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ORIGINAL ARTICLE

Postmortem redistribution of the heroin metabolites morphine and morphine-3-glucuronide in rabbits over 24 h

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Abstract The interpretation of postmortem drug levels is complicated by changes in drug blood levels in the postmortem period, a phenomena known as postmortem drug redistribution. We investigated the postmortem redistribution of the heroin metabolites morphine and morphine-3-glucuronide in a rabbit model. Heroin (1 mg/kg) was injected into anesthetised rabbit; after 1 h, an auricular vein blood sample was taken and the rabbit was euthanised. Following death rabbits were placed in a supine position at room temperature and divided into three groups namely (1) immediate autopsy, (2) autopsy after 30 minutes and (3) autopsy 24 h after death. Various samples which included femoral blood, cardiac blood, lung, liver, kidney, vitreous humour, subcutaneous and abdominal fat, liver, bone marrow and skeletal muscle were taken. The samples were analysed with a validated LC-MS/MS method. It was observed that within minutes there was a significant increase in free morphine postmortem femoral blood concentration compared to the antemortem sample (0.01±0.01 to 0.05±0.02 mg/L).Various other changes in free morphine and metabolite concentrations were observed during the course of the experiment in various tissues. Principal component analysis was used to investigate possible correlations between free morphine in the various samples. Some correlations were observed but gave poor predictions (>20 % error) when back calculating. The results suggest that rabbits are a good model for further studies of postmortem redistribution but that further study and understanding of the phenomena is required before accurate predictions of the blood concentration at the time of death are possible.

Keywords Postmortem redistribution · Morphine · Morphine-3-glucoronide · Animal model · Heroin · Rabbit

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Introduction

Drug overdoses are one of the biggest killers in the UK and USA with opiates being the largest group of drugs involved in overdoses [1]. Heroin (3,6-diacetylmorphine) and its metabolite morphine are the opiate drugs that are of greatest concern with an estimated 13–18 million people abusing them worldwide [2]. Following administration, heroin is rapidly deacetylated to 6-monoacetylmorphine (6-MAM) with a t½ of ~5 min [3]. 6-MAM is then rapidly metabolised to morphine (t½ 5–40 min) [3], with morphine being further metabolised to either the inactive metabolite morphine-3-glucuronide (M3G) or to the active metabolite morphine-6-glucuronide (M6G) [3]. Following unexplained deaths, forensic toxicologists and forensic pathologists are required to interpret the level of drugs detected in postmortem samples in order to determine the possible influence, if any, of the drug causing death; however, the interpretation of postmortem drug levels has been described as a 'toxicological nightmare' due to postmortem redistribution [4]. Postmortem drug redistribution is a term used to describe drug concentration changes in the body following death, mainly thought to be due to passive diffusion [5]. In this phenomenon, the concentration of drugs

in the blood and other tissues can change up to 15-fold after death [6]. It is thought that all drugs will be affected to some extent by postmortem redistribution and the main contributing factors are (1) the time between death and sampling, (2) the site(s) of sampling (with femoral blood being the sample that is least affected by postmortem redistribution), (3) potential postmortem metabolism/production by either body enzymes or bacteria and finally, (4) physicochemical properties of the drug (such as pKa, LogP and most importantly volume of distribution (Vd)) [5]. According to Hilberg et al. [7], all molecules with a Vd equal to or greater than 3 L/kg are liable to undergo postmortem redistribution. However, there are some molecules which appear to be an exception to this rule such as mirtazapine a drug with a Vd of ~5 L/kg has not been shown to exhibit any postmortem redistribution [8]. If the mechanisms governing postmortem redistribution could be defined, this would help the interpretation of postmortem drug levels.

Although previous studies have been carried out investigating the postmortem redistribution of morphine and metabolites in animal models [9–11], no studies have investigated the postmortem redistribution of the heroin metabolites morphine, M3G and M6G from heroin. The rabbit model was chosen as unlike rats, rabbits have a gall bladder [12] allowing morphine to undergo enterohepatic recirculation as would be found in humans. Rabbits also have vitreous humour that has similar chemical characteristics to those found in man [13], vitreous being an important matrix for the determination of the use of heroin [14]. Recent advances in analytical technology also allow reduced matrix volumes to be analysed [15] negating some of the concerns that have been raised about using smaller animal models in the past [11].

The aim of this work was to study the influence of both time and site on the postmortem redistribution of 6-MAM, morphine and M3G in rabbits, following intravenous injection in order to determine if the rabbit is a suitable model for investigating postmortem redistribution and also a good model for the study of the mechanisms of postmortem redistribution.

Materials and methods

Chemical reagents

All reagents and solvents were of analytical grade. Diamorphine hydrochloride (Wockhardt) was purchased from L E West Ltd (Barking, UK). Sodium carbonate, ethyl acetate, butyl acetate, ammonium carbonate and acetonitrile (all of analytical grade) were supplied by Fisher Scientific (Loughborough, UK). Horse plasma was supplied by TCS Biosciences (Buckingham, UK) and SAGM blood obtained from Ninewells Hospital, Dundee (ethical approval for use was obtained from local ethics committee). Blank samples of the following were purchased from local butchers: porcine vitreous humour, porcine liver, bovine muscle, bovine bone marrow, porcine heart, porcine lung, porcine kidney and bovine fat. Sterile potassium chloride concentrate 15 % *w*/*v* BP was supplied by B Braun (Melsungen, Germany).

Standards, calibrators and control preparation

Stock solutions of 6-mononacetylmorphine (6-MAM), morphine, morphine-3β-D-glucuronide (M3G), morphine-6-β-D-glucuronide (M6G), morphine-d3 (Md3) (all 1 mg/ml) and morphine-3-glucuronide-d3 (M3G-d3) (100 mg/L) were supplied by LGC Standards (Teddington, UK). Morphine, M3G and M6G stock solutions were used to prepare standard solutions at a concentration of 25 mg/L in methanol. Md3 and M3G-d3 solutions were used to prepare the internal standard solutions at a concentration of 1 mg/L in water. All stock solutions were stored in amber vials at 4 °C. The standard solutions were used to prepare a calibration curve range of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 mg/L in plasma along with low quality control (LQC) (0.025 mg/L) and high quality control (HQC) (0.5 mg/L) standards.

