ORIGINAL ARTICLE

BCPT
Basic & Clinical Pharmacology & Toxicology

Heroin metabolism in human blood and its impact for the design of an immunotherapeutic approach against heroin effects

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Abstract

Immunotherapeutic interventions that block drug effects by binding drug molecules to specific antibodies in the bloodstream have shown promising effects in animal studies. For heroin, which effects are mainly mediated by the metabolites 6-acetylmorphine (6-AM; also known as 6-monoacetylmorphine or 6-MAM) and morphine, the optimal antibody specificity has been discussed. In rodents, 6-AM specific antibodies have been recommended based on the rapid metabolism of heroin to 6-AM in the bloodstream. Since the metabolic rate of heroin in blood is unsettled in humans, we examined heroin metabolism with state-of–the-art analytical methodology (UHPLC–MS/MS) in freshly drawn human whole blood incubated with a wide range of heroin concentrations (1–500 μM). The half-life of heroin was highly concentration dependent, ranging from 1.2–1.7 min for concentrations at or above 25 μM, and gradually increasing to approximately 20 min for 1 μM heroin. At concentrations that can be attained in the bloodstream shortly after an i.v. injection, approximately 70% was transformed into 6-AM within 3 min, similar to previous observations in vivo. Our results indicate that blood enzymes play a more important role for the rapid metabolism of heroin in humans than previously assumed. This points to 6-AM as an important target for an efficient immunotherapeutic approach to block heroin effects in humans.

KEYWORDS
antibody, blood, heroin, immunotherapy, metabolism

1 | INTRODUCTION

Opioid use disorder and opioid induced fatal intoxications constitute a major public health problem. In 2020, an estimated 61 million people used opioids, representing 1.2% of the global population. Opioids remain the most lethal group of drugs, accounting for two thirds of all drug overdose deaths worldwide, with heroin playing a significant role.[1]

About 40% of all people in treatment for drug addiction report opioids as their primary drug of use.[1] The World Health Organization (WHO) recommends the

use of pharmacological treatments, preferably accompanied with psychosocial support.[2] At present, the main pharmacotherapies in use are the opioid agonist methadone and the partial agonist buprenorphine. The use of opioid maintenance treatment has been successful in many aspects; however, opioid agonists are addictive and can contribute to overdose deaths.[3–5] Some countries have also approved sustained release formulations of the opioid antagonist naltrexone.[6] Naltrexone treatment requires full detoxification, which may complicate treatment initiation among active users.[7] This has led to a search for alternative approaches not directly interfering with the opioid receptors in the central nervous system. One such approach is immunotherapeutic intervention, which aims to block the drug effects by binding the drug molecule itself rather than its molecular target. The principle is simple and based on drug-specific antibodies binding the drug in the bloodstream. The large size of the drug-antibody complex prevents the drug molecules from crossing the blood brain barrier (BBB) and entering the brain, thereby attenuating their pharmacodynamic effects.[8]

A challenge when developing an immunotherapy against heroin is the rapid metabolism of heroin into several active compounds. Heroin itself has low affinity for opioid receptors[9,10] but is rapidly metabolized to 6-acetylmorphine (6-AM; also known as 6-monoacetylmorphine or 6-MAM), morphine and morphine glucuronides. The first metabolite, 6-AM, is reported to be responsible for the acute effects of heroin in animal research studies, while the second metabolite, morphine, is probably responsible for later effects and of less importance for the immediate rewarding effects.[11–14]

Studies in humans have reported 6-AM and heroin concentrations in blood of the same order of magnitude shortly after intravenous (i.v.) injections of heroin.[15,16] The fast disappearance of heroin in blood was suggested to represent fast distribution of heroin to tissues[17] and rapid metabolism to 6-AM in tissues or blood.[15] This might have implications for immunotherapeutic intervention as to whether antibodies with specific binding of heroin and/or 6-AM would be most suitable for protection against heroin effects. If the major part of the i.v. injected heroin is distributed to the brain prior to metabolism, anti-heroin antibodies would be essential to prevent heroin from reaching the brain and exerting its effects through its metabolites. If, on the other hand, a substantial amount of heroin is being metabolized to 6-AM in blood and tissues prior to brain entry, anti-6-AM antibodies would probably be more efficient in preventing heroin effects.

