

Pharmacokinetics of heroin and its metabolites in vitreous humor and blood in a living pig model

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Abstract Vitreous humor (VH) is an alternative matrix for drug analysis in forensic toxicology. However, little is known about the distribution of xenobiotics, such as opioids, into VH in living organisms. The aim of this study was to simultaneously measure heroin and metabolite concentrations in blood and VH after injection of heroin in a living pig model. Six pigs were under non-opioid anesthesia during the surgical operation and experiment. Ocular microdialysis was used to acquire dialysate from VH, and a venous catheter was used for blood sampling. Twenty milligrams of heroin was injected intravenously with subsequent sampling of blood and dialysate for 6 h. The samples were analyzed by ultra-performance liquid chromatography–tandem mass spectrometry. Heroin was not detected in VH; 6-monoacetylmorphine (6-MAM) and morphine were first detected in VH after 60 min. The morphine concentration in VH thereafter increased throughout the experimental period. For 6-MAM, C_{\max} was reached after 230 min in VH. In blood, 6-MAM reached C_{\max} after 0.5 min, with a subsequent biphasic elimination phase. The blood and VH 6-MAM concentrations reached equilibrium after 2 h. In blood, morphine reached C_{\max} after 4.3 min, with a subsequent slower elimination than 6-MAM. The blood and VH morphine concentrations were

in equilibrium about 6 h after injection of heroin. In conclusion, both 6-MAM and morphine showed slow transport into VH; detection of 6-MAM in VH did not necessarily reflect a recent intake of heroin. Because postmortem changes are expected to be small in VH, these experimental results could assist the interpretation of heroin deaths.

Keywords Heroin · Pharmacokinetic · Vitreous humor · 6-MAM · Ocular microdialysis · Pigs

Introduction

Heroin is a highly lipid soluble opioid prodrug with negligible affinity and efficacy to μ -opioid receptor [1–3]. After intake, heroin is rapidly and completely converted to 6-monoacetylmorphine (6-MAM) spontaneously or by serum and tissue cholinesterases, and by tissue carboxyesterases, among others. 6-MAM is further metabolized to morphine that is in turn metabolized in the liver to the inactive morphine-3-glucuronide (M3G) and the active morphine-6-glucuronide (M6G), as well as several other minor compounds (for reviews see [4–6]). In man, the blood elimination half-life for heroin is less than 5 min, for 6-MAM around 15–20 min, for morphine 2–3 h, and for its glucuronides, 4–6 h [7]. The most intense phase of heroin effects, the “rush”, takes place shortly after intake. At this stage 6-MAM is the predominant metabolite in both blood and brain [7–10]. Later, 1–2 h after intake, morphine and M6G are the main metabolites able to mediate heroin’s effects.

In forensic toxicology, results from postmortem body fluids or tissues are used to interpret the role of drugs in each death. Postmortem redistribution of drugs is a major challenge in this interpretation, making the estimation of

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the true concentrations at the time of death difficult, especially in cases with long postmortem intervals and potentially substantial drug redistribution. Vitreous humor (VH) is used as an alternative specimen for toxicological interpretation, and it is assumed that this medium is less prone to postmortem redistribution, making the detected concentration more representative for the one present at the time of death. When trying to estimate a blood concentration from the one detected in VH, toxicologists fall short, because no studies have investigated the kinetics of heroin and metabolites in both blood and VH in vivo. Therefore, the penetration and detection times for drugs and metabolites in VH as compared to those in blood are unknown.

In fatal cases where heroin metabolites are detected, interpretation of the toxicological findings from different specimens is especially important, because the presence of metabolites and their concentrations can provide important information not only about the cause of death, but also about the time between intake of heroin and death. How heroin and its metabolites distribute into different body compartments after administration has previously been well described for blood and urine in man [6]; the distribution of heroin and its metabolites into the brain has been also reported in animal models [8, 9, 11]. However, the distribution into VH has been studied less thoroughly, and such knowledge is important when interpreting the death involving heroin.

