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Morphine Perfused Rabbits: A Tool for Experiments in Forensic Entomotoxicology

ABSTRACT: In order to establish an animal model for entomotoxicological studies, the kinetics of morphine elimination from blood after a single intravenous injection of morphine and the concentrations of morphine in tissues following a continuous perfusion were studied. The aim of these experiments was to obtain controlled morphine tissue concentrations similar to those encountered in fatal human heroin overdoses. These tissues can be used as a food source for developing fly larvae in entomotoxicological studies. In the single injection experiment, seven rabbits were administered 1 or 2 mg/kg body weight of morphine chlorhydrate via the main ear artery. Blood samples of 200 μL were removed regularly via a catheter. Morphine concentration was determined using RIA techniques. Morphine was found to be first rapidly distributed and then slowly eliminated, following a two-exponential equation. Elimination of morphine from blood can be described as a two-compartment model. Constants of the equation were determined using the Kaleidagraph® program. Using those constants, the main pharmacokinetics parameters were calculated. Results of these parameters showed the following: clearance from 13.3 to 16.2 L.h-1, half-life of the distribution phase from 0.6 to 0.9 min, and half-life of the elimination phase from 21 to 26 min. These results were used to calculate the rate of perfusion of morphine for rabbits to obtain desired, controlled, and constant concentrations of morphine in tissues. In the second experiment, three rabbits received a perfusion of morphine intravascularly at a rate of 2 mg/kg/h for a period of 3 h. These rabbits were sacrificed and analyses performed on several abdominal and thoracic organs. Results showed that the concentrations of morphine differed according to the organ analyzed, but were reproducible for organs between animals. These concentrations were similar to those normally encountered in cases of human death due to heroin overdoses.

KEYWORDS: forensic science, forensic entomology, pharmacokinetics, morphine, heroin, entomotoxicology, rabbits

It has been well established that maggots can be used as alternate specimens for toxicological analyses in cases of badly decomposed bodies or in cases when other tissues are not available for analysis. There have also been several forensic case studies detailed in which maggots were successfully used for toxicological analyses (1–5). In a series of papers from 1989 to 1997, Goff et al. have described the influence of some drugs and toxins on the growth rates and patterns of larvae and their relationships to the estimation of the postmortem interval (6–11). One major factor which has served to limit the growth and precision of this relatively new area of forensic investigation has been the absence of standardized experimental conditions.

The present studies were undertaken to establish an animal experimental model which will provide controlled level of drugs in the blood and tissues. The domestic rabbit was chosen as the experimental animal and the drug was morphine, the main active metabolite of heroin. One major aim of this study was to develop a technique to produce levels of morphine in the viscera of the animal model close to those usually encountered in cases of fatal human heroin overdoses and have these level reproducible. Tissues obtained from this model can be used as a food source for rearing of larvae for further entomological analyses.

Materials and Methods

This study was divided into two sections. In the first experiment, the pharmacokinetics of morphine was studied after a single rapid injection of morphine to allow calculation of the perfusion flow needed to reach a given blood plateau level. In the second experiment, a continuous flow of morphine was perfused in order to study the concentrations of the drug in visceral organs.

In the first experiment, seven domestic rabbits were administered doses of morphine hydrochloride as follows: 6 rabbits (R1: 2.36 kg; R2: 2.03 kg; R3: 2.54 kg; R4: 2.23 kg; R5: 2.92 kg; and R6: 3.05 kg in weight) received 2 mg/kg (1.6 mg/kg free base equivalent) of body weight; 1 rabbit (R7: 2.55 kg in weight) received 1 mg/kg (0.8 mg/kg free base equivalent) of body weight; and 1 control rabbit (R8: 2.52 kg body weight) received 2 mL of isotonic saline solution. Morphine, supplied by the Francopia Society (Gentilly, France), was administered in 2 mL of isotonic saline and injected directly into the vascular system through the main artery of the left ear. Injection was made using a metal needle (0.70 mm diam) which was removed following the injection. Blood samples were removed during the experiment from a plastic catheter (0.70 mm diam) placed into the central main artery of the right ear. In rabbits R1 and R2, a 200 μL blood sample was taken before the initial injection and additional samples taken at 10 min intervals for 3 h following the injection. From rabbits R3, R4, R7 and R8, 200 μL blood samples were taken immediately before the injection, every minute for the first 20 min following the injection and then every 10 min from 10 min following injection until 3 h following injection. For rabbits R5 and R6, 200 μL blood samples were taken immediately before the injection of the drug and then every minute for 20 min following injection. Following collection, blood samples were placed into 500 μL Eppendorf tubes and immediately centrifuged. Samples were frozen and stored at −20°C until analysis.