There is limited knowledge on the metabolism of heroin in different human tissues. A study performed by Way and Kemp in 1965[18] suggested that esterase enzymes in blood were important for the deacetylation of heroin to 6-AM, while liver enzymes were more important for the metabolism of 6-AM to morphine. Three different in vitro studies of heroin metabolism in human blood have reported heroin half-lives of 3, 9 and 15 min, respectively,[19–21] the latter two being considerably longer than the reported half-lives of 2.4–7.6 min in heroin users.[15,16] The in vitro studies were performed 30–45 years ago with limited information on blood quality and less sensitive and precise opioid analysis techniques.

In the present study, we re-examined human heroin metabolism in freshly drawn human blood with a wide range of relevant heroin concentrations using state-of-the-art analytical methodology. The aim was to gain further insight into the contribution of metabolism in human blood for heroin's in vivo pharmacokinetics. This knowledge is crucial when determining the specificity of an antibody to obtain efficient immunotherapeutic inhibition of heroin effects.

2 | MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[22]

2.1 | Human blood

Blood samples were collected from a total of 13 healthy volunteers (six men and seven women) by venous puncture using BD Vacutainer® Lithium Heparin Tubes (VWR International, Oslo, Norway). The blood was immediately aliquoted in tubes stored on ice and the in vitro experiments started within 60 min after blood collection.

2.2 | Materials

Heroin-HCl (94.5%) was purchased from Lipomed AG (Arlesheim, Switzerland). 6-Acetylmorphine-HCl (6-AM-HCl; 88.9%) was purchased from Lipomed AG, and 6-AM (100%) was purchased from Chiron (Trondheim, Norway). Morphine (100%) was purchased from Lipomed AG. The internal standards heroin d9 (100%), 6-AM d6 (100%) and morphine d3 (100%) were obtained from Cerilliant (Round Rock, TX, USA). 0.9% Saline was purchased from B. Braun (Melsungen, Germany). Acetonitrile for HPLC (≥99.9%, far UV gradient grade) was purchased from J. T. Baker, Avantor Performance

Materials Poland S.A. (Gliwice, Poland). Methanol CHROMASOLV™ LC-MS Ultra (≥99.9%) was from Honeywell Research Chemicals (Seelze, Germany). Ammonium formate AnalaR NORMAPUR® (≥97%) was purchased from VWR International AS (Oslo, Norway).

2.3 | Drug solutions

Drug solutions of heroin-HCl (10.2–5083 μM) and 6-AM-HCl (10.2–101.7 μM) for in vitro metabolism studies were prepared in 0.9% saline, stored on ice and used the same day. For analytical analyses, stock solutions of heroin-HCl and the internal standard heroin-d9 were prepared in acetonitrile and stored at −20°C. Stock solutions of 6-AM, morphine and the internal standards 6-AM d6 and morphine d3 were prepared in methanol and stored at −20°C. Stock solutions for calibration samples and quality control samples were prepared independently. Working solutions for calibrators and quality control samples for heroin and 6-AM/morphine (mixture) were prepared by dilution of stock solutions in 5 mM ammonium formate buffer (pH 3.1). Working solutions for internal standards were prepared as a mixture (heroin d9, 6-AM d6 and morphine d3) by dilution of stock solutions in 5 mM ammonium formate buffer (pH 3.1) to 1, 10 and 30 μM. The final concentration of internal standards in the samples were 0.5, 5 or 15 μM, depending on the heroin/6-AM content added to the samples.

2.4 | In vitro studies of heroin and 6-AM metabolism

Undiluted whole blood was aliquoted (110 μL) in plastic tubes and preincubated in a water bath at 37°C for 15 min. To each tube, 12 μL drug solution (10.2–5083 μM heroin or 10.2–101.7 μM 6-AM) was added to obtain final concentrations of 1–500 μM heroin or 1–10 μM 6-AM, mixed for 2–3 s at 2400 rpm on a vortexer (VELP Scientifica Vortex Mixer) and incubated at 37°C for different time periods (0–180 min). The samples were mixed on a vortexer every 30 s (0–10 min experiments) or every 30 min (0–180 min experiments) during incubation. The reaction was stopped by adding a mixture of ice-cold precipitation solution and internal standard (500 μL acetonitrile/methanol [85/15], 78 μL 13 mM ammonium formate buffer pH 3.1, and 50 μL internal standard). The samples were mixed on a vortexer for 30 s, immediately placed at −20°C for at least 10 min to enhance precipitation, followed by centrifugation (4700 g, 4°C, 10 min). The organic phase was transferred to glass tubes or Captiva 96-well plates (Agilent Technologies, Santa Clara, CA, USA) and evaporated to dryness at 40°C under a stream of nitrogen. The samples were reconstituted in ice-cold mobile phase (5 mM ammonium formate/ acetonitrile [97/3], pH 3.1), shaken and centrifuged (4700 g, 4°C, 10 min). The supernatants were transferred to autosampler vials or 96-well plates.