Most previous studies investigating the distribution of heroin and metabolites in both blood and VH have used postmortem samples [12, 13]; antemortem in vivo kinetics of drugs in VH have been studied for only a very few drugs related to forensic toxicology in living animal models using rabbits [14–16]. A study by Crandall et al. [17] compared concentrations of morphine in VH and blood for one time point in a porcine model. One of the main problems in the study of the kinetics of drugs in VH is the limited amount of VH available for sampling. This limits the number of time points and forces the use of different animals for the different time points increasing the spreading of the data. Some of the challenges associated with sampling from VH can be overcome by the use of ocular microdialysis, and this technique has previously been used to investigate drug distribution into the aqueous and vitreous body of the rabbit eyes [18–21] (for review see Boddu et al. [22]).

With more antemortem knowledge about both blood and VH kinetics of heroin and metabolites, the interpretation of postmortem cases is easier and more exact. A better understanding of the pharmacokinetics of these substances in VH might further open new perspectives about the utility of VH as a complementary sample in forensic toxicology. The aim of this study was, therefore, to investigate the concentration–time profiles of heroin, 6-MAM, morphine,

and M3G in both VH and blood after intravenous (i.v.) administration in the living organism using ocular microdialysis. This could further elucidate the usefulness of VH as a forensic material. Pigs were chosen as the preferred specimen for this study, because their physiology resembles that of humans [23]. It is previously indicated that formation of higher amounts of M3G could be expected in pig as compared to those in humans, and formation of M6G is not expected [24–26].

Materials and methods

Chemicals and reagents

Heroin hydrochloride, heroin-*d*₃, 6-MAM, 6-MAM-*d*₃, 6-MAM-*d*₆, morphine-*d*₃, M6G, M6G-*d*₃, M3G, and M3G-*d*₃ were obtained from Lipomed (Lipomed GmbH, Arlsheim, Switzerland); morphine from NMD (NMD Gros-sisthandel AS, Oslo, Norway); and morphine-*d*₆ from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Standard compounds were stored according to supplier recommendations. HPLC-grade methanol and acetonitrile were purchased from Labscan Ltd. (Poch SA, Gliwice, Poland); analytical grade ammonium formate and formic acid from Merck (Whitehouse Station, NJ, USA). All water used was provided by a MilliQ A10 purification system (Merck, Darmstadt, Germany). Stock and working solutions were prepared as described previously [11].

Animals and conditions

Six Norwegian Landrace pigs (*Sus scrofa domesticus*) of either sex (weight 45 ± 5 kg) supplied from the Centre for Comparative Medicine, Oslo University Hospital, Rikshospitalet were used. They were housed separately at standard housing conditions (08:00–20:00 lights on), with free access to food and water. The experimental protocol was approved by the Norwegian National Animal Research Authority (Authorization Number 6425) and carried out in accordance to Norwegian regulations and international standards.

In vivo experiment

Anesthesia was induced with intramuscular (i.m.) injection of ketamine (20 mg/kg), azaperone (3 mg/kg), and atropine (0.02 mg/kg). The anesthesia was maintained with i.v. ketamine (1–2 mg/kg) and pentobarbital (1–3 mg/kg) until a tracheotomy through a neck midline incision was performed, and the pig was mechanically ventilated by an anesthesia machine (Leon *plus*, Heinen + Löwenstein;

Bad Ems, Germany). Thereafter, general anesthesia was continued by an infusion of ketamine (2–2.5 mg/kg/h) together with inspired isoflurane at a concentration of 1.0–1.5 % using a Tec 7 gas analyzer (Heinen + Löwenstein). The inspired oxygen fraction was set to 0.35 and ventilation adjusted to keep expired end-tidal carbon dioxide concentration between 4.5 and 5.5 kPa. The right internal jugular vein was cannulated with a triple lumen central venous catheter (CVC) and advanced to vena cava superior for pressure registration and for heroin administration and blood sampling. The administration of heroin and collection of blood samples were obtained from different lumens of the CVC to avoid cross contamination of samples. The right carotid artery was cannulated for continuous blood pressure registration. A bladder catheter was placed by a mini-laparotomy to extract urine samples and measure diuresis and core temperature. Additional monitoring of the pig included electrocardiography, heart rate, and SpO₂. Fluid administration during the experiment was made with i.v. Ringer's acetate at a rate of 20 mL/kg/h.