Study design

New Zealand ex breeder white rabbits (Harlan, UK) with a mean weight (± SEM) of 4.26±0.13 kg were anaesthetised by inhalation of 3–5 % isoflurane with oxygen, (anaesthesia was maintained throughout the procedure). Following anaesthetisation, rabbits were administered i.v.1 mg/kg of diamorphine (prepared as 2 mg/ml stock in saline) and via the left auricular vein. One hour after the administration of the diamorphine, an antemortem blood sample was taken from the right auricular vein. Immediately following the sampling, the rabbit was euthanised with 1 ml i.v. potassium chloride (15 % *w*/*v*), and death was confirmed by the lack of a heartbeat. The potassium chloride was estimated to only alter the circulating blood volume by ~0.4 % based on rabbit total blood volume data [16]. Following death, the rabbit was placed in a supine position and kept at room temperature. The rabbits were then divided into one of the three groups: (1) immediate autopsy, (2) autopsy 30 min after death and (3) autopsy 24 h after death. Each group comprised of three rabbits with the sample size based on previous animal studies of postmortem redistribution [17–20]. Time points were selected in order to look at rapid postmortem changes and the effects of postmortem changes over 24 h. The experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. As far as the authors are aware, there is no pharmacokinetic data on the heroin metabolite morphine following heroin administration in rabbits; for this reason, we based the length of time between heroin administration and animal euthanasia on the morphine pharmacokinetics in a

rabbit [21]. In order to allow maximum possible distribution whilst still having a measurable amount of morphine in the blood, we selected a time of 1 h between injection of the morphine and euthanasia.

Sample collection

All tissue samples were stored in Sterilin 20 ml tubes, and all fluid samples (vitreous and blood) were stored in 5 ml ISS 2.5 % sodium fluoride/potassium oxalate tubes.

Femoral blood—the abdomen was opened along the midline sagittal plane, and the inferior vena cava, descending aorta and left and right femoral veins were exposed. Mixed femoral venous blood (left and right) was drawn via syringe using a 19G×1½″ (1.1×40 mm) needle after clamping the vena cava immediately below the renal artery. Lung—upper right lobe was wiped clean with tissue paper and then excised. Mixed cardiac—retrieved by removing the heart from the animal and sampling the blood that pooled in the chest cavity. Heart—after removal of the heart, the heart was wiped clean with tissue paper; the left free wall and septum were excised and dried with tissue paper. Right kidney—exposed and excised, and the renal fascia and fat layer removed. The kidney was then cleaned with tissue paper. Vitreous humour—extracted from both eyes using 19G×1½″ (1.1×40 mm) needle. Subcutaneous fat—the skin was separated from the underlying muscle above the right flank to reveal the subcutaneous fat pad and the sample was excised. Abdominal fat—a sample of fat was excised from the abdominal cavity. Liver—wiped clean and the right lobe of the liver was excised and then cleaned again with tissue paper. Bone marrow—mix extract from the left femur, tibia and fibula bones. Skeletal muscle—the left thigh muscle was exposed and approximately 5 g excised. After collection, all samples were stored at −20 °C until analysed.

Sample preparation

Tissue sample homogenates were prepared according to a previous published procedure by Flanagan et al. [22]. Tissue samples were homogenised with an Ultra-Turrax T25 homogeniser (IKA, Janke& Kunkel, Germany). In brief, 1–3 g of tissue was weighed accurately and homogenised in 4 volumes of deionised water. For fat/bone marrow, homogenisation was with two parts of methanol and four parts of hexane. The methanol/hexane homogenates were then centrifuged, and the top hexane layer discarded.

Sample extraction

The sample extraction was based on a previously published method by Taylor and Elliott [15].

For biological samples, quality controls and calibrators, 150 μL was diluted with 150 μl equine plasma. Fifty microlitre of M-d3 and 50 μl of M3G-d3 internal standard (IS) solutions were then added to each sample. One millilitre of 0.5 M ammonium carbonate solution was added to each sample and vortexed.

Solid phase extraction (SPE) was performed using a Varian Bond Elut LRC-C_{18}, 200 mg cartridges (Agilent, Wokingham, UK). The cartridges were conditioned using 2 ml of methanol followed by 2 ml of water and finally 1 ml of 0.5 M ammonium carbonate solution. Then, 1 ml of either biological sample, quality control or calibrator was loaded onto the SPE cartridges and allowed to drain to waste. The cartridges were then washed using 5 ml of 0.005 M ammonium carbonate solution and allowed to dry for 5 min. One millilitre of 70:30 ACN:H_2O solution was then loaded onto the cartridges and allowed to elute into a 7-ml clean glass vial. The eluent was collected and evaporated to dryness under air at 45 °C and reconstituted with 100 μl of freshly made LC-MS mobile phase (96 % Phase A: 4 % Phase B). Finally, the entire sample was transferred to an LC-MS vial for analysis.

Instrumental and chromatographic conditions

Qualitative LC-MS-MS analysis of 6-monoacetylmorphine, morphine, morphine-3-glucuronide and morphine-6-glucuronide

Qualitative analysis for 6-MAM, morphine, M3G and M6G was performed using an ABSciex 3200 QTRAP coupled to an Agilent 1200 series HPLC system consisting of a quarternary pump, degasser and an autosampler (Warrington, UK). The injection volume was 20 μl. Quantitative analysis was based on gradient elution. With the following mobile phases: mobile phase A, 1 ml of 1 mM ammonium formate; 1 ml of formic acid and 998 ml distilled water. Mobile phase B, 1 ml of 1 mM ammonium formate; 1 ml of formic acid; 499 ml of distilled water and 499 ml of acetonitrile. The method had a flow rate of 0.6 ml/min the gradient elution started at 97 % phase A and 3 % phase B held for 3 min and was then ramped to 5 % phase A, 95 % phase B over 5 min, held for 3.5 min and then reduced to 97 % phase A, 3 % phase B over 0.5 min. The overall run time was 15 min. The column used for analysis was Phenomenex Synergi 4 μm Polar-RP 80A column (150 mm×2 mm×4 μm) protected by a Phenomenex Security Guard column (Macclesfield, UK). A column temperature of 40 °C was used throughout. Pure standards of each analyte were first infused into the mass spectrometer to determine the most intense ion transitions that were consequently used for compounds identification (and quantitation) applying an MRM scan. The MRM transitions selected for identification were 6-MAM—328/328 and 328/193, morphine—286/286 and 286/165, M3G—462/462 and 462/286, M6G—462/462 and 462/286.