In all experiments, samples were included to control for non-enzymatic degradation of heroin during incubation and degradation during sample preparation. To examine non-enzymatic degradation, control samples with heroin or 6-AM were incubated at 37°C with blood being replaced by 0.9% saline. To control for degradation during the sample preparation, '0 s samples' were prepared by mixing precipitation solution with blood prior to the addition of heroin and compared with '0 s saline' control samples. The experiments were performed in duplicates and repeated four to five times using blood from different individuals.

2.5 | Opioid analysis

Heroin, 6-AM and morphine were analysed by a UPLC–MS/MS method modified from previous studies.[12,23] The analysis was performed on an Acquity UPLC coupled to a Xevo TQ MS (Waters, Milford, MA, USA). The analytes were chromatographically separated on an Acquity HSS-T3 C18 column (2.1 mm i.d., 100 mm, 1.8 μm particles; Waters) kept at 65°C with a mobile phase consisting of 10 mM ammonium formate buffer (pH 3.1; solvent A) and methanol (solvent B) delivered at a flow rate of 0.5 mL/min. The separation was performed using a 7.5-min gradient with the following profile: 0% B from 0–0.5 min; 0%–10% B from 0.5–2.7 min; 10%–20% B from 2.7–3.3 min; 20%–80% B from 3.3–4.6 min; 80%–100% B from 4.6–4.61 min; 100% B from 4.61–6.6 min; and 0% B from 6.61–7.5 min. The calibration standards for heroin were handled separately from the metabolites to examine the stability of heroin during sample preparation and analysis. The inter-assay variability was lower than 15% for all compounds.

2.6 | Data analysis

Data are presented as mean ± S.E.M. or mean + S.E.M., and figures created using SigmaPlot Version 14.5. (Systat Software Inc., San Jose, CA, USA). The one compartment i.v. bolus model of Kinetica Version 5.1. (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for pharmacokinetic calculations to estimate half-lives. The sequential metabolism of heroin to 6-AM and morphine was modelled using the designer module of

Kinetica, and the corresponding metabolic rates calculated. The data were analysed using IBM SPSS Statistics Version 26.0. (IBM Corp, Armonk, NY, USA). Paired two-sided Student's t-test or general linear model (GLM) followed by post hoc pairwise contrasts with Bonferroni correction were used to evaluate statistical differences.

3 | RESULTS

We first studied heroin metabolism at concentrations of 1 and 10 μM (Figure 1), representative of the concentrations measured in heroin users shortly after i.v. injections of heroin.[24] After addition to freshly drawn human whole blood, heroin was rapidly converted into 6-AM and morphine. From the concentration *versus* time curves, it could be observed that the sum of heroin and metabolites was quite stable throughout the incubation period of 90 min. It could also be observed that the conversion of heroin to 6-AM was faster at incubation with 10 μM heroin compared to 1 μM (Figure 1).

As the blood concentration of heroin can be very high at the injection site immediately after an i.v. bolus injection, we also measured the heroin concentration over a broad range of concentrations (1–500 μM) during 10 min of incubation. For the total 10-min period, heroin disappeared faster with increasing heroin concentrations, reflected by steeper lines in the semilog plot (Figure 2A) and shorter half-lives (Figure 2B). The heroin disappearance rates decreased with time as the concentrations gradually became lower, as can be observed by a flattening of the curves (Figure 2A).

For initial heroin concentrations between 10 and 100 μM, the half-lives for the first minute of incubation were significantly shorter compared with the total 10 min incubation period (Figure 2B). The shortest half-lives of heroin were observed during the first minute of incubation, ranging from 1.2 to 1.7 min for heroin concentrations at or above 25 μM, increasing to 2.7, 12.9 and 19.5 min when lowering the initial heroin concentrations to 10, 5 and 1 μM, respectively (Figure 2B).