A microdialysis guide cannula (AT6.14.iC, AgnThós, Lidingö, Sweden) was carefully inserted into the eye through a 19-gauge syringe needle. The needle was carefully inserted about 3 mm below the corneal scleral limbus through the pars plana; the needle was then retracted and the guide cannula inserted in its place. The guide dummy was then retracted and the microdialysis probe (AT6.14.4, AgnThós) was placed through the guide into the vitreous body. The probe position was adjusted, by visual inspection through the lens, such that the 4 mm long microdialysis membrane (outer diameter, 0.6 mm) resided well suspended in the mid-VH, and was then secured with surgical tape (Fig. 1).

Before implantation of the microdialysis probe, the probe was connected by fluorinated ethylene propylene-tubing to a syringe containing a physiological Ringer's solution with deuterated recovery calibrators (6-MAM-*d*₃, morphine-*d*₆, M3G-*d*₃) for each compound under study.

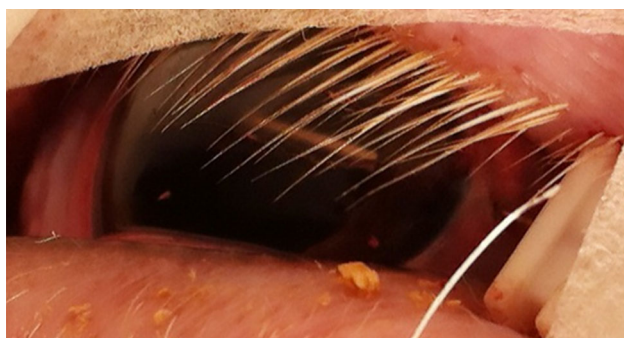


Fig. 1 Microdialysis guide penetrating the pars plana with the probe extending through the guide shaft into the vitreous chamber of the eye

The syringe containing the perfusion fluid was placed in a syringe microinfusion pump (CMA 400; CMA Microdialysis, Solna, Sweden). The pump perfusion flow was set to 0.7 μ L/min. After implantation of the microdialysis probe the pig received an i.v. bolus injection (2 mL) of 20 mg heroin through the CVC, followed by flushing with 10 mL physiological saline solution. Dialysate was collected at 10 min intervals, into vials containing 5 μ L internal standards (ISs), during the entire experimental session. That is, 7 μ L dialysate was collected per vial, giving a total sample volume of 12 μ L including the ISs. The vials were placed in a fraction collector (CMA/470; CMA Microdialysis) cooled to 6 °C during collection. Concurrently, 0.1 mL blood samples were taken through the CVC at 1, 2, 5, 7, 10, 15, 30, and then at every 30 min up to a maximum of 360 min after the injection of heroin. In one pig, samples from the living pig were only taken until 180 min after the injection, and postmortem samples were collected for another 150 min (postmortem data is not included in the succeeding data processing). In the remaining pigs, samples from the living animals were collected for 300–360 min after injection of heroin. The exact time point for each sample was recorded.

Two samples from the VH, in the contralateral eye of the one holding the microdialysis probe, were collected by syringe aspiration at different time points during the experiment. After euthanasia, and removal of the microdialysis probe, an additional aspiration of VH was performed from the eye holding the probe. The aspirated samples were taken to compare to the microdialysis samples (dialysates).

The blood samples were prepared in accordance with a previously established method with some modifications [9, 27]. In brief, blood samples were drawn using a syringe and transferred to microcentrifuge tubes containing 100 μ L ammonium formate buffer (5 mM, pH 3.1) with 4 mg/mL NaF and 17.8 IE/mL heparin sodium, handled on ice, and immediately frozen on a freezing block at –80 °C (placed in a –80 °C freezer overnight and placed on ice in a Styrofoam box just before sampling). The block was switched with a new block from the –80 °C freezer after the first 60 min).

Each pig was finally euthanized by an i.v. injection of 1 g pentobarbital and 100 mmol potassium chloride through the CVC.

Chemical analysis

Dialysate required no further sample preparation; the VH sample analysis was done according to our previously established method with minor modifications [11]. Briefly, the samples were analyzed on a Waters Xevo TQ-S ultra-performance liquid chromatography (UPLC)–tandem mass

spectrometry (MS/MS) system equipped with an Aquity HSS T3-column (Waters, Milford, MA, USA). This system was more sensitive than the system used in Gotta et al. [11]. We, therefore, determined new lowest levels of detection (LODs) and lowest levels of quantification (LLOQs) for 6-MAM, morphine, and M3G. Each LOD was determined by evaluation of the signal/noise (S/N) ratio for diluted series of the lowest standard, with an acceptance criteria of $S/N > 3$.