Quantitative LC-MS-MS analysis of morphine, morphine-3-glucuronide and morphine-6-glucuronide

Quantitation was based on the liquid chromatography with mass spectrometry (LC-MS-MS) method of Taylor and Elliott [14]; analysis was isocratic, (97 % phase A, 3 % phase B) with a run-time of 5 min using an ABSciex 3200 QTRAP MS/MS. The scan was performed in positive mode using ion spray ionisation (voltage 5500 V). Ion source temperature was set at 700 °C, DP 20 V, EP 10 V. The MRM transitions selected for quantitation were the following, for morphine 286/165 (against Md3 289/152), for M3G and M6G 462/286 (both against M3G-d3 465/289), the MRM scan was run in unscheduled mode.

Method validation and matrix effects

The method was validated for all samples being investigated (blood, vitreous humour, liver, muscle, bone marrow, heart, lung, kidney and fat) according to the previously published guidelines of Peters et al. [23]. The calibration curves for morphine, M3G and M6G were linear (R^2>0.99) with a 1/x weighting factor on seven points. Quality control samples of 0.025 mg/L and 0.5 mg/L were used. According to validation results, the limit of detection (LOD), calculated based on a signal to noise ratio of 3:1, for morphine, M3G and M6G were 0.004, 0.003, and 0.004 mg/L, respectively, and the limit of quantitation (LOQ) based on a signal to noise ratio of 10:1 was 0.01 mg/L for all analytes. Accuracy and bias (both inter-day (n=5) and intra-day (n=30) were within the acceptable ranges (±15 %) apart from heart (27 %). The matrix effects of morphine, M3G and M6G were evaluated by the methods of Matuszewski et al. [24] at both 0.025 and 0.5 mg/L. The concentrations of the solid matrices were calculated according to the method of Flanagan et al. [22]. Morphine, M3G and M6G results for QC samples in all biological matrices were within ±20 % of the expected concentration apart from heart>±27 %.

Statistical analysis

The intra-day precision for each compound was assessed by measuring samples spiked with standard mixture composed all analytes three times a day, whilst the inter-day precisions were evaluated once a day on three consecutive days. For validation, the inter-day and intra-day accuracy and precision were calculated using one-way ANOVA with day as the grouping variable. The principal component analyses were constructed using Minitab (version 16).

A note on terminology used in the study

Free drug concentration In pharmacological studies, the term "free" drug refers to the unbound drug concentration that not bound to plasma proteins [25]. However, in postmortem studies involving morphine, it is common to refer to free morphine as the concentration of unconjugated morphine quantitated with total morphine referring to the concentration of "free" morphine and conjugated morphine.

Results and discussion

In this study, we investigated the effect of postmortem interval on the postmortem distribution and redistribution of heroin and its metabolites, (6-MAM, Morphine and M3G) in various fluids (antemortem blood, postmortem femoral blood, postmortem mixed cardiac blood and vitreous humour) and tissues (liver (right lobe), left cardiac muscle, bone marrow, lung (right apex), kidney (right), abdominal fat and subcutaneous fat) following intravenous injection.

The samples were selected as ones that were the least likely to be affected by postmortem redistribution based on data from previous studies [26]. As expected from previous rabbit studies, M6G was not detected as rabbit metabolism favours the glucuronidation of morphine in the 3 rather than the 6-position [27]. This is a major limitation of the study but still allows the investigation of the redistribution of both morphine and M3G. Further studies would need to be carried out to investigate the postmortem redistribution of oral ingestion of morphine.

Statistical analysis of results

The results obtained for biological matrices and animals underwent statistical analysis applying the 'Principal Component Analysis' (PCA). PCA is an unsupervised multivariate procedure which is a well-known linear data compression and feature extraction technique [28]. It derives new, uncorrelated variables that are linear combinations of the original variable set ordered by reducing variability. PCA is mainly used to reduce the dimensionality of a data set whilst retaining as much information as possible by eliminating the lowest-ranking variables. It is a simple and fast method but remains a linear approach, so any nonlinear correlation between variables will not be retained. The scores produced may be plotted in two or three dimensions to inspect the data. Therefore, it might be possible to relate the data using statistical methods such as principal component analysis (PCA). Statistical analysis showed that there are limited correlations between triplicate rabbits for the same time point, hence showing the increased complexity of dealing with animal models as they are all individual with varied metabolism. PCA (Fig. 1) carried out on morphine data at the three time points (T=0 min, T=30 min and T=24 h), shows that there are some linear correlation between antemortem blood and bone marrow, abdominal fat, cardiac blood and femoral blood at T=0 and 30 min. At

