

Does the preparation for intravenous administration affect the composition of heroin injections? A controlled laboratory study

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ABSTRACT

Aims To study whether the preparation procedure, and its acidic and heating conditions, used by heroin users to prepare heroin for intravenous administration affects the final composition of the fluid to be injected. **Methods** Samples from different seizures of illegal heroin provided by the Norwegian police were prepared by adding water and ascorbic acid before heating under controlled conditions in the laboratory. Further, three seizures were prepared with different amounts of ascorbic or citric acid relative to their diacetylmorphine content. Pure diacetylmorphine base or salt was also submitted to the procedure applying two different heating intensities. The seizures and the final product after preparation were analysed for diacetylmorphine, 6-acetylmorphine and morphine using liquid chromatography with tandem mass spectrometry (LC-MS-MS). **Results** After preparation, a decrease of 19.8% (25th and 75th percentiles = -29.2 and -15.3) in the initial diacetylmorphine content was observed. Both the 6-acetylmorphine and morphine content increased but, due to their low content in the initial product, diacetylmorphine still represented 83.9% (25th and 75th percentiles = 77.3 and 88.0) of the sum of these three opioids in the final solution. The loss of water during preparation caused an increase in the concentration of diacetylmorphine, 6-acetylmorphine and morphine, depending on the heating intensity applied. The content of these opioids was affected by the quantity and type of acid added in relation to the heroin purity and the level of diacetylmorphine dissolved being proportional to the amount of ascorbic acid, but not citric acid, in the sample with high heroin purity. **Conclusions** Preparation of heroin for intravenous injection appears to change the amount or concentration of diacetylmorphine and its active metabolites, 6-acetylmorphine and morphine in the final product, depending on heroin purity, amount and type of acid used or heating conditions. These circumstances can contribute to unintentional variations in the potency of the final injected solution, and therefore affect the outcome after injection.

Keywords 6-acetylmorphine, ascorbic acid, drug preparation, heroin, i.v. injection, morphine.

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INTRODUCTION

Diacetylmorphine (DAM) is an acetylated derivative of morphine, mainly illegally produced by chemical treatment of the air-dried milky latex extracted from the opium poppy (*Papaver somniferum*) [1] and distributed as

illicit heroin.¹ Heroin presents a high degree of harm, including a high risk of overdose [2,3]. In the last years, an increase in heroin consumption has been accompanied by a high rise in opioid overdose fatalities [4,5], many involving fentanyl surreptitiously added to illicitly produced heroin [6]. Intravenous (i.v.) injection is a common route

¹In the present article, diacetylmorphine (DAM) is used when referring to the pure compound and heroin when referring to illegally synthesized diacetylmorphine normally blended with impurities.

of administration among heroin users [7], associated with a high risk of dependence and overdose [8]. In illegal heroin, DAM can be found in salt (white heroin) or base (brown heroin) form [9]. For i.v. injection, both forms have to be dissolved in water. Whereas the salt form is soluble in water, frequently with the help of heating [10], heroin in base form is quite insoluble in water and has to be prepared for injection by heating the heroin in an acidic solution [10,11].

After intake of heroin, DAM is very rapidly metabolized to the active metabolite 6-acetylmorphine (6-AM), which is further metabolized to morphine, both in animals and humans [12–14]. Past [15–18] and recent studies [19–21] have shown that DAM can be considered a prodrug, its acute effects being mediated by these metabolites, predominantly by 6-AM [19]. We have earlier shown that the major part of the active metabolites reach the brain after DAM has been metabolized to 6-AM in the periphery [12,21].

Heroin is considered to be unstable in aqueous solution [22] and can undergo degradation at high temperatures [23]. Therefore, preparation of base heroin for i.v. administration is a procedure which may potentially alter the chemical composition of the final product, affecting the quality or strength of the acute effects, and therefore the outcome. We wanted to assess if the preparation of heroin for i.v. injection can modify the composition of the final fluid to be injected. For this purpose, we aimed to study under controlled conditions: (1) if exposition of samples from seizures of illegal heroin to the procedure employed by users for preparing their doses for i.v. injection affects the contents of DAM, 6-AM, and morphine in the resulting liquid, (2) if variations in the acidic conditions (amount and type of acid added) would affect the final composition, and (3) if the heating conditions during preparation would affect pure DAM or 6-AM.

