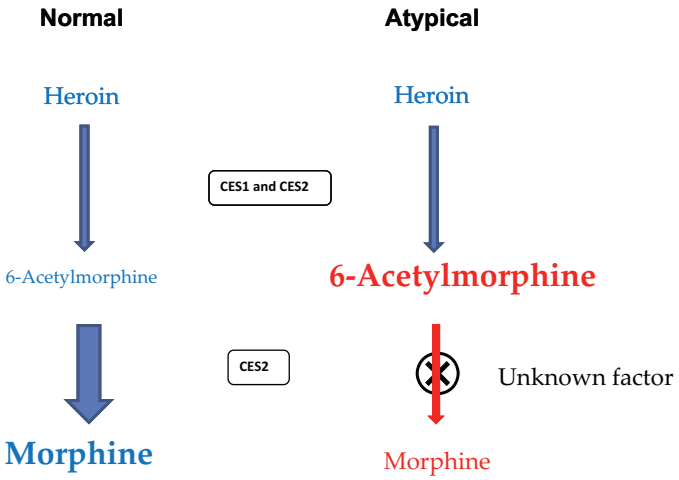

Fig. 1. Simplified presentation of heroin metabolism showing the normal and atypical metabolic pattern. The first step from heroin to 6-acetylmorphine (6-AM) can be enzymatic and chemical. The second conversion of 6-AM to morphine is catalyzed by carboxylesteraseses. In the subjects showing an atypical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

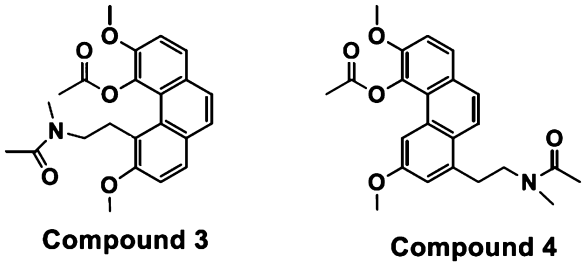

Fig. 2. Chemical structures of Compounds **3** and **4** formed from thebaine during the production of heroin.

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

6-AM and morphine according to a previously described LC–MS/MS method with direct injection [23].

Single experiments were also performed with liver microsomes and cytosol preparations for loperamide. Microsomes and cytosols were prepared as previously described elsewhere [25].

2.4. Investigation of urine using LC–MS/MS (targeted investigation)

The targeted investigation was performed on an LC system which consisted of an ACQUITY UPLC connected to a Xevo TQ MS mass spectrometer (Waters, Milford, MA, USA) or a Quattro Premier XE. Three different chromatographic systems were needed in order to cover all the different analytes studied (See Table 1). The tandem mass spectrometer was operated with MassLynx™/Target Lynx™ Software version 4.1 (Waters) in the positive electrospray mode using selected reaction monitoring (SRM). The capillary voltage was 1.0 kV and the extractor voltage was set to 3 V. The source temperature was 120 °C and the desolvation temperature was 350 °C. The cone gas flow was 50 l/h, desolvation nitrogen gas flow rate was 1000 l/h and the collision argon gas flow rate was 0.15 ml/min. In Table 2, the individual transitions for each analyte and the internal standard are presented.

Forty atypical samples (defined as MTOT <1000 ng/ml and 6-AM >2 ng/ml) and 40 reference samples (MTOT >1000 ng/ml and 6AM >2 ng/ml) were investigated with LC–MS/MS. Sample preparation was performed according to an earlier reported procedure [23]. In brief, 25 μl of urine were diluted 5-fold with ultrapure water containing deuterated internal standards M3G-d_3 (360 ng/ml), MDMA-d_5, Buprenorphine-d_4 (500 ng/ml respectively) and salicylic acid-d_6 (250 ng/ml). Stock solutions of each analyte were prepared in methanol. Calibrators were prepared in blank urine from these stock solutions to the following concentrations: 1, 5, 15 and 30 μM for cocaine, lidocaine and procaine and 1, 5 and 15 μM for heroin and loperamide. For salicylic acid, calibrators were made at 100, 250, 500 and 750 ng/ml in blank urine.