When incubating blood with heroin concentrations in the range of 25 to 500 μM, more than 67% of the initial heroin amount was transformed into 6-AM and to a minor extent morphine within 3 min (Figure 3). In comparison, only 10%–25% of the initial heroin dose was metabolized during 3-min incubation when adding heroin at concentrations of 1–5 μM (Figure 3). During the 10-min incubation period, heroin was mainly metabolized to 6-AM, with less than 10% of the added heroin dose being transformed to morphine (Figure 3). Calculation of the rate constants for the metabolism of heroin into 6-AM showed the highest metabolic rate at 100 μM initial dose (Figure 4), gradually decreasing with lower heroin concentrations. In comparison, the rate constants for the metabolism of 6-AM to morphine were much lower and appeared to be less dependent on substrate concentration.

In separate experiments, the metabolism of administered 6-AM (1 or 10 μM) and formation of morphine was also studied (Figure 5). The concentration *versus* time curves at both concentrations displayed a 6-AM half-life of about 90 min in human blood, that is, substantially longer than the half-life of heroin (Figure 2B). The sum of 6-AM and morphine concentrations were
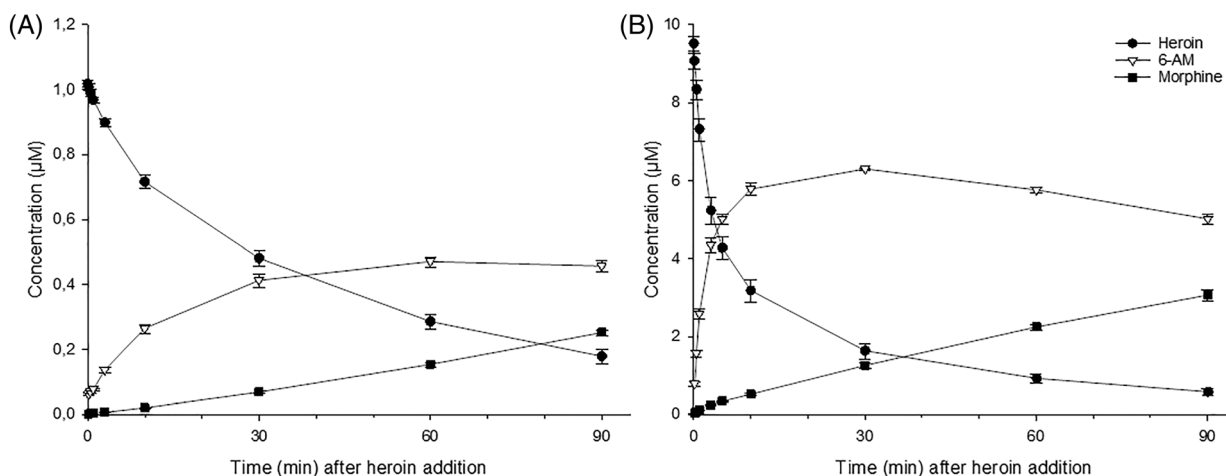
FIGURE 1 In vitro concentration curves of heroin, 6-AM and morphine in human blood at 37°C as function of time (min) after addition of (A) 1 μM heroin and (B) 10 μM heroin. All values are presented as mean ± S.E.M. ($n = 4$–5).
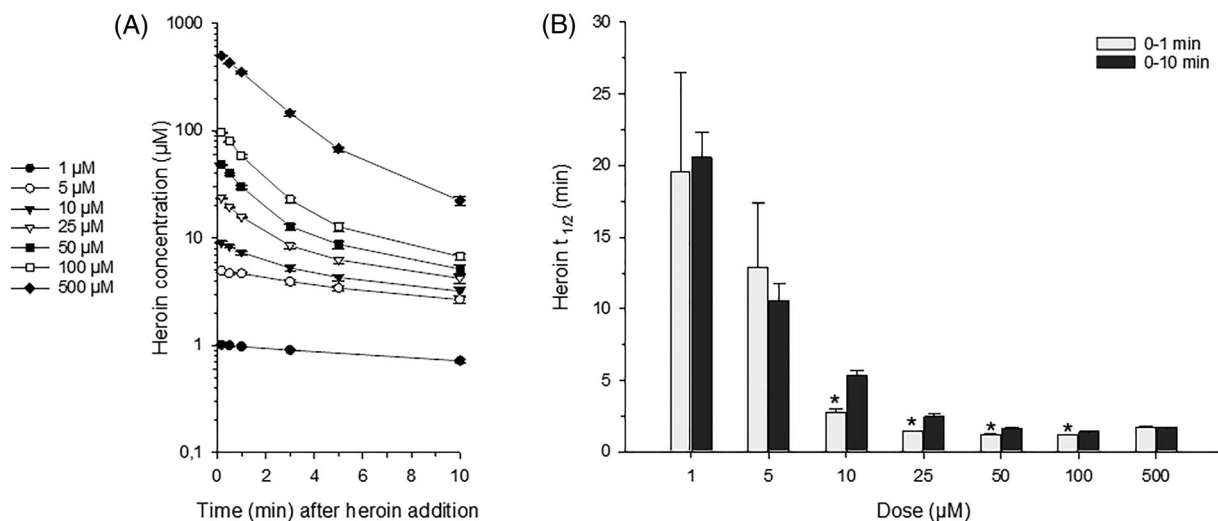
FIGURE 2 (A) Semilog plot of heroin concentrations (mean $\pm$ S.E.M.) after incubation of heroin (1–500 μM) with human blood at 37°C as function of time (min) ($n = 4$–5). (B) Half-lives (mean + S.E.M.) of heroin in human blood were calculated using Kinetica 5.1. one compartment i.v. bolus model ($n = 4$–5). Significant differences between 0–1 and 0–10 min are reported with * ($p < 0.05$ *versus* 0–10 min, paired two-sided Student's *t*-test).