Sample preparation of the aspirated VH samples was performed according to a previously published method [27]; however, the chromatographic conditions were the same as in the previously published method for urine [28]. Briefly, a 100- μL aliquot of VH was added 50 μL IS solution (0.5 μM in water), and liquid-liquid extraction with 500 μL acetonitrile/methanol (85:15) was performed. Blood samples were prepared for analysis similarly to a previously published method [27]. Briefly, 50 μL IS solution (0.5 μM in water) was added to the blood samples before protein precipitation plus liquid-liquid extraction with 500 μL acetonitrile/methanol (85:15). High-performance liquid chromatography (HPLC)-MS/MS analysis was performed with a Waters Quattro Premier coupled to a XE MS/MS system equipped with a Xterra[®] MS C18 column (150 \times 2.1 mm internal diameter, 3.5 μm particle size) (Waters). Chromatographic conditions were in accordance with a previously established method [9].

Statistical analyses

Kinetic version 5.1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for processing of the data. Results are presented from fitting curves from this program, in addition to individual data in some figures. The compartment model was selected based on the best goodness of fit as determined by the lower Akaike Information Criterion for each drug.

The calculated fitted results were used in a noncompartmental analysis to calculate the area under the concentration-time curve from time zero to last sample time (AUC_{Last}), the estimated maximum concentration (C_{max}) and the time, T_{max} , to reach C_{max} for all the different substances. The half-lives ($t_{1/2}$) were calculated for heroin, 6-MAM and morphine in blood. Additionally, $t_{1/2}$ was calculated for 6-MAM in VH. The AUC calculations were based on a mixed log linear model implemented in the Kinetic software, with an i.v. bolus model using the extrapolated zero concentration functions for heroin in blood after i.v. administration, and an extravascular model for the metabolites in blood and all compounds in VH. All data below LLOQ were discarded from the dataset before processing in Kinetic.

Results

Validation of the analytical method

Although most analytical conditions were almost the same as described previously [11], the UPLC-MS/MS system is somewhat different and more sensitive. In the previous report, the dialysate samples of rat brain extracellular fluid were analyzed; in the present study, the dialysate samples of pig VH were dealt with.

To check the sensitivity of the new instrumental system, the lowest reference standard solutions were diluted down to 6.0 pM; at this concentration the S/N ratios were as high as 13.5, 58.3, and 6.0 for 6-MAM, morphine and M3G, respectively. The performance of the lowest reference standard (100 pM) determined the LLOQ. The variation (% relative standard deviation) and accuracy (% bias) were 0.4 and 0.7 % for 6-MAM, 1.6 and 2.9 % for morphine, and 9.6 and 6.0 % for M3G, respectively, at this concentration, well within the widely accepted criteria of ± 20 % [29].

Recoveries for each microdialysis probes in VH were calculated by retrodialysis using isotope analogs as described by Gotta et al. [11]. This procedure was used for calculating the concentration of unbound analyte in the VH. The average recovery values for the deuterated recovery calibrators were 38 ± 9 % [mean \pm standard deviation (SD)] for 6-MAM- d_3 , 31 ± 6 % for morphine- d_6 , and 28 ± 10 % for M3G- d_3 ($n = 5$ each).

The validation data for blood samples has been described in our previous report [9].

Blood pharmacokinetics

The concentration-versus-time profiles of heroin and its metabolites in blood and VH of pigs are shown in Fig. 2, and the calculated pharmacokinetic parameters are listed in Table 1. The heroin level in blood declined below LLOQ already after about 15 min, with a biphasic concentration-versus-time curve (Fig. 2a). The initial alpha phase (ultra-rapid distribution, between 0 and 2 min) had an apparent $t_{1/2}$ of 0.4 min and the terminal elimination phase (between 5 and 15 min) a $t_{1/2}$ of 1.9 min. The extrapolated C_0 for heroin was 116 μM . Based on the population-calculated data, 6-MAM in blood reached a C_{max} at 2.29 μM and T_{max} after 0.5 min. The decline in 6-MAM blood concentration curve was also biphasic, with an initial alpha phase (between 0.5 and 5 min) with an apparent $t_{1/2}$ of 2.0 min, and a terminal elimination phase (between 30 and 270 min) with a $t_{1/2}$ of 27.2 min (Fig. 2b). In the alpha phase, heroin was still present and, therefore, influenced the concentration curve for 6-MAM, whereas virtually all heroin was