2.5. Analysis of ethanol and ethyl glucuronide

Ethyl glucuronide (EtG) is a biomarker for ethanol intake, and remains detectable in the urine longer than ethanol itself [26,27]. To screen for ethanol intake we analyzed both EtG and ethanol (EtOH) in the urine samples. DRI ethyl alcohol enzyme assay and DRI EtG immunoassay (Thermo Fisher Scientific, Waltham, MA, USA) were applied on an Olympus AU 640 (Beckman Coulter, Indianapolis, Indiana, USA) according to the manufacturer's instructions, with a cut-off of 500 ng/ml for EtG and 5 mM for EtOH. Eighty urine samples displaying a normal metabolic pattern and 35 urine samples displaying an atypical metabolic pattern were investigated.

Table 1
Presentation of the three different chromatographic systems used for analytical investigations of urine samples.

System 1. Column: BEH 2.1 × 100 mm, 1.7 μm			
Time (min)	Flow rate (ml/min)	Mobile phase A 0.1% formic acid	Mobile phase B Methanol
0.50	0.35	90	10
2.10	0.35	70	30
4.50	0.35	5	95
5.50	0.35	5	95
5.51	0.35	90	10
6.5	0.35	90	10
System 2. Column: BEH Shield RP 18 2.1 × 50 mm, 1.7 μm			
Time (min)	Flow rate (ml/min)	Mobile phase A 0.1% formic acid	Mobile phase B Acetonitrile
0.30	0.35	90	10
2.00	0.35	1	99
2.10	0.35	90	10
3.00	0.35	90	10
System 3. Column: HSS T3 2.1 × 100 mm, 1.8 μm			
Time (min)	Flow rate (ml/min)	Mobile phase A 0.1% formic acid	Mobile phase B Acetonitrile
0.50	0.20	50	50
6.00	0.20	5	95
7.50	0.20	5	95
7.51	0.20	50	50
9.00	0.20	50	50

2.6. Investigation of urine using high resolution mass spectrometry (non-targeted investigation)

For the non-targeted investigation an LC coupled to a high resolution mass spectrometer (HRMS) was used. The LC–HRMS analysis was performed on a Dionex Ultima 3000 coupled to a Thermo Scientific Q Exactive mass spectrometer (Fremont, CA, USA). The mass spectrometer was operating in positive mode and was equipped with a heated electrospray ionization source (HESI-I). The spray voltage was 3.0 kV, capillary temperature 250 °C, heater temperature 400 °C, S-lens RF level 55, sheath gas flow rate 55 arbitrary units, auxiliary gas flow rate 18 AU and sweep gas flow rate 1. The full scan acquisition ranged from 90 to 1350 *m/z*. The mass spectrometer was operated at 70 000 resolution power. The chromatography was carried out on an ACQUITY UPLC HSS T3 (2.1 × 100 mm, 1.7 μm particles) (Waters, Milford, MA, USA) kept at 50 °C. A multi-step gradient was used with mobile phase A containing 2 mM ammonium formate and 0.2% ammonia solution (25%) and mobile phase B containing 100% methanol with the same amount of ammonium formate and ammonia. Gradient was operated at a flow rate of 0.300 ml/min with a total run time of 18 min. The software used was SIEVE 2.1 (Thermo Scientific). The

Table 2
Transitions monitored in selected reaction monitoring mode for the different investigated compounds.

Analyte	Precursor ion (*m/z*)	Quantitative product ion (*m/z*)	Qualitative product ion (*m/z*)	Retention time (min)	Chromatographic system
Cocaine	304.2	82.0	105.0	3.25	1
Compound **3**	396.3	99.7	354.1	2.81	3
Compound **4**	396.3	281.0	354.1	3.23	3
Heroin	370.2	165.0	268.2	3.15	1
Lidocaine	235.2	86.1	134.1	2.76	1
Loperamide	477.3	266.2	115.0	4.24	1
Procaine	237.2	120.0	100.1	1.49	1
Salicylic acid	136.9	92.7	–	1.74	2
Buprenorphine-d_4	472.4	59.0	–	3.87	1
MDMA-d_5	199.1	165.0	–	2.44	1
Salicylic acid-d_6	140.9	96.8	–	1.73	2

identification criteria were correct retention times and a mass error <5 ppm.