MATERIAL AND METHODS

Samples from 17 different heroin seizures provided by the Norwegian National Criminal Investigation Service (KRIPOS), the criminal investigation agency of the Norwegian police, were used. The heroin seizures were apprehended in different Norwegian counties between April 2012 and May 2013. All the seizures were categorized as heroin base after Fourier-transform infrared (FTIR) spectroscopy testing by KRIPOS, and varied in colour and texture, from fine white to granulated dark brown powder. Their mean purity (15.4%) also approximately reflects the mean purity of heroin seizures in Norway during the last years (2017–20) [24].

Composition of heroin seizures

For chemical characterization, samples from the seizures were analysed for their content of DAM, 6-AM, morphine, codeine, acetylcodeine and acetaminophen using a previously validated and published method [25] (see Supporting information). The presence of other compounds, such as caffeine, barbiturates, or benzodiazepines, was examined but not quantified. The purity of the heroin seizure was determined by the percentage (w/w) of DAM content in the sample.

Experimental procedures

Opioid content in samples from heroin seizures after preparation for i.v. administration

To analyse possible changes due to preparation for i.v. injection (aim 1), the preparation for i.v. injection was modelled by placing a 250-mg sample of the seizures (median = 250.6; 249.5–251.3) on a metallic spoon and adding 40 mg ascorbic acid (Apotekproduksjon AS, Oslo, Norway) and 750 µl tap water. The amount of heroin and ascorbic acid chosen are in the range usually used by injecting drug users [11]. The spoon was held in a stand and heated for 40 seconds with a lighter placed below so that only the tip of the flame was in contact with the bottom of the spoon, preventing the liquid from boiling. The spoon, with its content, was weighed before and after heating in order to calculate the water lost due to preparation, considering a water density of 1 g/ml. After weighing, the resulting solution was sampled for analysis with a syringe through a cotton swab, circumventing solid residues. The samples were later analysed using the same published method as above [25] (see Supporting information).

To estimate the changes induced by the preparation procedure, a theoretical concentration (mM) before sample preparation was calculated from the amount (µmol) of each substance in the sample to be prepared, derived from the composition of the seizure, and the volume (µl) of water added, simulating an unmodified, total dissolution of the compounds. The amount (µmol) in the sample after preparation was calculated from the concentration (mM) measured in the final solution and the volume (µl) of the liquid remaining in the spoon after preparation. ‘Total DAM-related opioids’ denotes the sum of DAM, 6-AM and morphine. The results are presented as medians with 25–75 percentiles in parentheses or after semicolon.

The compounds before and after preparation were statistically compared using a Wilcoxon’s signed-rank test for related samples. Pearson’s correlation was used for the correlations presented. A $P < 0.05$ was considered statistically significant. The IBM SPSS Statistics package version 25 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis.

Effect of acidic conditions on opioid content after preparation for i.v. administration

To study the effect on the final solution of the amount and type of acid added (aim 2), three seizures with different heroin purity (3.3, 14.9 and 32.1%) were subjected to the same procedure as above, but different molar amounts (1 : 3, 1 : 2, 1 : 1) of ascorbic acid were added in proportion to the moles of DAM present in the sample; that is, to heroin purity (Supporting information, Table S1). Moreover, the high purity seizure (32.1%) was also prepared using citric acid in these same proportions. After preparation, the samples were collected and analysed as described above. In this experiment, 3-acetylmorphine (3-AM) was also analysed in the samples treated with ascorbic acid. One preparation was performed for each experimental treatment, therefore no statistics were estimated.

Effect of heating intensity on DAM and 6-AM

In order to examine if DAM and 6-AM were altered by heating intensity (aim 3), 750 µl tap water containing 16.1 mg (43.3 µmol) DAM base and 40 mg ascorbic acid, or barely 16.7 mg (39.5 µmol) DAM.hydrochloric acid (HCl) or 2.1 mg (5.2 µmol) 6-AM.HCl (all of standard analytics purity from Lipomed AG, Arlesheim, Switzerland) was heated in a metallic spoon for 45 seconds with the help

of a lighter, either avoiding (moderate heating) or allowing (intense heating by heightening the power of the flame) the water to boil. The amounts of DAM and 6-AM used were selected to reflect their ratios in the seizures. As only one measure was taken for each experimental treatment, no statistics were estimated.