Fig. 2 Blood and vitreous humor (VH) concentrations of heroin, 6-monoacetylmorphine (6-MAM), morphine, and morphine-3-glucuronide (M3G) after intravenous administration of 20 mg heroin. *Points* represent the observed concentrations (the *different symbols* representing the different animals), *lines* show the fitted values calculated in the Kinetica software. **a–d** Observed and fitted values for each separate compound in blood ($n = 6$) (for heroin, $n = 5$). **e–g** Observed and fitted values for each separate compound in VH ($n = 5$)

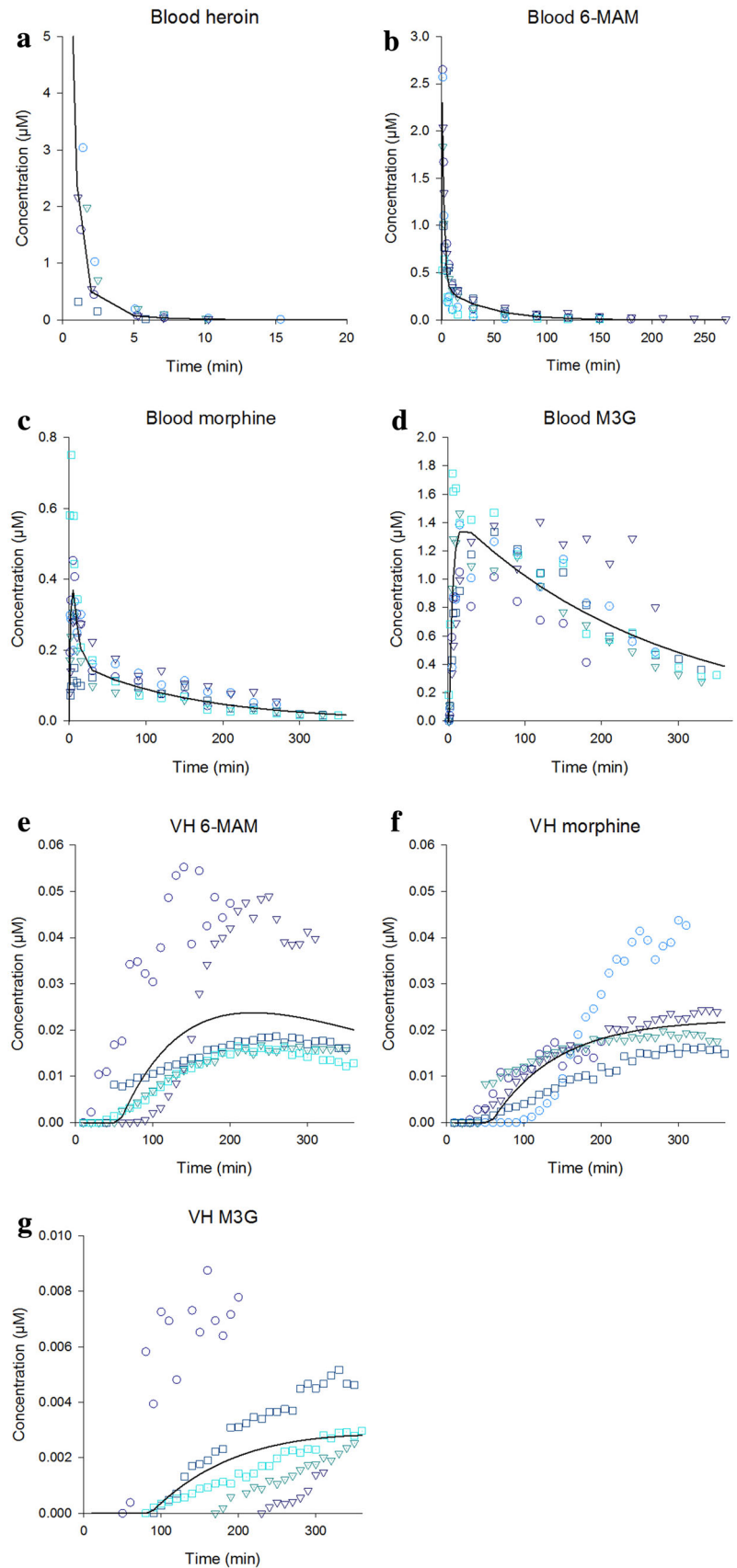


Table 1 Pharmacokinetic parameters for blood and vitreous humor (VH) dialysate after intravenous injection of 20 mg heroin to each pig