The samples investigated as the control group ($n = 25$) contained 6AM >2 ng/ml and a total morphine of >5000 ng/ml. The samples for the atypical group ($n = 19$) contained 6-AM >10 ng/ml and a total morphine of <300 ng/ml.

2.7. Statistical analysis

All statistical tests were performed using GraphPad Prism v. 6.00 and values of $p < 0.05$ were considered statistically significant. Fishers exact test was used to evaluate if there was any statistically significant difference in the presence of EtOH, EtG, cocaine, heroin, lidocaine, loperamide, procainamide or salicylic acid between patients with "atypical" or "normal" metabolic pattern. The Mann–Whitney U Test was used to evaluate differences in peak areas of the different substances in normal and atypical samples.

3. Results

The analytical findings of 6-AM and MTOT in the randomly selected ($n = 695$) urine samples are presented in Table 3. The majority of samples contained high concentrations of 6-AM (>100 ng/ml) and MTOT (>1000 ng/ml). An atypical sample was first defined as having MTOT value below1000 ng/ml but with detected 6-AM (>2 ng/ml). The number of atypical heroin samples was then 125 (18%). These were from 84 individuals, and 29 of them provided samples with both atypical and "normal" patterns. If an atypical sample instead was defined more strictly as having MTOT below 300 ng/ml and 6-AM above 10 ng/ml, the number of atypical samples were 80 (11.5%). These atypical samples were from 55 individuals and 23 of them provided samples with both atypical and "normal" patterns.

The 6-AM/MTOT ratio was plotted in a frequency diagram and revealed a bimodal distribution (Fig. 3). The atypical pattern detected this way had a 6-AM/MTOT ratio of >0.26. A number of 80 (11.5%) samples displayed this atypical pattern. These atypical samples were from 57 individuals and 21 of them provided samples with both atypical and "normal" patterns.

The incubation system provided a mean conversion rate of 6-AM to morphine of 86%, with variability between the 10 individuals of 52%.

The inhibition from various compounds was found to be concentration-dependent and the results from using an inhibitor concentration of 61 μM are presented in Table 4. The strongest inhibition effect was seen for Compound **3**, Compound **4** and loperamide. The uncertainty in the measurement for Compound **3** was 5.4% ($n = 3$) and the variability between individuals was 8.8% ($n = 10$). For the known carboxylesterase inhibitor loperamide, the uncertainty in the measurement was 4.8% ($n = 3$) and the variability between individuals was 14.4% ($n = 10$).

Table 3
Presentation of analytical findings in the 695 samples being positive for opiates in the screening (cutoff 300 ng/ml) and with detected 6-AM (>2 ng/ml).

6-AM	MTOT <300 ng/ml	MTOT 300–1000 ng/ml	MTOT >1000 ng/ml	MTOT >2000 ng/ml
<10 ng/ml ($n = 101$)	13	11	77	69
10–100 ng/ml ($n = 148$)	24	10	114	105
>100 ng/ml ($n = 446$)	56	11	379	377

MTOT refers to total morphine as summarized from free and glucuronide conjugated morphine.