RESULTS

Composition of heroin seizures

The heroin purity of the seizures ranged from 0.66 to 32.1% (6.6–321 mg/g DAM), with a median of 14.9% (10.6–22.3). There were significant positive correlations between the content of DAM and 6-AM, morphine or acetylcodeine, whereas a significant negative correlation was found between DAM and acetaminophen (Fig. 1).

Opioid content in samples from heroin seizures after preparation for i.v. administration

After preparation, the DAM content showed a significant reduction of 19.8% (−29.2 to −15.3), while the content of 6-AM and morphine showed an increase of 32.5% (19.9–57.7) and 11.7% (0.5–5078), respectively, in relation to the initial contents in the samples (Table 1). No significant associations were observed between heroin purity and the changes in DAM ($r = -0.101$), 6-AM

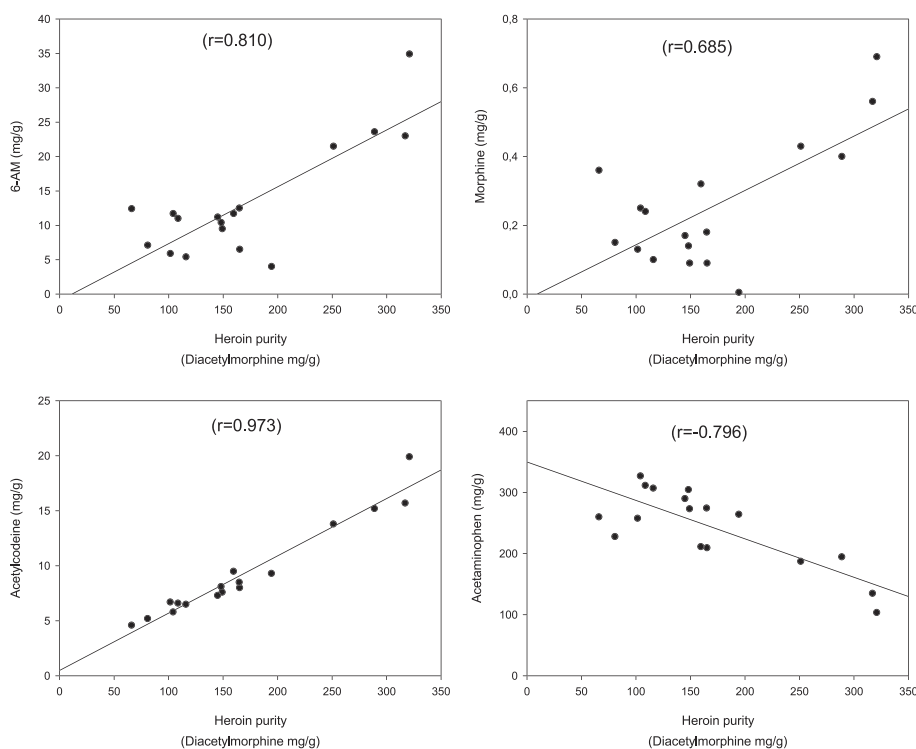


Figure 1 Relationship (Pearson's correlation) between heroin seizure purity, measured as diacetylmorphine (DAM) content (in mg/g seizure), and 6-acetylmorphine (6-AM), morphine, acetylcodeine or acetaminophen content (in mg/g seizure) in the 17 seizures from the Norwegian police (KRIPOS) used in the present study. All four correlations were statistically significant ($P < 0.05$)

Table 1 Total amount (in μmol) in the fluid before and after preparation of heroin seizures for intravenous (i.v.) injection. Results are given as median, with the 25 and 75 percentiles in parentheses. The amount before preparation was calculated from the values obtained from the analysis of the seizures for each substance corrected for the amount used.