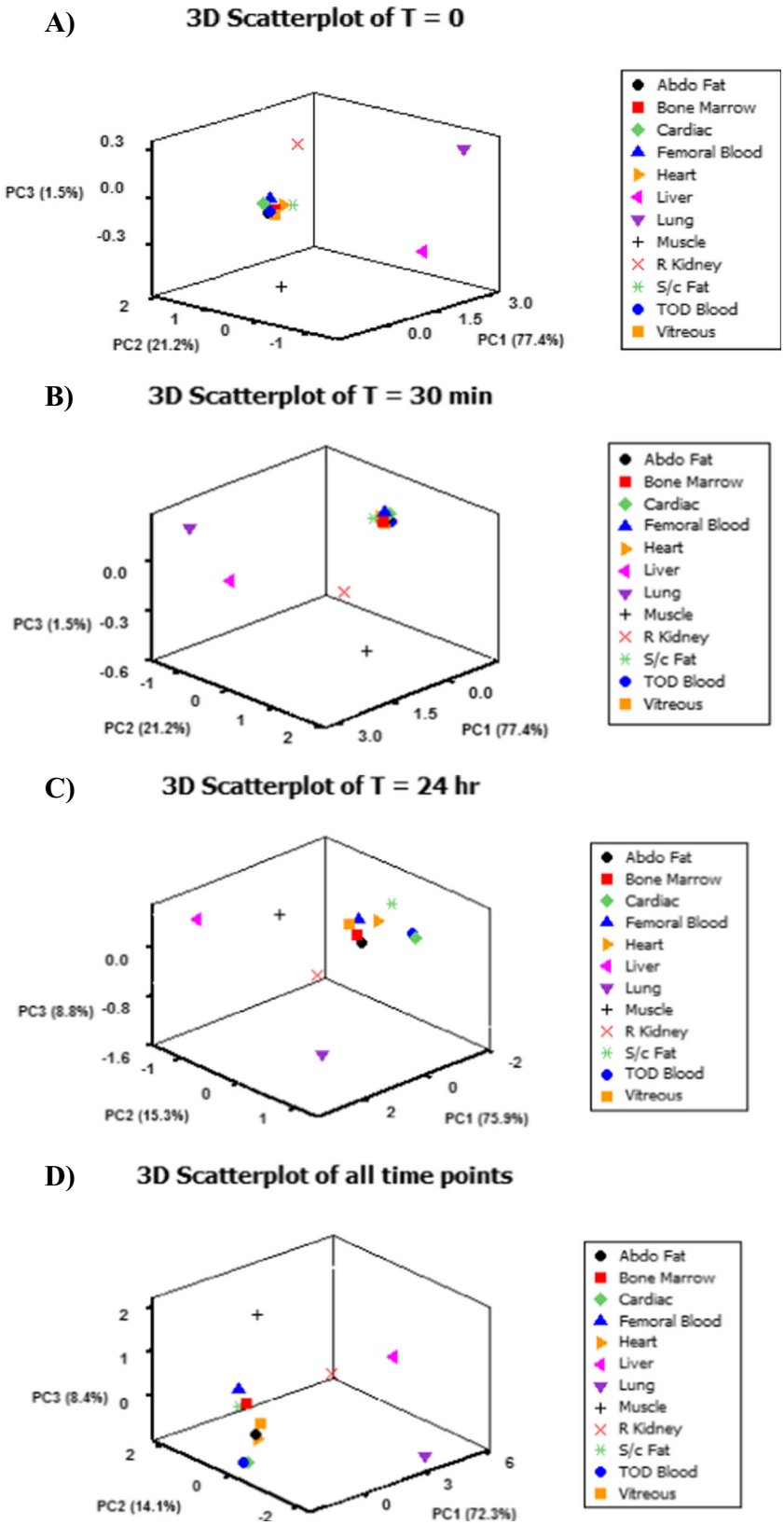

Fig. 1 3D Scatter Plot for free morphine concentration for all biological matrices analysed. **a** Immediate postmortem. **b** Postmortem after 30 min **c** Postmortem after 24 h. **d** All Postmortems combined

24 h, only cardiac blood shows some linear correlation. When data at all-time points were analysed, it was observed that only cardiac blood had some linear correlation to time of death blood (T=0). Although these correlations looked promising,

predictions were poor with over 20 % errors in predicted values. This highlights the complexities of interpreting postmortem toxicology results.

Detection of 6-MAM in rabbit tissues

6-MAM is considered the most reliable biomarker for confirmation of use of heroin. Our results (as shown in Table 1) show that 6-MAM was detectable in all antemortem samples. This was to be expected as studies on the pharmacokinetics of heroin in humans have shown that 6-MAM has a t½ of ~40 min before metabolism to morphine [29]. 6-MAM was only detected in two matrices throughout the 24 h duration of the experiment, vitreous humour and muscle. These are matrices which have previously been found to be particularly good for the investigation of 6-MAM in postmortem toxicological investigations as they lack esterase enzymes which continue to metabolise 6-MAM even after death [30, 31]. Heart, liver, lung and kidney were the tissues that proved to be the least useful for the confirmation of heroin after death as at best only 1/3 of samples were positive for 6-MAM after death. These matrices have previously been shown to have high levels of carboxylesterase one of the enzymes responsible for the metabolism of 6-MAM [32]. 6-MAM in bone marrow appeared to be stable for between 30 min to 24 h with only 1/3 of samples positive at 24 h. These results suggest that 6-MAM is not as stable in rabbit bone marrow as other species (rats and human), where 6-MAM has been shown to be detectable up to 2 months after death. The reason for these differences are currently unclear [33, 34]. 6-MAM levels appeared to be stable in femoral blood (100 %) and mixed cardiac rabbit blood (2/3) over 24 h. Human studies indicate that 6-MAM is unstable in femoral blood as observed in eight cases in which femoral blood was taken on admission of the body to the mortuary, and then at autopsy (an average of 64 h later), there was a decrease in mean 6-MAM levels from 0.031 to 0.015 mg/L. As we did not quantitate 6-MAM levels and also due to the differing time scales of sampling between the human study and this study, it is not possible to compare this study with the human data. However, a study in horse blood (which has a similar esterase make up to rabbits (containing both Acetylcholine esterase (AChE) and carboxylesterase (CES) rather than humans which only contain AChE) has been compared with human blood. In horse blood, it was found that 6-MAM was more stable than in human blood with identical conditions [35]. It is unclear to why there are differences, but it could be due to differing enzyme kinetics and enzyme expression patterns. These data re-enforce the previous recommendations that vitreous is the matrix of choice for the postmortem detection of 6-MAM and in cases without vitreous, muscle should be the sample of choice for the determination of heroin use.

Table 1 Frequency of positive results for 6-monoacetylmorphine (6-MAM) expressed as a percentage

Tissue	At death	0.5 h Postmortem	24 h Postmortem
Antemortem blood	100	100	100
Femoral blood	100	66.6	100
Mixed cardiac blood	66.6	66.6	66.6
Vitreous	100	100	100
Liver	33.3	0	0
Muscle	100	100	100
Bone marrow	100	100	33.3
Heart	33.3	0	0
Lung	33.3	0	33.3
Right kidney	0	0	33.3

Antemortem morphine and morphine-3-glucuronide levels

The ability to accurately calculate the antemortem value of a drug at death from postmortem data would be the ideal scenario in forensic toxicology as standard pharmacokinetic equations could then be used to calculate the dose of drug taken or administered and clinical data could be used to asses potential toxicity. For this reason, immediately prior to death, an antemortem blood sample was taken with which to compare matrices sampled postmortem. Our results, as expected from previous studies [11] showed a noticeable but non-significant variation between the individual antemortem samples between rabbits, (0.01 to 0.139 mg/L for free morphine and 0.08 to 0.672 mg/L for M3G). This variation was also seen when looking at total morphine values (morphine+ M3G) with values of 0.106 to 0.690 mg/L. These results also make it likely that the high variation in postmortem morphine values may not just be a postmortem artefact but due to individual variances found in life [11].

The postmortem femoral blood sample is considered to be the sample that is least affected by postmortem redistribution [5]. However, both human [36] and animal studies [9] have shown that a rise in free morphine is observed when comparing antemortem to immediate postmortem sample. This was mirrored in our study where the free morphine concentration of the femoral blood sample taken immediately after death was found to be at a higher concentration compared to the antemortem sample (mean 0.05 and 0.01 mg/L, respectively). It is thought that the rapid change in free morphine is due to rapid changes in blood pH [37] (cardiac blood 7.34±0.02 to 6.74±0.05 within 5 min [9]) in the body. It is a limitation of this study that due to small sample volumes it was not possible to measure the pH of the postmortem blood samples. pH changes will alter the ionisation state of the morphine molecules allowing greater diffusion and thus increased changes

along any potential concentration gradient [9, 38]. pH changes in the postmortem environment apart from changes in ionisation states may also alter the plasma protein binding of drugs (such as morphine) due to changes in protein structure and protein denaturation increasing the amount of morphine available to freely diffuse in the postmortem environment [39].