In the second experiment, three domestic rabbits (R9: 3.32 kg; R10: 3.50 kg; R11: 3.45 kg body weight) received 2 mg/kg of body weight of morphine hydrochloride per hour for a period of 3 h using a continuous perfusion through a plastic catheter in the main central artery of the left ear. This vessel was used instead of a vein as it is easier to access in rabbits. A control rabbit (R12: 3.45 kg body weight) received only isotonic saline solution (Perfuflex, Fresenius France). The perfusion was placed in a bottle located 1.75 m above the rabbits in order to reverse the blood flow in the artery, thus assuring a correct diffusion of the solution in the body of the rabbit. The perfusion was checked regularly during the experiment to assure that a constant flow was maintained. Rabbits were sacrificed in a carbon dioxide chamber 3 h following the initiation of the perfusion. Immediately after death, the four rabbits were necropsied and 5 g of each of the following organs sampled: lung, liver, muscle, spleen, heart, and fat. The samples were homogenized in a Poter-Elvehjem homogenizer and then immediately centrifuged. Two aliquots were made of the supernatant and these were frozen at $-20°C$ and stored until analysis.

Samples were analyzed for morphine using a sensitive and specific radioimmunoassay (RIA) method (Coat-a-count® Serum morphine RIA, Behring Diagnostic, Rueil, France. Detection limit: 1 ng/mL). Samples anticipated to have concentrations of morphine higher than the highest calibration (250 ng/mL) were diluted with human serum before assay. Analyses were repeated twice following the manufacturer's instructions.

Blood morphine concentrations versus time were analyzed using KaleiGraph® software. A two-compartment model was used to fit the data: $C(t) = A \exp(-\lambda_1 t) + B \exp(\lambda_2 t)$. Here $C(t)$ stands for the blood concentration at time t, $\lambda 1$ and $\lambda 2$ are the rate constants for the first (distribution) and second (elimination) phases of the decline, respectively, and A and B are the corresponding zero-time intercepts. Clearance (Cl) was calculated by dividing the dose administered (Do) by the area under concentration versus the time curve (AUC): $Cl = Do/AUC$. The half-life ($t1/2$) for the distribution (α) and the elimination (β) phases were calculated as $(\ln 2)/\lambda_1$ and $(\ln 2)/\lambda_2$, respectively.

Results

Analyses of blood and tissue samples showed the presence of morphine in all ten animals dosed with the morphine hydrochloride, while samples from the two control rabbits (R8 and R12) were negative.

In the first experiment, after a single intravascular injection of morphine hydrochloride in rabbits R1 to R7, morphine was observed to be initially rapidly distributed and then slowly eliminated following a 2-exponential curve. These two phases are illustrated in Fig. 1 from data from rabbit R7. Similar curves were obtained for the other rabbits. Semilogarithmic curves from data derived from rabbits R1, R2, R5 and R6 demonstrated two straight lines for each rabbits (Fig. 2). This clearly indicates that the distri-
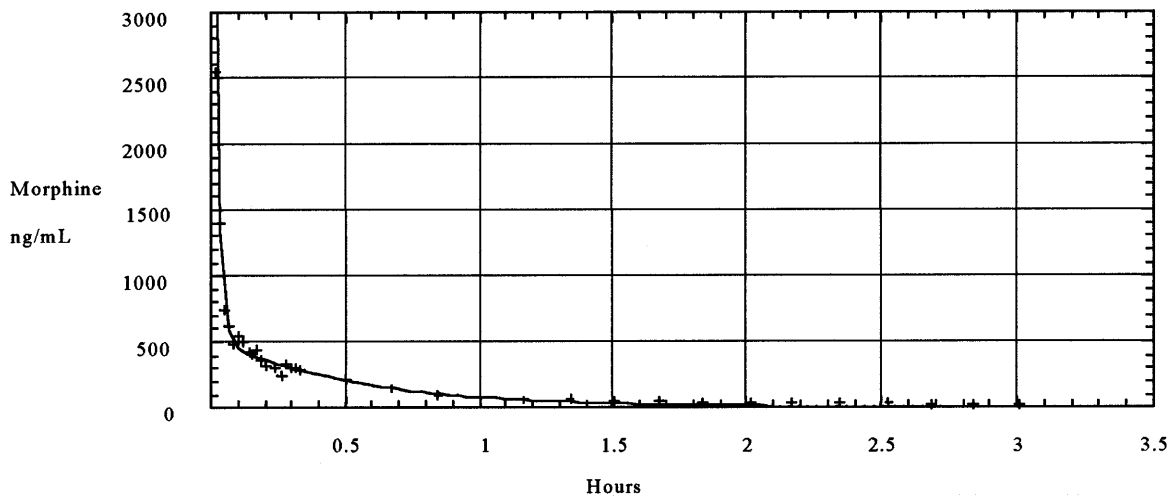