approximately constant over the incubation period of 180 min at both initial concentrations of 6-AM.

## 4 | DISCUSSION

4.1 | Heroin metabolism in blood

The major finding in the present study is the extremely rapid metabolism of heroin to 6-AM in human whole blood, in particular at concentrations higher than 10 μM. When incubated in freshly drawn blood, heroin rapidly disappeared and 6-AM and morphine were formed in quantitative amounts. The half-life of heroin was in the range of 1.2–1.7 min for concentrations between 25 and 500 μM, increasing gradually to approx. 2.7, 12.9 and 19.5 min for 10, 5 and 1 μM, respectively. To our knowledge, no previous studies have examined the rate of heroin metabolism in fresh human whole blood over a broad range of different initial heroin concentrations.

Our data did not support clear-cut first order kinetics, but rather a mix of several first order disappearance rates. Owen and Nakatsu reported four different enzymes responsible for the conversion of heroin to 6-AM in human blood, with different capacities and K_m values (K_m: substrate concentration at which the reaction rate is 50% of the velocity maximum) ranging from 38 to 2000 μM and relative contribution depending on the heroin concentration.[25] Studies of purified erythrocyte acetylcholine esterase (AChE) and plasma butyrylcholine

esterase (BChE),[26,27] both engaged in heroin metabolism, found K_m values around 350 and 110 μM, respectively, as well as differences in catalytic efficiency. Human recombinant AChE and BChE displayed K_m values of 2170 and 120 μM, respectively.[28] Thus, the presence of multiple enzymes with different K_m values and catalytic capacities is a likely explanation for the concentration-dependent half-lives of heroin observed. Product inhibition by 6-AM[26] and deterioration of blood esterase capacity during incubation in vitro, may also contribute to increasing heroin half-lives with time.
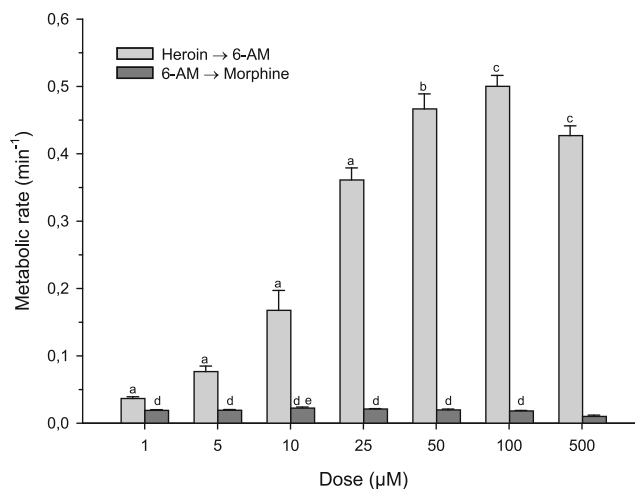
4.2 | The impact of rapid metabolism in blood for heroin pharmacokinetics in vivo