	<i>Before preparation</i>	<i>After preparation</i>	<i>% difference</i>	
DAM	100.7 (69.2–122.0)	77.8 (50.8–93.7)	–19.8 (–29.2 to –15.3)	< 0.001
6-AM	8.4 (4.7–9.5)	9.7 (7.8–13.0)	+32.5 (19.9 to 57.7)	< 0.001
Morphine	0.15 (0.1–0.3)	0.38 (0.1–11.1)	+11.7 (0.5 to 5078)	0.007
6-AM + morphine	8.7 (4.8–9.8)	19.8 (8.0–23.2)	+57.0 (32.2 to 150.5)	< 0.001
Total DAM-related opioids	108.0 (76.1–128.5)	98.5 (60.6–105.6)	–11.5 (–20.8 to –4.5)	< 0.001
Codeine	0.02 ^a (0.02–0.04)	0.0 ^b (0.0–0.1)	–67.4 (–100.0 to –12.4)	< 0.001
Acetylcodeine	5.6 (4.5–6.9)	0.8 (0.7–1.0)	–84.9 (–87.4 to –80.9)	< 0.001
Acetaminophen	431.3 (335.5–493.8)	348.1 (270.6–370.0)	–21.5 (–27.5 to 15.9)	0.068

^aCodeine was detected in only eight of the 17 seizures analyzed. ^bCodeine was detected after preparation in three samples in addition to the eight already detected before preparation. Total DAM-related opioids is calculated as the sum of DAM, 6-AM and morphine. Exact statistical significance (*P*-value, Wilcoxon's signed-rank test) between after and before preparation is shown in italic type. DAM = diacetylmorphine; 6-AM = 6-acetylmorphine.

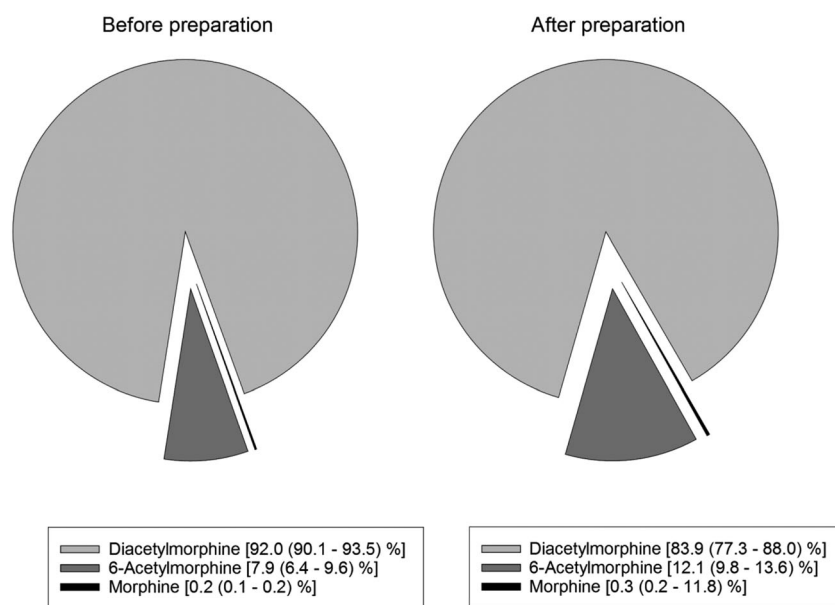


Figure 2 Median percentage (25th and 75th percentiles in parentheses in legends) of diacetylmorphine (DAM), 6-acetylmorphine (6-AM) and morphine of the total diacetylmorphine (DAM)-related opioids (sum of diacetylmorphine, 6-AM and morphine) before and after preparation of samples from heroin seizures for intravenous (i.v.) injection

($r = 0.238$) or morphine ($r = -0.222$) after preparation. The amounts of both codeine and acetylcodeine were significantly lower after preparation, but the procedure did not significantly affect acetaminophen levels.

The changes in the content of opioids also implied alterations in the relative composition of the sample (Fig. 2). Thus, the DAM content changed from representing 92.0% (90.1–93.5) of the total DAM-related opioids to 83.9% (77.3–88.0%) after preparation, whereas 6-AM increased from 7.9% (6.4–9.6) to 12.1% (9.8–13.6) and morphine from 0.2% (0.1–0.2) to 0.3% (0.2–11.8).

The amount of DAM lost after preparation (19.1 μmol ; 10.0–33.3) was not covered by the combined increases in

6-AM and morphine (5.9 μmol ; 1.9–12.8). This implies that a median of 12.0% (4.9–23.0) of the DAM in the samples was not found in the solution after preparation, neither as 6-AM nor morphine. The percentage of the DAM lost during the preparation procedure did not show any significant correlation ($r = 0.176$) with the purity of the heroin seizure (Fig. 3).