Drug	Parameter	Blood	VH dialysate
Heroin	AUC _{Last} [$\mu\text{M} \times \text{min}$]	26.4	–
	C_0 (μM)	116 ^a	–
	T_{max} (min)	0.0	–
	$T_{1/2}$ alpha-phase (min)	0.4	–
	$T_{1/2}$ elimination-phase (min)	1.9	–
6-MAM	AUC _{Last} [$\mu\text{M} \times \text{min}$]	18.7	5.9
	C_{max} (μM)	2.29	0.0238
	T_{max} (min)	0.5	230
	$T_{1/2}$ alpha-phase (min)	2.0	–
	$T_{1/2}$ elimination-phase (min)	27.2	336
Morphine	AUC _{Last} [$\mu\text{M} \times \text{min}$]	26.1	4.9
	C_{max} (μM)	0.37	0.0217 ^b
	T_{max} (min)	4.3	360 ^b
	$T_{1/2}$ elimination-phase (min)	109	–
M3G	AUC _{Last} [$\mu\text{M} \times \text{min}$]	285	0.5
	C_{max} (μM)	1.36	0.0028 ^b
	T_{max} (min)	20.2	360 ^b

All parameters are based on population simulation data in Kinetica

^a C_0 calculated in Kinetica must be considered as uncertain

^b C_{max} and T_{max} at the end of experiment presented

cleared from the blood during the terminal elimination phase. The morphine concentrations increased gradually, with a C_{max} of 0.37 μM after 4.3 min (T_{max}) (Fig. 2c). Morphine further had a $t_{1/2}$ of 109 min in the terminal elimination phase between 270 and 360 min, i.e. when no 6-MAM was detected in blood. The blood levels of M3G increased even slower than morphine, with a C_{max} of 1.36 μM after 20.2 min (T_{max}) (Fig. 2d).

Ocular/vitreous humor pharmacokinetics

The concentration-versus-time profiles in VH are also shown in Fig. 2, and the calculated pharmacokinetic parameters are listed in Table 1. Heroin was not detected in VH dialysate. 6-MAM appeared in VH dialysate 56.8 min (range 20–80 min) after injection of heroin. Thereafter the 6-MAM concentrations increased gradually and reached a C_{max} of 0.0238 μM after 230 min (T_{max}) (Fig. 2e). The apparent $t_{1/2}$ for 6-MAM was 336 min. Similarly to 6-MAM, morphine was first detected in VH 57.2 min (range 30–100 min) after the heroin injection. Thereafter, the concentration increased gradually throughout the experimental procedure to reach a concentration of 0.0217 μM after 360 min (Fig. 2f). M3G first appeared in VH after about 85.9 min (range 60–240 min), thereafter the levels increased slightly during the total experimental

procedure to reach a concentration of 0.0028 μM after 360 min (Fig. 2g).

To show the microdialysis technique superiority over traditional aspiration, aspirated VH samples were also collected. A total of 18 VH samples were collected by aspiration. Only eight of these did not show too much contamination by blood, making the results reliable. For these samples, the mean concentration ratio of aspirated samples to dialysates at the same time point were 2.4 ± 1.8 (mean \pm SD) for morphine and 2.1 ± 3.0 for 6-MAM. The results from aspirated samples were not used any further, because of the possible blood contamination.

Relationship between drug concentrations in blood and vitreous humor

The blood/VH concentration ratio for morphine according to the fitted curve was 160 after 1 h, 7 after 2 h and decreased to approximately 1 after 6 h (Fig. 2c, f). The blood/VH concentration ratio for 6-MAM according to the fitted curve was 64 after 1 h, 1 after 2 h and ≤ 1 for the remaining experimental period (Fig. 2b, e). The blood/VH concentration ratio for M3G was >100 for the total experimental period (Fig. 2d, g).