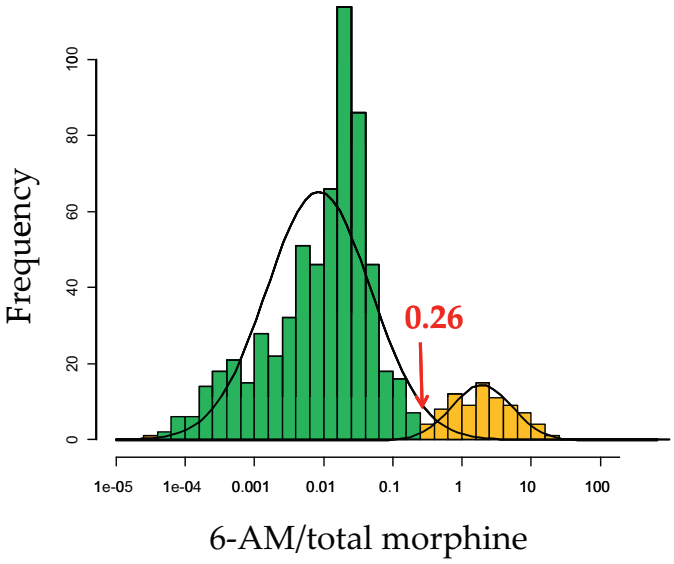

Fig. 3. Frequency diagram of the distribution of 6-AM/total morphine ratios in 695 urine samples collected from the routine flow of samples sent for urine drug testing. The samples were selected by a positive urine screening result using the CEDIA opiate reagent (cutoff 300 ng/ml) and presence of 6-AM (cutoff 2 ng/ml) in the LC–MS/MS confirmation method. A bimodal distribution was revealed and 80 samples were determined as atypical i.e. 6-AM/total morphine ratio of >0.26 with a median concentration of 6-AM at 328 ng/ml and varied in a range of 41–7270 ng/ml. A number of 615 samples displayed a normal metabolic pattern, i.e. 6-AM/total morphine ratio of <0.26, with a median concentration of 6-AM at 265 ng/ml and varied in a range of 2.1–21977.

The inhibitory effect was weak for other carboxylesterase substrates. Caffeine displayed an inhibitory effect similar to some of the known carboxylesterase substrates. However, inhibition by caffeine displayed an inter-individual variability of 102% ($n = 10$) between the individuals. The inhibitory effect varied from 0 to 68% between the individual liver homogenates.

Both liver microsomes and cytosol exhibited activity consistent with morphine formation from 6-AM (data not shown). For the single experiments using loperamide as the inhibitor with the liver microsomes the inhibitory effect was 54% and with the cytosol preparation the inhibitory effect was 67%.

The presence of possible inhibitors was investigated using different sets of patient samples according to procedures described above. Patient samples ($n = 80$) with the ratio 6-AM/total morphine <0.26 ng/ml, i.e. "normal metabolic pattern" were screened for EtG and EtOH. EtG was present in 40% of these samples and EtOH was present in 5%. In the samples with a ratio 6-AM/total morphine >0.26 ng/ml ($n = 35$), i.e. "atypical metabolic pattern", EtG was present in 34% and EtOH was present in 14% of the samples. Statistical analysis using Fishers exact test showed no significant difference between the two groups for presence of EtH ($p = 0.13$) or EtG ($p = 0.68$). In another set of samples, 40 atypical samples using the criteria 6AM/MTOT >0.26 were compared with 40 "normal" samples 6AM/MTOT < 0.26. The samples were analyzed with LC–MS/MS for the presence of possible inhibitor candidates (Table 5). The analytical concentration limit was set to 1 μM for all samples except salicylic acid, which had a limit of 750 ng/ml, and the results are shown in Table 5. Few analytical findings were made in general and only salicylic acid was more prevalent in the atypical group (Table 5). Statistical analysis using Fishers exact test showed no significant difference between the two groups for the presence of cocaine, heroin, lidocaine, loperamide, or procainamide, but for salicylic acid, there was a statistical significant difference between the groups ($p < 0.05$). A total of 44 samples were investigated with LC–HRMS using a

Table 4
Inhibition of the de-acetylation of 6-AM in human liver homogenates using a 10-fold excess of inhibitor. Liver from 10 individuals were studied in triplicate.

Study samples	Cocaine $n=3\times10$	EtOH $n=3\times10$	Caffeine $n=3\times10$	Comp **3** $n=3\times10$	Comp **4** n=3× 10	Lid $n=3\times10$	Lop $n=3\times10$	Proc $n=3\times10$	ASA $n=3\times10$
Mean inhibition value (% ± SD)	14 ± 7	19 ± 13	21 ± 22	85 ± 7	60 ± 11	22 ± 13	61 ± 8	9 ± 8	13 ± 14

Abbreviations used Lid = lidocaine; Lop = loperamide; Proc = procaine; ASA = acetyl salicylic acid. The mixture was pre-incubated at 37 °C for 5 min with inhibitor and then for a further 15 min with putative inhibitors.

non-targeted approach. The selection of samples was made according to the criteria taken from the frequency plot in Fig. 3. Nineteen samples from the atypical group had a 6-AM/MTOT ratio >0.26 and 25 patient samples from the "normal" group had a ratio of <0.26. The samples were processed with the Sieve software program. Most of the detected components were more abundant in the "normal" group. As expected, a significantly higher abundance for morphine-3-glucuronide and morphine-6-glucuronide were seen in the "normal" group. Table 6 summarizes the results obtained from the non-targeted investigation and presents the incidence of urine samples attributed to the above-mentioned compounds together with mean peak areas. The mean peak area of Compound **3** and of the metabolites ATM3/ATM4 (that were not chromatographically separated) were significantly higher in the atypical samples studied compared to the normal samples; statistical analyses showed $p < 0.05$. There was no statistical significant difference between the two groups for the peak areas of the other substances.