Postmortem morphine levels

We wanted to investigate the possible concentration changes of morphine over time in the postmortem period. In order to minimise the variability between the differing animals, the postmortem concentrations of free morphine were normalised to the antemortem blood concentration for each animal (the free morphine levels are shown in Table 2). The changes in normalised free morphine concentration are shown in Table 3 and Fig. 2. In the postmortem period, mixed cardiac blood, vitreous and abdominal fat were the three matrices in which the largest concentration changes of free morphine were observed over 24 h with increases of +432, +181 and +154 %, respectively. Intermediate changes (~30–42 % change) were observed over 24 h with bone marrow, heart and right kidney free morphine concentrations all increasing and muscle and subcutaneous fat free morphine concentrations both decreasing; finally, there were minor free morphine concentration changes for femoral blood, liver, and lung (less than 10 % change in concentration). Investigations of the phenomena of postmortem redistribution in animal models have demonstrated that changes in drug concentration can occur in as short a time period as 5 min [11]. Reasonably, rapid changes in free morphine concentrations were observed in our studies, as 30 min after death changes in free morphine concentration were observed, mainly decreases of between −30 and −71 %. In our study, the only two matrices in which increases in free morphine concentration were observed were femoral blood (+26 %) and abdominal fat (minimal change of +2 %). The postmortem redistribution of morphine has been studied in humans and animals but has not previously investigated the postmortem temporal changes of metabolites from heroin. However, these studies do show similar results in free morphine changes to those that we have obtained in this study following the injection of heroin. Studies using animal models to investigate free morphine changes have given mixed results; we observed an increase in free morphine cardiac blood concentrations over 24 hr (+432 %). This is similar to rat studies [9] and [10] in which +68 and +270 % increases in free morphine concentrations changes were observed, but this was in contrast to studies in pigs [11] in which a decrease of 8.2 % in free morphine was seen in left ventricular blood. Limited data is available from humans on the time course of free morphine changes postmortem; in one study of 11 cases, postmortem femoral blood was taken on mortuary admission and then again at autopsy (average 64 h); this result showed a slight decrease in the free morphine concentration (admission 0.262 mg/L, autopsy 0.253 mg/L) [40]. In a slightly larger study in 32 deaths, left ventricular blood and femoral blood were sampled as soon after death as possible (between 3–144 h) and then at autopsy (3–43 h later). In most cases, no significant changes in either femoral or left ventricular blood free morphine concentrations were observed over time. However, increases were observed in individual cases, mainly in cases in which higher initial free morphine concentrations were observed. On average, the concentration of free

Table 2 Concentration values of free morphine and morphine-3-glucuronide (M3G) by sampling site and time of postmortem interval

	Free morphine				Morphine-3-glucuronide			
	Postmortem interval (h)				Postmortem interval (h)			
	0	0.5	24	Mean	0	0.5	24	Mean
Control antemortem blood (mg/L)	0.01±0.00	0.05±0.04	0.01±0.00	0.02±0.01	0.34±0.03	0.28±0.20	0.30±0.05	0.31±0.04
Postmortem femoral blood (mg/L)	0.05±0.02	0.13±0.06	0.04±0.02		0.45±0.14	0.49±0.27	0.07±0.03	
Postmortem mixed cardiac blood (mg/L)	0.02±0.00	0.05±0.04	0.07±0.04		0.20±0.01	0.21±0.07	0.10±0.03	
Vitreous (mg/L)	0.03±0.00	0.04±0.02	0.06±0.01		0.01±0.00	0.02±0.01	0.12±0.03	
Liver (mg/kg)	0.28±0.08	0.38±0.25	0.26±0.05		0.48±0.02	0.41±0.28	0.10±0.01	
Muscle (mg/kg)	0.27±0.17	0.16±0.10	0.11±0.02		0.13±0.02	0.04±0.02	0.09±0.02	
Bone marrow (mg/kg)	0.04±0.01	0.10±0.04	0.04±0.01		0.05±0.01	0.05±0.02	0.04±0.01	
Heart (mg/kg)	0.03±0.00	0.10±0.06	0.04±0.01		0.44±0.12	0.35±0.11	0.20±0.04	
Lung (mg/kg)	0.50±0.02	0.74±0.60	0.36±0.26		0.81±0.08	0.52±0.11	0.42±0.10	
Right kidney (mg/kg)	0.63±0.37	1.01±0.63	0.51±0.30		3.86±1.30	3.06±1.09	1.30±0.40	
Abdominal fat (mg/kg)	0.02±0.00	0.03±0.01	0.03±0.01		0.03±0.01	0.06±0.02	0.02±0.00	
Subcutaneous FAT (mg/kg)	0.06±0.01	0.07±0.05	0.04±0.02		0.06±0.01	0.06±0.05	0.04±0.02	

Values are mean±SEM (n=3)

Table 3 Changes in normalised free morphine and M3G concentration ½h postmortem and 24 h postmortem

Tissue	Free morphine		Morphine-3-glucuronide	
	% Change (0.5 h after death)	% Change (24 h after death)	% Change (0.5 h after death)	% Change (24 h after death)
Femoral blood	+26	−4	+77	−79
Mixed cardiac blood	−55	+432	+113	−46
Vitreous	−51	+181	+161	+1,002
Liver	−61	6	+295	−75
Muscle	−55	−42	+22	−19
Bone marrow	+42	+35	+89	−4
Heart	−14	+36	+171	−51
Lung	−71	−1	+66	−44
Right kidney	−41	+27	+63	−63
Abdominal fat	+2	+154	+273	−36
Subcutaneous fat	−30	−32	−34	−34

All samples were normalised to the antemortem sample

morphine in left ventricular blood samples was significantly higher than femoral blood samples. When looking at multiple studies, human cardiac concentrations of free morphine have been found to be higher than those observed in femoral samples [41]. Our study differed in the early postmortem period. We observed an initial drop of free morphine concentration after 30 min (−55 %). This was similar to the observation of Schmidt et al. [42] who reported an ~30 % decrease in postmortem free morphine concentration, compared to the antemortem free morphine concentration after 12 h in a rabbit model. Further comparisons with the data from this research are not possible as no detailed data was given. Postmortem falls in free morphine drug concentration could have various explanations: (1) stability of the drug (unlikely as morphine has been shown to be stable in long term storage conditions) [43], (2) enzymatic and bacterial degradation [44] or (3) diffusion. However, diffusion is thought to be the most likely explanation in postmortem free morphine changes. The