Due to warming, a loss of water occurred during preparation, resulting in a median final volume of 423 μl (400–463). Consequently, the concentrations of DAM, 6-AM and morphine in the final fluid were significantly higher than the theoretical concentration before preparation (Table 2).

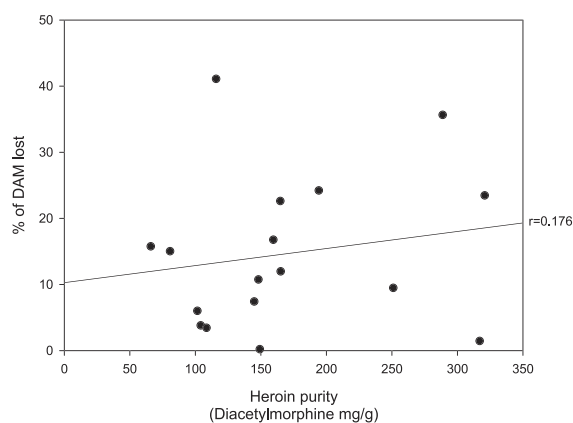


Figure 3 Relationship (Pearson's correlation) between the purity of heroin seizure, measured as diacetylmorphine (DAM) content (in mg/g), and the percentage of DAM lost during preparation. Percentage of DAM lost were measured as the percentage of the content of DAM plus the surplus of 6-acetylmorphine (6-AM) and morphine in the resulting fluid in relation to the amount of DAM in the sample before preparation. The correlation was not statistically significant ($P > 0.05$)

Effect of acidic conditions on opioid content after preparation for i.v. administration

In the seizure with low purity (3.3%), all DAM was dissolved with the three fractions of ascorbic acid used (Fig. 4). In the sample with 14.9% purity, close to the average purity of the seizures analysed, approximately 80% of the DAM was found after preparation when using a 1 : 3 molar dose of ascorbic acid, and an equimolar dose of ascorbic acid was needed to dissolve all DAM present in the sample. For the sample with high heroin content (32.1%), DAM was not completely dissolved even when adding an equimolar dose of ascorbic acid, but a relationship was observed between the amount of acid added and DAM dissolved. In comparison, up to 85% of DAM was dissolved regardless of the amount of citric acid used.

In the two seizures with 3.3 and 14.9% heroin purity, no relationship was found between the amount of ascorbic acid added and the amount of 6-AM and morphine after preparation (Fig. 4). Increased amounts of the metabolites

with increased concentrations of ascorbic acid, but not with citric acid, were observed after preparation of the sample with high purity (32.1%).

The amount of 3-AM was low in these three seizures, representing between 0.0016 and 0.0053% of the seizure content, or between 0.011 and 0.140% of the total DAM-related opioids. After preparation, 3-AM increased substantially in the samples with 32.1 and 14.9% purity, but still only represented between 0.08 and 0.13% of total DAM-related opioids in the sample.

Effect of heating intensity on DAM and 6-AM

When pure-grade DAM or DAM.HCl were exposed to different heating intensities, only small differences in content, between 2 and 7%, were observed in the final preparation (Fig. 5a). The content of 6-AM.HCl showed a decrease both after moderate and intense warming, the latter causing up to 15% loss. Heating induced an increase in the final concentrations of DAM and 6-AM, which were much larger under the higher intensity (Fig. 5b) as consequence of a greater loss of water.

DISCUSSION

Animal studies have revealed that for DAM to have an effect it has to be metabolized to the active metabolite 6-AM, which is further metabolized to morphine [19–21]. After administration of DAM to rodents, the levels of 6-AM in the brain are significantly higher than after administration of 6-AM itself [13,19,20], which can explain the differences observed in their behavioural outcome [26,27]. In this context, we wanted to investigate to what extent preparation of illegal heroin for i.v. injection affects the composition of the resulting solution.