Discussion

This study described the transfer of heroin metabolites into VH. To our knowledge, this is the first pharmacokinetic study on heroin metabolites in both blood and VH in the living animal. We observed a slow distribution from blood to VH for all of the heroin metabolites studied; heroin, however, was not detected in the VH. Importantly, the interval when 6-MAM can be detected was much longer in VH as compared to that in blood.

The strength of the present study is the tracing of the concentrations of heroin metabolites in VH in detail in a living animal. This was made possible by the use of the microdialysis technique, without regular aspiration demanding a large number of animals and incurring a high probability of blood contamination of the samples. Macha and Mitra [30] stated that microdialysis was superior to direct sampling techniques and could be applied to study ocular drug pharmacokinetics without rendering considerable physiological changes to the ocular barriers and the intraocular environment. We chose the pig model because it resembles the human being in both size and physiology [23, 31], and we found that the blood pharmacokinetics were comparable to data previously published on humans [6]. From the literature, it has also been indicated that glucuronidation and esterase-mediated hydrolysis in pigs is expected not to differ substantially from those of humans

[24, 25, 32]. One weakness of this model is the lack of formation of M6G, which is also used in the interpretation of postmortem cases. There was also some inconsistency in the experimental period with sampling from the living animal, because it was prolonged as compared with the handling of postmortem samples, after we discovered the slow transfer to VH in the first pig. In the first pig, where postmortem samples were also collected for 150 min after death, the blood/VH concentration ratios of 6-MAM, morphine, and M3G continued to fall during the post-mortem period (data not shown). This needs to be studied in more detail. The results in the present study were quite consistent among the animals, with only one pig showing higher concentrations of 6-MAM and M3G in VH (shown by dark blue circles in Fig. 2e, g), but not in blood (Fig. 2b, d). These findings might reflect the fact that individual differences in penetration could be expected, but their prolonged detection times in VH were still to be expected.

Our results are in accordance with the limited previous studies on kinetics of drugs of abuse in VH. In rabbit models, equilibrium between blood and VH for ethanol after its i.v. administration was attained after 10–20 min [14], for diazepam 60–120 min after its i.m. administration [15], and for MDMA 30 and 120 min after its i.m. administration [16]. Crandall et al. [17] reported that morphine appeared in the aspirated VH 20 min after i.v. administration of a fatal dose (about 120 mg) of morphine using a pig model. However, in our experience, it was difficult to avoid blood contamination of the aspirated VH samples, and we believe that it cannot be excluded that the results presented by Crandall et al. [17] are influenced by blood contamination. We also observed early and high levels of both morphine and M3G in our aspirated blood-contaminated VH samples (data not shown).

Although the pharmacokinetics of opioids in VH *in vivo* has not previously been studied in detail, a number of previous studies have investigated on the postmortem relationship between blood and VH concentrations for different drugs. Our results could explain the findings in two previous studies regarding heroin metabolite ratios of blood to VH. Bogusz et al. [12] compared concentrations in femoral blood versus VH in 12 human cases where deaths were attributed to intoxication with heroin. The concentrations of morphine were generally lower in VH than in blood, and they found a mean ratio of blood/VH of 4.2 for morphine; 6-MAM was most often not detected, or only trace amounts were found in either blood or VH (LOD 0.5 ng/mL). They concluded that lower concentrations of morphine and its glucuronides in VH as compared to those in femoral blood might reflect a slower distribution of drugs to VH, which was verified in our study (Fig. 2c, d, f, g) [12]. Wyman and Bultman [13] investigated 25 autopsy

cases identified as heroin exposures. They also found concentrations of morphine higher in femoral blood than in VH, with a mean blood/VH concentration ratio of 3.5. For 6-MAM, however, they found that its concentrations in VH, in general, were higher than in femoral blood, with a blood/VH concentration ratio of 0.2. They concluded that the possibility of detecting 6-MAM in postmortem specimens could be greatly improved when VH was included as a specimen for analysis in such cases [13]. This conclusion was strengthened by the experimental data in the present study (Fig. 2b, e), because the concentration of 6-MAM in VH exceeded the one in blood at a relatively early time point for 6-MAM, as compared with morphine as shown in Fig. 2b, e.