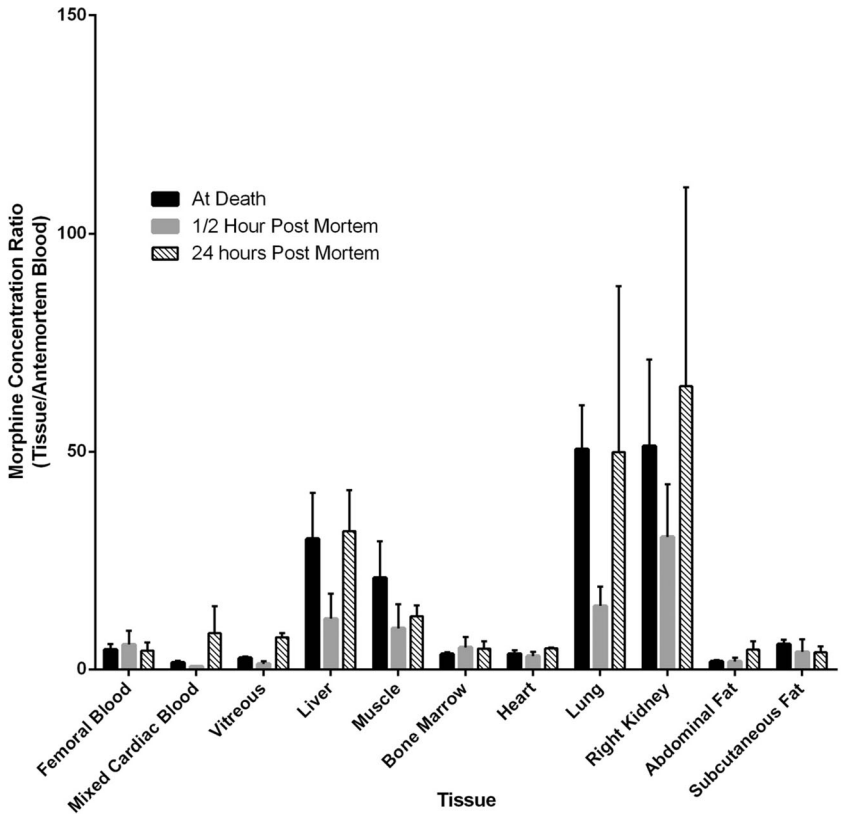

Fig. 2 Comparison of free morphine concentration in freshly sacrificed, 0.5 h postmortem and 24 h postmortem rabbits

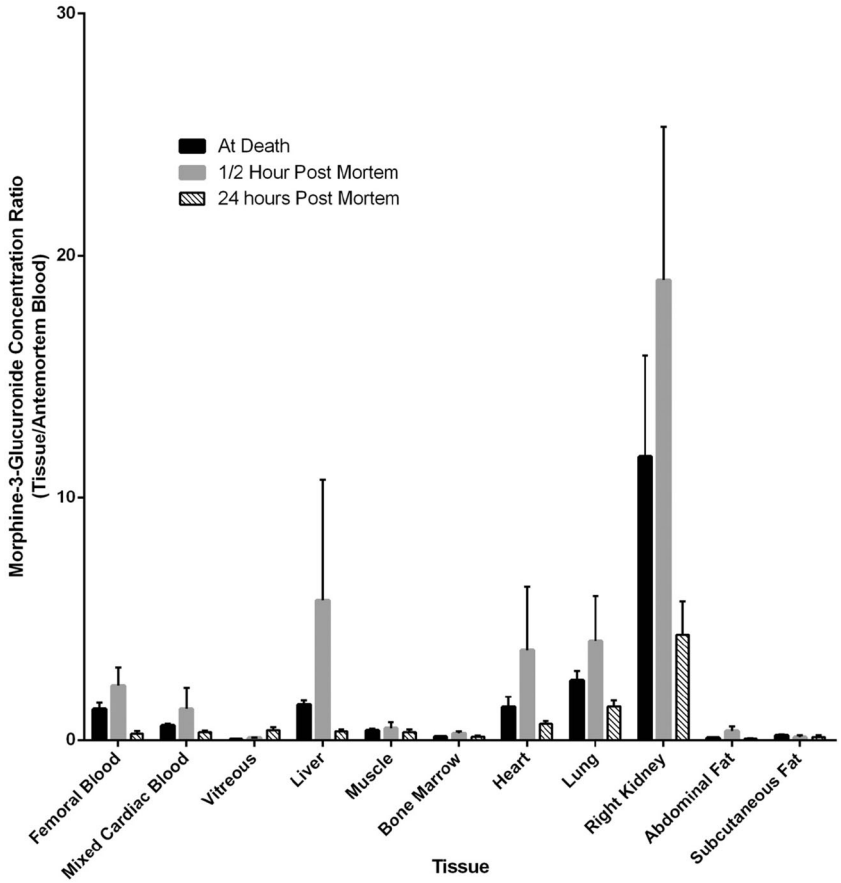

Fig. 3 Comparison of morphine-3-glucuronide (M3G) concentration in freshly sacrificed, 0.5 h postmortem and 24 h postmortem rabbits

decrease in free morphine concentration in the early postmortem period has been previously observed in a rabbit model of morphine postmortem redistribution and also in pig and humans studies investigating the postmortem redistribution of tetrahydrocannabinol (THC). It is likely that in these cases the equilibrium between blood and tissue concentrations may not have been reached. In our study, equilibrium may not have been reached 1 h after injection even though the kinetics before euthanasia (based on the information of [21]) seemed to indicate that the elimination phase was ongoing. It is also

Table 4 Concentration values of total morphine by sampling site and time of postmortem interval