DAM was the most abundant opioid in all the seizures, independently of their heroin purity. 6-AM content was at least one order of magnitude lower than DAM, in line with other studies [28–30]. The morphine content represented only approximately 0.02% of the seizure weight, and 3-AM was present merely in trace amounts, indicating a

Table 2 Concentration (mM) of DAM, 6-AM and morphine in the fluid before and after preparation of samples from heroin seizures for intravenous (i.v.) injection. Results are given as median, with the 25th and 75th percentiles in parentheses. The concentrations before preparation were calculated from the values obtained from the analysis of the seizures for each substance corrected for the amount used and water added.

	Before preparation	After preparation	% difference	
DAM	134.3 (92.3–162.7)	186.4 (134.1–232.1)	+41.5 (+31.4 to +51.4)	< 0.001
6-AM	11.3 (6.3–12.7)	24.7 (21.4–32.6)	+121.7 (96.7 to 185.4)	< 0.001
Morphine	0.20 (0.1–0.4)	0.79 (0.3–25.3)	+104.1 (64.5 to 9375)	< 0.001

Statistical significance (P -value, Wilcoxon's signed-rank test) between after and before preparation is shown in italic type. DAM = diacetylmorphine; 6-AM = 6-acetylmorphine.

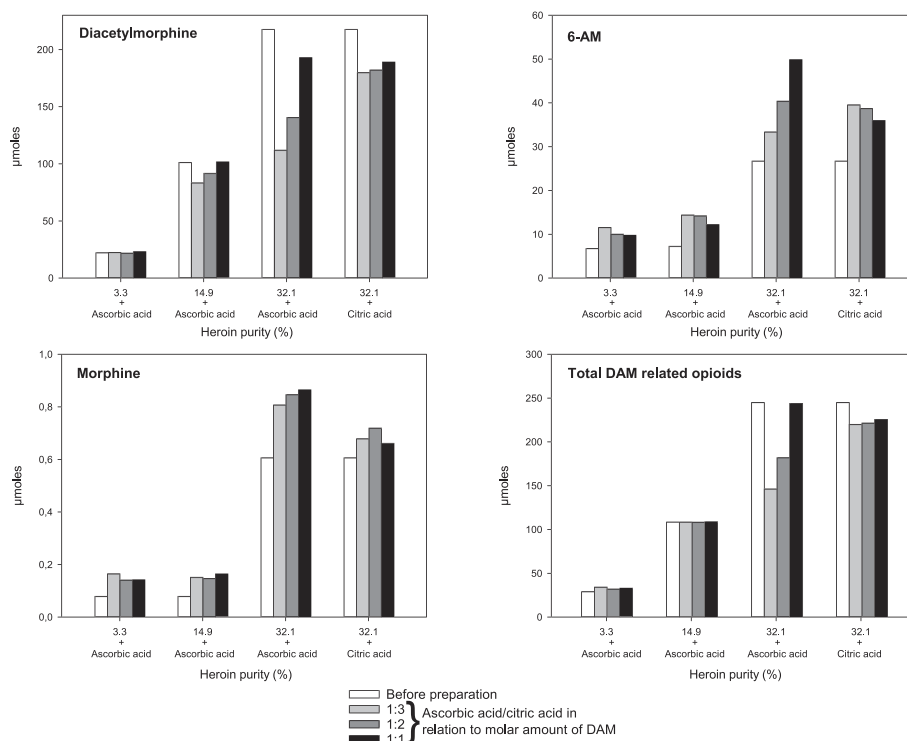


Figure 4 Content, in μmol , of diacetylmorphine (DAM), 6-acetylmorphine (6-AM), morphine and total DAM-related opioids (DAM + 6-AM + morphine) found in the fluid before and after preparation for intravenous (i.v.) injection. Samples from three heroin seizures of different purity (as measured by percentage of DAM content in seizures) were used and three different amounts of ascorbic acid or citric acid (only the high purity seizure) corresponding to 1 : 3, 1 : 2 and 1 : 1 of the moles of DAM present in the sample were added before preparation

high efficacy of the acetylation process during heroin production [31,32]. The proportions between the content of DAM and the content of 6-AM, morphine or acetylcodeine in the seizures were comparable to other seizures described elsewhere [1].