The heroin half-life in human whole blood reported in the present study was significantly shorter than previously reported.[19–21] In fact, the provided in vitro half-lives were of the same order of magnitude as that reported in humans in vivo.[15] This demonstrates that blood enzymes play a more important role for the rapid metabolism of heroin in humans than previously assumed. Similar findings have been observed in animal studies, with a half-life of heroin in rat whole blood in vitro ($t_{1/2}$: 0.28 $\pm$ 0.03 min; Figure S1 and Table S1 in Supporting Information) being of the same order of magnitude as the reported half-life in vivo in rats.[29] In Gottås et al.,[29] the observed half-life of 0.3 min in rats was attributed to distribution, in accordance with the literature available at the time.
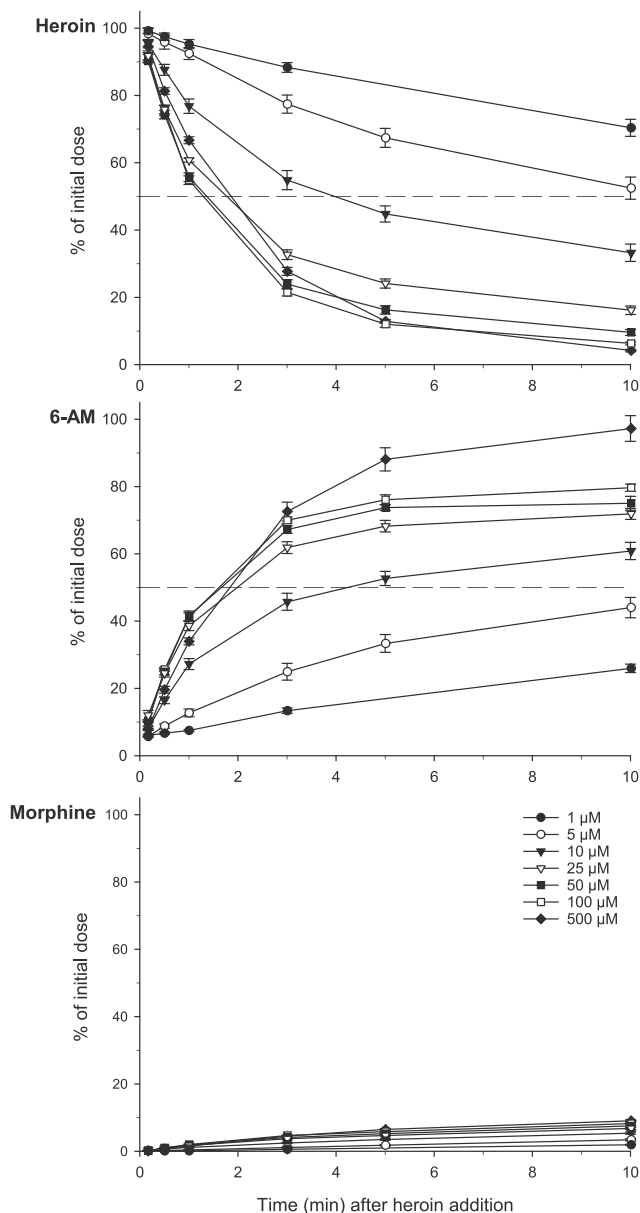
FIGURE 3 Heroin, 6-AM, and morphine in percentage of the added initial heroin dose (1–500 μM) as function of time (min) after incubation in human blood at 37°C ($n = 4–5$).

FIGURE 4 Metabolic rate constants (min^{-1}, mean + S.E.M.) for the conversion of heroin to 6-AM and 6-AM to morphine ($n = 4–5$). For the metabolism of heroin to 6-AM (light grey bars): a) $p < 0.05$ *versus* all the other doses, b) $p < 0.05$ *versus* all the other doses except 100 and 500 μM, c) $p < 0.05$ *versus* all the other doses except 50 μM (GLM pairwise contrasts wiht Bonferroni correction). For the metabolism of 6-AM to morphine (dark grey bars): d) $p < 0.05$ *versus* 500 μM; e) $p < 0.05$ *versus* 100 μM (GLM pairwise contrasts with Bonferroni correction).