	Total morphine			
	Postmortem interval (h)			
	0	0.5	24	Mean
Control antemortem blood (mg/L)	0.35±0.03	0.33±0.11	0.31±0.03	0.33±0.04
Postmortem femoral blood (mg/L)	0.50±0.09	0.62±0.19	0.11±0.03	
Postmortem mixed cardiac blood (mg/L)	0.22±0.01	0.26±0.06	0.17±0.05	
Vitreous (mg/L)	0.04±0.00	0.06±0.01	0.18±0.02	
Liver (mg/kg)	0.76±0.07	0.79±0.34	0.36±0.02	
Muscle (mg/kg)	0.41±0.12	0.20±0.06	0.20±0.01	
Bone marrow (mg/kg)	0.09±0.01	0.14±0.11	0.08±0.02	
Heart (mg/kg)	0.47±0.08	0.46±0.11	0.24±0.02	
Lung (mg/kg)	1.31±0.09	1.27±0.37	0.78±0.22	
Right kidney (mg/kg)	4.49±1.09	4.07±1.14	1.81±0.42	
Abdominal fat (mg/kg)	0.05±0.01	0.09±0.02	0.05±0.01	
Subcutaneous fat (mg/kg)	0.12±0.01	0.13±0.06	0.08±0.03	

Values are mean±SEM

possible that this equilibrium between blood and tissues was modified after death due to the arrest of circulation and the decrease in intracellular pH. In the later postmortem period (24 h), however, we observed increases in free morphine concentration in line with the rest of the studies. The redistribution potential of morphine can be seen with the 'reservoir' tissues for morphine. Higher concentration of free morphine (when compared to blood samples) have been observed in humans in lung [45], liver [46, 47], muscle [46] and myocardium [48], thus potentially setting up a concentration gradients in which morphine can diffuse. This human data is mirrored in the animal studies in which free morphine was again found in higher concentrations than femoral blood in liver, muscle, lung and kidney [this study, [9, 49]]. A more detailed picture of the postmortem redistribution of morphine and the potential for redistribution can be seen when tissue changes in free morphine concentration are investigated. Only one previous study looked at free morphine changes in tissue, and this study only measured free morphine in liver and kidney [9]. We observed similar free morphine concentration increases in the kidney after 24 h (+27 % compared to +18 % [9]) although in the liver we observed a slight increase in the concentration of free morphine (+6 %). This was very different to the significant increase of +400 % that was observed in rats.

Postmortem morphine-3-glucurionide levels

A limited number of studies have investigated the glucuronide metabolites of morphine [50, 51]. In this study, we also studied the concentration changes of M3G over time in various matrices. The results obtained are shown in Table 2. As with morphine, in order to minimise the variability between differing animals, the concentrations of M3G were normalised to the antemortem blood concentration of M3G. The concentration changes in normalised M3G are shown in Table 3 and Fig. 3. In all the samples, an increase in normalised M3G concentration was observed 0.5 h after death. The maximum changes in M3G concentration observed were in the liver (+295 %) and abdominal fat (+273 %). In only subcutaneous fat, a decrease in M3G concentration was observed (−34 %). Twenty-four hours after death in all samples, except vitreous in which a 1002 % increase in M3G concentration was observed, a decrease in M3G concentration was observed compared to the time of death samples. The largest decreases in M3G concentration were observed in the liver (−75 %) and femoral blood (−79 %), and a minimal change in M3G concentration was observed in the bone marrow (−4 %). It would be thought that as a glucuronide, M3G would be highly polar and also with a Vd of ~0.28 L/kg [41] M3G should not undergo significant postmortem redistribution. However like morphine, higher M3G concentrations were observed in tissues (liver, kidney, lung and heart), and they may act as depots of higher concentrations of M3G in which diffusion may occur into adjacent areas which have lower concentrations of M3G. An explanation for the postmortem redistribution of M3G may be that M3G has also been shown to exist in two conformational forms in which M3G is actually found to be far more lipophilic than expected and is only slightly less lipophilic than morphine [52]. It is conceivable that although M3G is more lipophilic than expected, the changes in concentration of M3G in the various matrices are likely to be a combination of diffusion and hydrolysis dependent on the concentration gradient and the metabolic enzymes present in the individual tissue. In vitreous and muscle, it is likely that the M3G concentration increase is due to diffusion as decreases in the free/total morphine ratio are observed over 24 h (vitreous 69 to 35, muscle 67 to 54; Table 6). However, changes in concentration could also be due to a greater rate of diffusion of free morphine into surrounding tissue compared to M3G.

Table 5 Changes in normalised total morphine concentration 0.5 h postmortem and 24 h postmortem

Tissue	% Change (½h postmortem)	% Change (24 h postmortem)
Femoral blood	+63	−71
Mixed cardiac blood	+25	−20
Vitreous	+93	+425
Liver	+84	−47
Muscle	−34	−43
Bone marrow	+113	+9
Heart	+50	−43
Lung	+58	−39
Right kidney	+44	−57
Abdominal fat	+182	22
Subcutaneous fat	−18	−24

Postmortem total morphine levels

In order to allow a better comparison of the results obtained in this study compared to previous studies, and also to investigate if the changes in free morphine concentration postmortem could be due to the hydrolysis of morphine glucuronides rather than postmortem redistribution, we calculated the total morphine levels (morphine+M3G). The results (normalised to antemortem total morphine levels) are shown in Table 4. The percentage changes to total morphine concentration are shown in Table 5 and Fig. 4. After half an hour postmortem, an increase was observed in the total morphine concentration compared to the samples taken immediately after death. The largest changes in total morphine concentration observed were in the fatty tissues, bone marrow and abdominal fat with increases of 113 and 182 %, respectively. This was not true of all