The preparation for injection resulted in a significant decrease in the DAM content, accompanied by an increase in 6-AM and morphine. Nevertheless, in the resulting solution DAM still remained the predominant opioid by far, its share being up to seven times larger than the sum of both active metabolites. The changes observed in the proportions between DAM and its active metabolites are relatively moderate, and it is uncertain if they are of sufficient magnitude to have a significant impact on the outcome of the heroin injection. Moreover, the combined increase in 6-AM and morphine did not totally cover the loss observed in DAM. The fraction of DAM lost was independent of the purity of the seizure, and seizures with very similar DAM content could greatly differ in the percentage of DAM lost. For example, two seizures with similar heroin purity (11.6 and 10.9%) showed a percentage net DAM loss of 41 and 3%, respectively. These two samples differed in their physical characteristics, the first being a light brown powder, the other dark brown and grainy. Also, the final products diverged, respectively producing either a quite

dark or a clear final solution, as noted by the experimenter. As even high-intensity heating did not appear to induce a loss of pure DAM, this 'heroin debt' might not be due to volatilization or formation by pyrolysis of by-products not detected in our analysis, but to non-solubilized DAM remaining in the solid residues resting in the spoon or trapped by the filter [33,34]. Filters are indispensable to avoid solid particles in the injected fluid that can lead to serious health complications [33,34] and are commonly used by heroin-injecting drug users, although being aware of some drug being retained on them [33–35]. Conversely, when testing the effect of acidic conditions using the lower amount of ascorbic acid, a larger proportion of DAM is dissolved than predicted by the amount of acid added [11]. This could indicate that certain substances present in the heroin, such as adulterants, could probably affect the overall solubility of DAM during preparation. As the possible sources for this 'heroin debt' have not been examined in the present study, further experiments are needed to clarify the contribution of non-solubilized DAM in the residues and filter or the impact of adulterants in DAM solubility.

Due to the heating needed to dissolve heroin, the preparation process involved a significant loss of water, eliciting a significant increase in the concentration of DAM, 6-AM and morphine in the liquid to be injected.

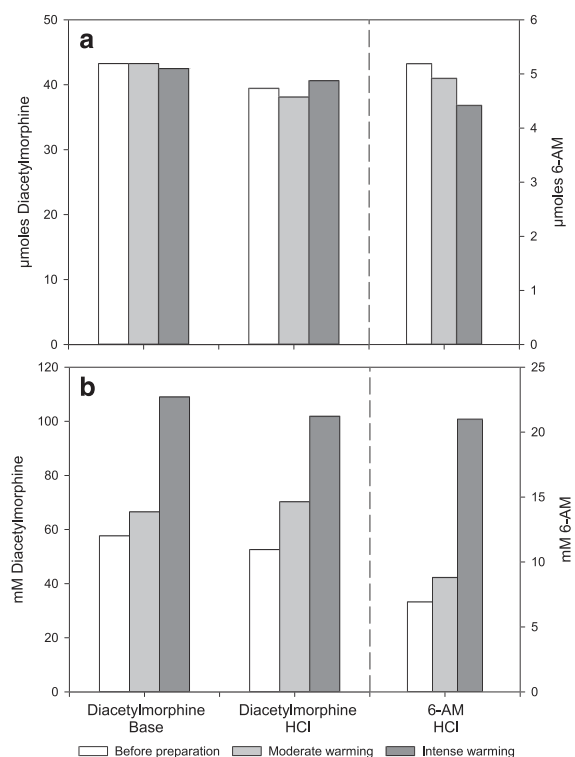


Figure 5 Content (μmol ; a) and concentration (mM; b) of diacetylmorphine (DAM) base, DAM.hydrochloric acid (HCl) and 6-acetylmorphine.HCl in the fluid after treating with moderate (not allowing to boil) or intense (with boiling) heating $43.3 \mu\text{mol}$ pure DAM base, $39.5 \mu\text{mol}$ pure DAM.HCl or $5.2 \mu\text{mol}$ pure 6-AM.HCl diluted in $750 \mu\text{l}$ tap water. For a full dilution of DAM base, 40 mg ascorbic acid were added to the solution before heating

This increase appears to be dependent upon the heating intensity, as observed with pure DAM. A higher opioid concentration can, plausibly, facilitate a faster delivery of compounds to the brain, which is known to increase their behavioural effects [36,37], and might have a potential impact on breathing [38].