A study of population pharmacokinetics attributed the rapid decline in heroin concentration in blood to rapid distribution to tissues, with an estimated distribution half-life of 2.4 min, whereas the elimination half-life was estimated to 7.6 min.[16] In the present study, we observed half-lives of heroin in blood even shorter than the estimated distribution half-lives, suggesting that heroin metabolism rather than distribution is responsible for the initial rapid heroin disappearance from blood. Accordingly, the distribution of heroin to the brain and other tissues may be less pronounced than previously

assumed. Furthermore, the capacity of the brain to hydrolyse heroin into 6-AM by AChE appears to be limited.[27] However, some studies have demonstrated human brain esterase activity at the endothelial component of the BBB,[30,31] suggesting that heroin may be hydrolysed into 6-AM upon brain entrance. Pharmacokinetic modelling of heroin distribution and metabolism in mouse brain tissue[32] also point to limited heroin metabolism in the brain. However, animal studies have demonstrated higher concentration of 6-AM than heroin in cerebral extracellular fluid at all times after i.v. heroin administration.[29] Taken together, these observations indicate that the dominant source of intracerebral 6-AM is either generated in the blood or from heroin being metabolized at the BBB upon brain entrance.

Our study also demonstrates a striking difference between the metabolism of heroin and 6-AM in blood. While the metabolism of heroin occurred at a rate comparable to the rate reported in heroin users, the in vitro metabolism of 6-AM in blood was markedly slower displaying a half-life about four times longer than the reported in vivo half-life of 22 min.[16] This points to the importance of esterases located in tissues, including liver carboxylases, for 6-AM hydrolysis.[18,33] Thus, distribution to tissues and in vivo metabolism outside the bloodstream appears to play a more important role for 6-AM disappearance than for heroin.
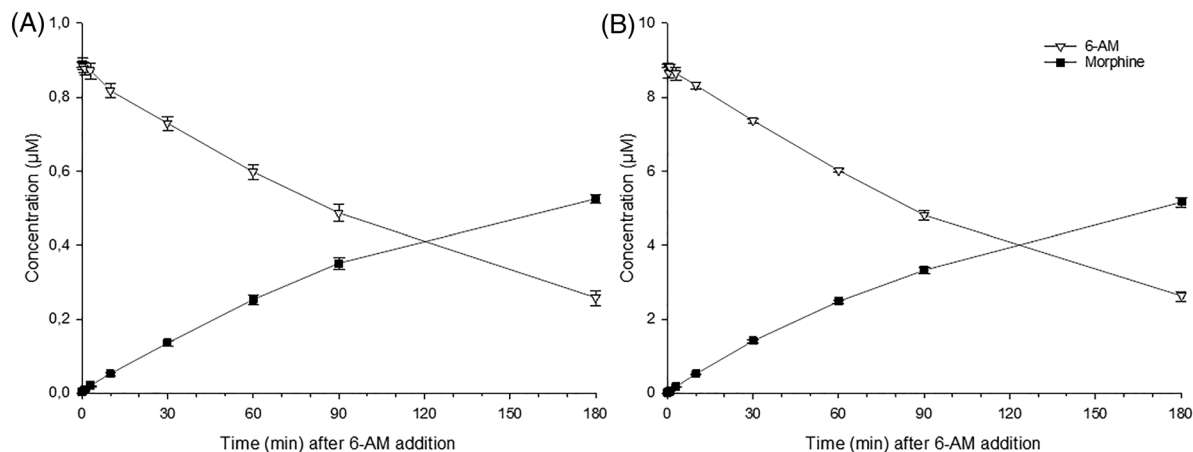
FIGURE 5 In vitro concentration curves of 6-AM and morphine in human blood at 37°C as function of time (min) after addition of (A) 1 μM 6-AM and (B) 10 μM 6-AM. All values are presented as mean ± S.E.M. ($n = 4$–5).