The present results raise questions about the physiology of transfer of drugs from blood into VH. The entry of systemic administered drugs into the VH is prevented by different barriers, e.g., the blood-aqueous barrier and the blood-retina barrier (BRB) [22, 33]. The penetration of drugs through membranes, including the BRB, depends on their size, shape, charge, and lipophilicity [34, 35]. The flow is further governed by hydrostatic and osmotic pressure, as well as the concentration differences across the membrane [34]. The differences in penetration into VH observed between drugs have been attributed also to protein-binding of the drugs studied [36]. VH is low in proteins, with only 1–3 % of the total serum protein concentration [16], and we assume that there are no relevant differences between humans and pigs in this regard. The protein binding of morphine in pig and human plasma does not show significant differences [37].

As seen in Fig. 2, a delay in diffusion was observed for all of the opioids under study, and especially for 6-MAM, its concentration in VH still increased after the concentration in blood became very low (Fig. 2b, e). Even though the mechanisms for this finding are not fully understood, some theories can be launched. It has been suggested that the cornea may act as a reservoir for drugs [21]; furthermore, some drugs might have an affinity for melanin pigment present outside the retina [38–40], possibly both delaying and prolonging the distribution of drugs into different compartments of the eye (for reviews see Gaudana et al. [41] and Bévalot et al. [42]). In a study by Pitkanen et al. [40], the most lipophilic beta blockers showed the highest permeability coefficients through the outer BRB; moreover, the longest VH diffusion time (permeation lag time) was seen for the most lipophilic drugs. They also suggested that this might be due to an increasing drug-melanin binding with increasing lipophilicity, because these drugs showed the greatest binding to melanin [39]. Whether the theories mentioned above also apply to the opioids in this study is only speculative; however, the binding of opioids to melanin in hair

has been documented [43]. Regarding the prolonged detection times for 6-MAM, it was previously suggested that esterases in VH showed lower activities and were more saturable as compared with those in blood [44, 45]. The mechanisms behind the decline in concentrations of morphine in VH have not previously been described, but it should be noted that prolonged detection times were observed for all of the metabolites.

How can our results assist in the practical interpretation of postmortem cases, considering the fact that drug concentrations in VH are expected to be subject to fewer postmortem changes [36, 46]? If the approximate time frame between intake and death is known, the true antemortem blood concentration could be calculated based on the concentration in the VH. Often, a very high blood concentration of morphine is seen together with a low concentration in VH, which might represent the true antemortem situation if death occurred within the first hour after intake of heroin; however, if some hours had elapsed, which is often the case with heroin deaths, the true antemortem blood morphine concentration would be expected to be closer to or lower than the morphine concentration observed in the VH.

Based on the final measured concentrations and the half-lives of 6-MAM, in the present study, the expected detection time for 6-MAM in VH could be more than 20 h in a living subject. Assuming limited postmortem changes in VH [36, 46], the detection of 6-MAM in VH postmortem could indicate that death occurred as many as 20 h or more after an intake of heroin. The dose and the LLOQ may, of course, influence this estimate. In postmortem cases, a negative 6-MAM in blood together with a positive 6-MAM in VH is often observed. In light of our findings, this could be explained by the considerably longer detection times for 6-MAM in VH as compared to those in blood. Because the present study also indicated longer detection times for morphine in VH, as compared to those in blood, it can be assumed that the detection times for other drugs are also longer in VH, as compared to those in blood.

It has previously been reported that a high blood/VH concentration ratio of drugs suggest rapid death [42]. The results from the present study could argue that the detection of 6-MAM or morphine in VH indicates that death did not occur within the first hour after intake of heroin. This finding is, however, complicated by the fact that heroin is often used on a daily basis, and therefore, detections in VH could be caused by intake, for instance, on the day before.

Conclusions

To our knowledge, this is the first report dealing with a pharmacokinetic study on heroin metabolites in VH using a living pig model. The pig resembles the human in both size

and physiology except from minor metabolic species differences. To get pharmacokinetic data from VH samples, the present microdialysis sampling using the pig model is most recommended. Such data of heroin metabolites obtained from the blood and VH samples collected from living pigs gave an important clue for interpretation of antemortem heroin poisoning in human victims by the postmortem analysis of heroin metabolites in both blood and VH samples.

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Compliance with ethical standards

Conflict of interest There are no financial or other relations that could lead to a conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines were followed for the care and use of the animals. This study did not involve samples collected from human participants.

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