Contrary to heroin salt, heroin base needs the addition of an acid to dissolve in water, ascorbic and citric acids being the most used [10,39]. We show that the proportion of ascorbic acid added in relation to the DAM content of the seizure (heroin purity) affects the quantity of DAM and the active opioids 6-AM and morphine present in the solution after preparation. In the 14.9 and 32.1% seizures, a clear relationship was observed between the proportion of ascorbic acid added and the level of DAM dissolved. Furthermore, Scott *et al.* [11] revealed that the use of either ascorbic or citric acid would notably affect the amount of DAM present in the fluid after heroin preparation. Indeed, when preparing heroin of high purity we observed that, in contrast to ascorbic acid, citric acid was able to dissolve a much higher proportion of DAM when using the lower molar

amount of acid. This is a foreseeable consequence of the higher acidic strength of citric compared to ascorbic acid [11].

These findings indicate that different elements during the preparation of heroin for i.v. injection can affect the amount and/or concentration of DAM and its active metabolites in the injected fluid. Variation in only one individual aspect of the process would probably not modify the composition to an extent sufficient to have a significant effect on the outcome after administration. However, unintentional combinations of these factors, e.g. changing the amount of acid or moving from ascorbic to citric acid while at the same time handling heroin of unfamiliar purity, are possible and together can substantially influence the composition of the final injected fluid. Users can consume different types of heroin and mingle the method of preparation [10]. Also, how the local market operates can prevent the final user acquiring information on the type or purity of the acquired heroin [40]. Furthermore, even after becoming a learned routine, the steps during preparation and injection are not standardized and not always under the control of the user [34,39,41]. This situation is expected to be more prevalent in the case of homeless heroin consumers, which also presents a higher overdose risk [42]. Additionally, it is not uncommon that unexperienced injectors refine their injecting technique by 'trial and error' [34]. These dynamics can generate deviations in the preparation process leading to unintentional variations in the final product and dose administered. These unintentional variations might result, in a worst-case scenario, in an injection which can lead to fatal consequences, primarily if tolerance to opioids has changed or heroin is consumed in combination with other drugs [43]. In this context, a recent study shows that high inter-administration variability in the quantity of opioid used is associated with a higher risk of overdose, probably coupled with a reduction in tolerance [44]. These unintentional alterations in the prepared heroin can be a relevant contributory element in polydrug overdoses, as they can involve users more prone to taking higher risks [45].

In conclusion, our results show that the preparation of illegal heroin for i.v. administration decreases the amount of DAM and increases its active metabolites 6-AM and morphine, DAM still remaining the most abundant of the opioids in the final solution for injection. The different elements in the preparation procedure, e.g. the amount and type of acid used or the heating intensity, can affect the final product. Alterations in one individual step of the process would probably not be sufficient to significantly affect the outcome after injection, although unsystematic simultaneous variations in several elements can unintentionally alter the quantity or the concentration of DAM and the related active opioids 6-AM and morphine in the injected heroin and, therefore, the amount and/or the time

lapse of active compounds reaching the brain. These accidental variations, concurrently with known risk factors such as polydrug use or tolerance [43], can be an additional element which might contribute to overdose and influence its random character.

Declaration of interests

None.

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Author contributions

Jannike Andersen: Conceptualization; investigation; methodology. **Inger Lise Bogen:** Conceptualization; investigation; methodology. **Ritva Karinen:** Formal analysis; methodology; validation. **Gerd-Wenche Brochmann:** Data curation; formal analysis; methodology. **Jørg Morland:** Conceptualization; funding acquisition; investigation; methodology; resources. **Vigdis Vindenes:** Conceptualization; funding acquisition; investigation; resources. **Fernando Boix:** Conceptualization; formal analysis; investigation; methodology; project administration; resources; visualization; writing-original draft and writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Amount (in μmol and mg) of ascorbic or citric acid added to 250 mg samples of three heroin seizures of different purity (3.3, 14.9, and 32.1%). Different molar amounts (1:3, 1:2, 1:1) of acid were added proportional to the molar content of DAM in the sample. One sample of each seizure was used for each molar amount of acid. Citric acid was added only to the seizure with the highest purity (32.1%). To ensure sufficient precision a 290 $\mu\text{mol}/\text{ml}$ solution of the acid was prepared and diluted with water to give the desired amount for each sample.