4. Discussion

The present study confirmed previous observations that a subset of urine samples collected from heroin users display an atypical pattern of an elevated 6-AM/morphine ratio. By using a frequency plot of the 6-AM/morphine ratio, a bimodal distribution was demonstrated for the first time, which gives further support to the significance of the atypical pattern. A frequency of 11.5% of atypical cases was observed, which is in agreement with previous reports [5,9–11]. However, the selection of samples may have influenced the measured frequency as one inclusion criterion was the presence of 6-AM, which might not be the case in all urine samples collected after heroin intake. In a recent study, 82% of samples classified as resulting from heroin intake contained 6-AM [23]. On the other hand, some true cases with atypical pattern may have been missed by the first selection criterion of being positive for morphine in the screening. Taken together and considering other previous publications [7–11], it seems to be without doubt that samples with atypical patterns exist, indicating a situation with an inhibited inactivation of 6-acetylmorphine in a rather substantial number of authentic cases. The atypical pattern seems not to be solely related to a genetic polymorphism in the enzymes involved as the same individual can produce both "normal" and atypical patterns. In the *in vitro* study, a marked inter-individual variability (52%) in the conversion rate of 6-AM to morphine was observed among the 10 subjects, indicating that a polymorphic factor may play a role.

Enzymes considered to be mainly involved in the metabolic transformation of heroin via the intermediate 6-AM to morphine are different esterases. Carboxylesterase-2 (CES2) is the enzyme mainly responsible for the conversion of 6-AM to morphine. [1,28,29]. The carboxylesterases are also involved in the metabolism of other drugs such as cocaine, which is mainly metabolized to benzoylecgonine by CES2 [29]. However, in the presence of ethanol, CES1 also catalyzes the transesterfication of cocaine forming the pharmacologically active but also toxic metabolite cocaethylene, i.e. a concomitant intake of ethanol and cocaine, is connected with an increased risk of toxicity [30,31]. Thus, it could be hypothesized that concomitant intake of ethanol and heroin might cause a changed metabolic pattern of heroin. Indeed, a previous report from 23 lethal cases attributed

Table 5
Results from the target analysis of a number of selected compounds with carboxylesterase inhibitory capability. The number of detected samples containing the different compounds is presented. Statistical analyses using Fishers exact test showed no significant difference between the two groups for presence of cocaine, heroin, lidocaine, loperamide, or procaine, but for salicylic acid, there was a statistical significant difference between the groups (*$p < 0.05$).

Study samples	Cocaine	Heroin	Lidocaine	Loperamide	Procaine	SA
Atypical ($n=40$)	–	2	3	–	–	6[a]
Normal ($n=40$)	3	2	6	–	1	–

Abbreviations for SA = salicylic acid.
[a] Median value of SA for the atypical group is 33 500 ng/ml.

Table 6
Non-targeted investigation of authentic urine samples using LC–HRMS and by targeted investigation with LC–MS/MS. Median peak values (25–75‰ within parenthesis). The peak area of Compound **3** was significantly higher in the a typical samples studied compared to the normal samples. There was no statistically significant difference between the two groups for the peak areas of the other substances. LC–MS/MS was used for analyzing Compounds **3** and **4** and ATM 3/4. For Lidocaine, Cocaine, Caffeine and Acetylcodeine peak areas are divided by 100 000.