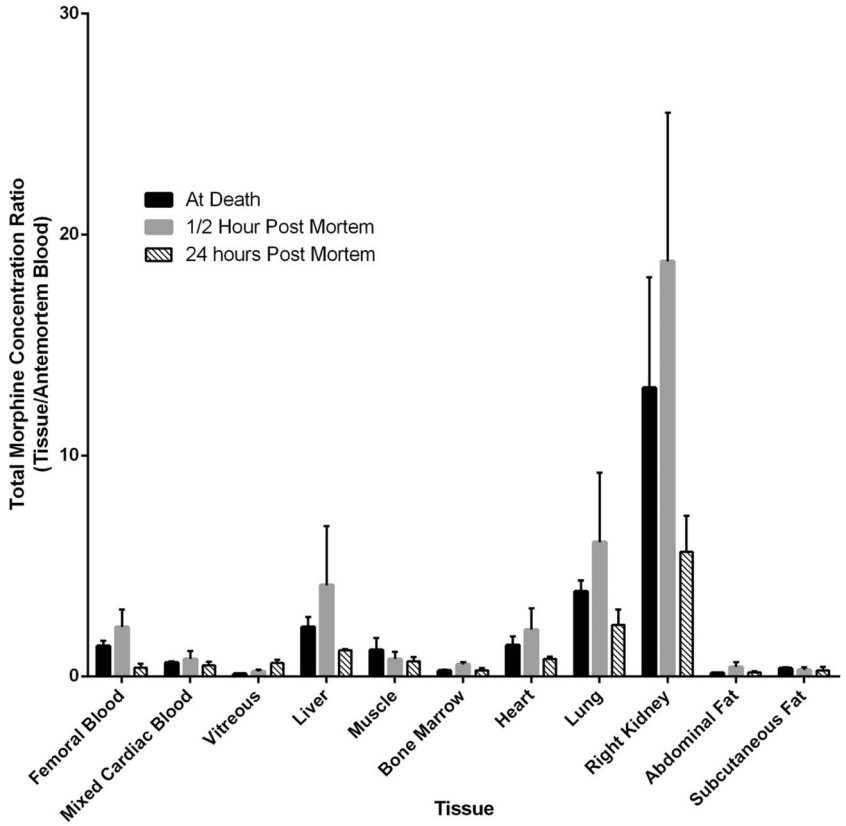

Fig. 4 Comparison of total morphine concentration in freshly sacrificed, 0.5 h postmortem and 24 h postmortem rabbits

fatty tissue as in subcutaneous fat concentrations of total morphine showed little change (+18 %) in the half hour following death. The only other tissue where a decrease in total morphine concentration could be observed was muscle with a decrease of 34 %. However, 24 h after death, the total morphine concentration had decreased in most of the tissues compared to the values of total morphine concentration obtained close to the time of death. The only tissues that saw an increase in total morphine concentration were vitreous humour (+425 %) and abdominal fat (+22 %). It has previously been suggested from both in vivo and in vitro studies that observed increases in free morphine concentration postmortem could be due to hydrolysis of morphine glucuronides rather than postmortem redistribution [9, 11, 14] where an increase in the free/total morphine concentration ratio would be seen with increasing hydrolysis. In order to investigate this, we calculated free/total morphine ratios. As shown in Table 6, there were increases in the free/total morphine ratio 24 h postmortem for femoral blood (10 to 37), cardiac (7 to 39), liver (37 to 72), right kidney (14 to 28), and abdominal fat (35 to 65). Vitreous humour was the only matrix in which the free/total morphine ratios decreased in the 24 h following death (69 to 35). The role of hydrolysis in postmortem redistribution of morphine is unclear; however, our results agree with those found in a rat model of postmortem redistribution where in both blood and liver, the free/total morphine ratio increased over time [9]. In our study for the 24 h postmortem interval, the liver free/total morphine ratio increased from 15 to 41, and in the 96 h postmortem interval, liver free/total morphine ratio increased from 20 to 86. This increase in ratio was mirrored with cardiac blood with the free/total morphine ratio increasing from 26 to 69. However, we felt that the changes in free/total morphine ratio and thus hydrolysis of morphine glucuronides were only partially responsible for the increases of free morphine over time due to the 1000 % concentration increase in free morphine concentration in the liver. In a pig model of postmortem

Table 6 Free/total morphine ratios

Tissue	0 h Postmortem	½h Postmortem	24 h Postmortem
Antemortem blood	3	17	3
Femoral blood	10	21	37
Mixed cardiac blood	7	21	39
Vitreous	69	66	35
Liver	37	49	72
Muscle	68	78	54
Bone marrow	43	69	50
Heart	7	23	18
Lung	38	59	47
Right kidney	14	25	28
Abdominal fat	35	34	65
Subcutaneous fat	48	56	54

redistribution of morphine [11], increases in free/total morphine ratios were observed after 24 h (cardiac blood left ventricle 11 to 76, femoral blood 22 to 37 and femoral artery 18 to 33); however, after 96 h, the samples only exhibited a slight increase in free/total morphine ratios from 5 min postmortem (cardiac blood left ventricle 11 to 20, femoral blood 22 to 26 and femoral artery 18 to 23) in the other samples collected; right ventricle showed a decrease in the free/total morphine ratio at both 24 h 22 to 17, and 96 h 22 to 18. Together, these results show that although hydrolysis of M3G does occur and may have a role in the differing concentrations of free morphine found in the various tissue postmortem, it cannot be easily predicted what will happen in any specific case in a specific tissue and that further investigation into the phenomena is required.

Possible movement of drugs in the postmortem environment

There are three current theories as to how morphine specifically and drugs in general redistribute in the postmortem environment. They are (1) postmortem pH changes which modify the equilibrium of the drug in tissue compartments [9], (2) passive diffusion of the drug down a concentration gradient (either from neighbouring tissue, from the gastrointestinal tract or through blood vessels) [4] and (3) conversion of morphine glucuronide(s) to morphine [53]. If there is movement of morphine postmortem, it would be expected to move from areas of high free morphine concentration to areas of low free morphine concentration. As described above, morphine was found to have numerous tissue depots from which it could diffuse from (liver, muscle, lung, and kidney) to adjacent organs and tissues. From the data in Table 2, morphine appears to be moving from the heart tissue to the cardiac blood as there is an increase in the concentration in the cardiac blood over time (from 0.02 mg/L (time 0) to 0.07 mg/L (24 h) with the free morphine in the heart muscle increasing from 0.03 mg/L (0 h) peak at 0.10 mg/L (½h) then decreasing to 0.04 mg/L (24 h). The increase in the cardiac muscle free morphine concentration could be from the lung as a decrease is seen over 24 h (from 0.5 to 0.36 mg/L). However, an increase in lung free morphine concentration is observed from time of death to 0.5 h (0.5 to 0.74 mg/L). It is possible this could be redistribution from the lower lobes of the lung, but as there was no sampling from the other areas in the lung, this was not possible to confirm. In the muscle, a decrease in the concentration of free morphine was observed over 24 h (0.27 to 0.11 mg/L); it is possible that free morphine diffused from the thigh muscle into the adjacent blood vessels. This could account for the increase in femoral blood free morphine that was seen in the first half hour following death (from 0.05 to 0.13 mg/L); however, the femoral blood concentration of free morphine then decreased in the following 23.5 h. It is possible the free morphine in the femoral blood then diffused into tissues with a lower morphine concentration.

Conclusions

Although previously, there have been doubts about the use of smaller animals to investigate the mechanisms of postmortem redistribution [11]; rats and rabbits still offer the most convenient method of carrying out controlled studies for understanding of postmortem redistribution. This study confirms the use of vitreous as the matrix of choice for evaluating use of heroin and further recommends the use of muscles tissue in cases where vitreous is unavailable.

Conflict of interest All authors have nothing to disclose.

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