4.3 | Immunotherapeutic prevention of the effects of heroin

Detailed knowledge about the metabolic rate and site for the conversion of heroin to its more active metabolites is of major importance for the development of an efficient immunotherapeutic approach to block heroin effects. This study indicates that 6-AM is the major active metabolite in human blood within a few minutes of heroin injection. While heroin displays low affinity and efficacy at μ opioid receptors,[9,10] it may serve an important role when it comes to efficient transport to the brain demonstrated by significantly higher brain 6-AM levels after heroin injections compared to 6-AM injections in rodents.[14,29] Animal studies have shown that binding of 6-AM is an efficient strategy to block heroin effects.[14,34–36] Raleigh and co-workers demonstrated that an active vaccine generating antibodies with high specificity towards both 6-AM and heroin inhibited heroin self-administration in rats, although the heroin concentration in brain was not significantly reduced.[35] Our research group found dose-dependence between the amount of anti-6-AM monoclonal antibodies (mAbs) administered and the magnitude of inhibition of heroin-induced locomotor activity[14] and heroin-induced reward.[36] Stowe and co-workers showed that antibodies with specificity towards heroin and morphine were unable to prevent acquisition of heroin self-administration, while antibodies specific for 6-AM did.[34] Thus, several lines of evidence point to the importance of binding 6-AM instead of heroin. A recent study concluded that mAbs binding heroin and not 6-AM are critical for the reduction of heroin analgesia[37]; however, uncertainties regarding the pharmacokinetics of heroin when given intraperitoneally could challenge this conclusion.

After an i.v. dose of heroin, the immediate local concentrations in blood would depend on the amounts of heroin dissolved in the injection solution and the speed of injection, two factors known to be subject to variability.[38] An i.v. dose containing about 270 μmol (100 mg) pure heroin would give rise to maximum heroin concentrations in the range of 270–540 μM immediately after injection when diluted in 0.5–1 L blood. When diluted in a total blood volume of 5 L, a theoretical concentration of approximately 54 μM would be reached when disregarding drug metabolism and distribution to tissues. Based on our present findings, a blood concentration of 54 μM would be reduced to 50% within 1.2 min. This suggests a blood concentration of 20 μM heroin within 1.5–2 min after injection, with blood 6-AM concentrations expected to be in the same order of magnitude. This is in accordance with Rentsch et al., reporting blood concentrations of 6-AM surpassing heroin concentrations already 1 to 2 min after i.v. heroin administration.[15]

The calculations above have relevance when choosing antibody specificity and estimating the antibody levels needed to successfully interfere with the effects of heroin. In a human experimental study on anti-methamphetamine antibodies, the highest antibody dose tested (20 mg/kg) resulted in serum antibody concentrations of about 3.5 μM, which can be estimated to correspond to 1.75 μM (0.25 g/L) in whole blood. Therapeutic antibodies used for other purposes, for example, for protection against the toxic effects of the bacterium *Bacillus anthracis* (Anthrax), have been dosed up to 40 mg/kg in healthy subjects,[39,40] representing antibody concentrations of 3.5 μM (0.5 g/L) in human whole blood. Safety studies revealed that most adverse events observed after injection of 40 mg/kg mAb in humans were transient and mild to moderate in severity, and did not differ significantly between the mAb and

placebo groups.[40] If we consider a maximum antibody concentration in whole blood to be 3.5 µM as suggested above, a heroin concentration of 7 µM could be bound by mAb because of the two antigen binding sites per IgG molecule. This drug-binding capacity is too low to neutralize the total amount of heroin measured in heroin users shortly after i.v. injections of heroin.[15,24] However, a study performed in our laboratory demonstrated that anti-6-AM mAb, using a drug:mAb ratio capable of binding 5% of the injected opioid dose, reduced 6-AM levels in the brain and heroin-induced locomotor activity by more than 50% within 20–25 min after administration of heroin (2.5 µmol/kg) in mice.[14] This indicates that 6-AM antibody levels even below those giving complete protection might have important clinical effects. This finding has also been reported for other drugs of abuse and has been shown after partial antibody binding of fentanyl,[41] methamphetamine[42] and phencyclidine.[43]

5 | CONCLUSION

In summary, the present results indicate that the metabolism of heroin by human blood plays a more important role for the rapid conversion of heroin to the active metabolite 6-AM than previously assumed. By in vitro studies, we have shown that the metabolism in blood can account for a half-life of heroin down to 1.2 min for concentrations reported shortly after heroin intake. Since 6-AM is a key mediator of heroin effects, 6-AM sequestration by antibodies appears essential for an efficient immunotherapeutic interference with heroin effects in humans, although concerns with respect to the drug binding capacity of actual antibody levels exists. However, animal studies using mAbs specific for 6-AM at levels assumed to be safe in humans have shown substantial protection against heroin effects, and future clinical studies should explore whether similar protection can be obtained in humans.

CONFLICT OF INTEREST STATEMENT
The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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