Study samples	Lidocaine	Cocaine	Caffeine	Acetylcodeine	Compound **3**	ATM 3/4
Atypical	$n=17$	$n=$ **6**	$n=$ **19**	$n=$ **18**	$n=$ **17**	$n=$ **15**
Normal	$n=25$	$n=$ **11**	$n=$ **25**	$n=$ **22**	$n=$ **23**	$n=$ **15**
Atypical median peak area (25–75%)	100 (5–63)	28 (22–78)	18 (2–109)	1.6 (0.4–6.7)	46* (21–98)	46 (20–84)
Normal median peak area (25–75%)	140 (6–79)	56 (41–59)	28 (9–58)	2.6 (1.1–4.6)	20* (13–37)	70 (24–150)

* Mann–Whitney U test showed $p < 0.05$.

to either heroin or heroin and alcohol intoxication showed an association between ethanol concentrations in blood and high concentrations of 6-AM, indicating a possibly inhibited conversion from 6-AM to morphine in the presence of ethanol [32]. However, in our *in vitro* model, ethanol was not a strong inhibitor of the conversion of 6-AM to morphine. In addition, in our patient samples, only a fraction of the atypical samples had a concomitant presence of ethanol or the alcohol biomarker EtG. Notably, among the patients with a normal metabolic pattern, the concomitant presence of ethanol and EtG exhibited the same proportion as among patients with atypical pattern. Thus, according to our data, ethanol alone cannot explain the atypical pattern of heroin metabolism.

The *in vitro* study using liver homogenates revealed that the 6-AM conversion to morphine can be inhibited. Activity to convert 6-AM to morphine was present both in microsome and cytosol preparations. Several possible substrates/inhibitors of CES were studied and affected this conversion. This supports our initial hypothesis that the occurrence of the atypical pattern can be caused by a carboxylesterase inhibition from substance(s) ingested together with heroin. Apart from ethanol, other candidates may be salicylic acid from aspirin and caffeine and products formed from thebaine during heroin production. Salicylic acid is the metabolite of aspirin formed by carboxylesterase involvement.

It should be noted that the inhibition by caffeine displayed a very large inter-individual variability. Thus, in some subjects the inhibition of caffeine might be highly significant and might contribute to the atypical pattern of heroin metabolism.

The production process of heroin from opium also generates other acetylated alkaloid products. Acetylcodeine, which is *O*-acetylated and acetylcodamine, which is *N*-acetylated, are two examples. Thebaine is converted to phenanthrene derivates during the acetylation process with acetic anhydride. This reaction results in a formation and migration of the ethylamide side chain. The migration of the side chain results in two phenanthrene compounds, originally designated as Compounds **3** and **4** (Fig. 2) [33]. Both Compounds **3** and **4** are present in illicit heroin. Chen et al. [34] recently suggested that the metabolites of these compounds could possibly be used as heroin biomarkers due to the fact that cleavage of amides (as the N-acetyl) is more resistant than ester acetyl cleavage. In the present study, both Compounds **3** and **4** were investigated in the *in vitro* system and found capable of inhibiting the 6-AM conversion to morphine. Interestingly, Compound **3** was the strongest inhibitor of all compounds studied. Compounds **3** and **4** become O-deacetylated *in vivo*, with the acetamido group being preserved, to form ATM3 and ATM4 respectively [34]. Consequently, the presence of these compounds was investigated. Compound **3** was present in low amounts (<1 ng/ml), but in almost all samples investigated. Compound **4** were not detected in any of the samples. This might suggest that the locality of the acetamido side chain in Compound **3** could partially hinder enzymatic deacetylation of the C-4 O-acetyl group. While for Compound **4** the C-4 O-acetyl group is not sterically hindered by its acetamido side chain and therefore more likely undergo deacetylation. These findings were unexpected since Chen and co-workers did not find Compound **3** in urine samples during their investigation. The peak areas for Compound **3** were significantly higher ($p < 0.05$) in the atypical group. The metabolites ATM3 and ATM4 were not chromatographically separated in our system but the peak areas were significantly higher in the atypical samples studied ($p < 0.05$). This provides a weak association of the thebaine-derived compounds to the observed atypical pattern and needs to be further investigated. These compounds are possible to separate chromatographically [34].

5. Conclusion

The present study confirmed earlier observations that a subset of urine samples collected from heroin users display an atypical pattern of an elevated 6-AM/morphine ratio. By using a frequency plot of the 6-AM/morphine ratio, a bimodal distribution was demonstrated for the first time, which gives further support to the significance of the atypical pattern. The inhibition of liver carboxylesterase activity is a possible mechanism causing the atypical pattern of heroin metabolism and one candidate compound is the result of the heroin production process (Compound **3**). It is most likely that several other substances may contribute to the observed atypical pattern. 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.

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