FIG. 1—*Morphine blood concentration evolution calculated from the rabbit R7 after a continuous perfusion of 2 mg/kg during 3 h (blood samples every minute during 20 min, then every 10 min until 3 h after the initial injection).*
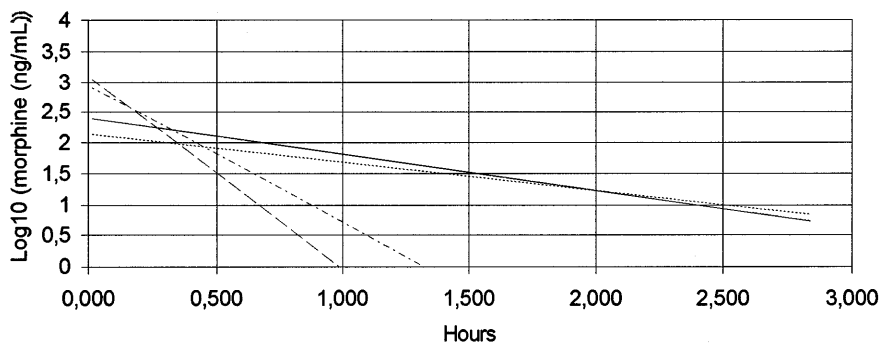

FIG. 2—*Logarithmic curves corresponding to the distribution (two lower lines) and elimination (two upper lines) phases. The two upper lines correspond to rabbits R1 and R2 and the two lower lines to rabbits R4 and R5.*

TABLE 1—*Constants of the equation $C(t) = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$.*

Rabbit	Dosage*	A†	λ_1‡	B†	λ_2‡	R^2
R6	2.55	2.79	68.5	0.230	1.98	0.994
		(0.171)§	(3.46)	(0.0108)	(0.199)	
R7	5.1	4.39	45.4	0.361	1.60	0.977
		(0.336)	(3.85)	(0.0461)	(0.428)	
R8	5.1	5.74	54.6	0.542	1.94	0.993
		(0.327)	(2.83)	(0.0305)	(0.224)	

* Dosage of morphine injected (mg).
† Zero-time intercept (mg·L^{-1}).
‡ Rate constants for the first and second phase of the decline (h^{-1}).
§ Margin of error calculated by the software.

TABLE 2—*Kinetics of morphine calculated from A, λ_1, B, λ_2 constants.*

Rabbit	AUC*	Cl†	$t^{1/2}(\alpha)$‡	$t^{1/2}(\beta)$§
R3	0.157	16.2	0.606	21
R4	0.322	15.8	0.912	26
R7	0.383	13.3	0.756	21.5

* Area under the concentration versus time curves (mg·h·L^{-1}).
† Clearance (L·h^{-1}).
‡ Half-time of the distribution phase (min).
§ Half-time of the elimination phase (min).

TABLE 3—*Morphine concentrations in tissues (ng/g).*

Rabbit	Liver	Kidney	Lung	Heart	Spleen	Fat	Muscle
R9	2637	3502	2848	1970	4970	238	2773
R10	1751	2557	3457	1801	5033	478	2566
R11	2116	3961	3463	2197	6192	694	2969

bution and elimination phases both correspond to a bi-exponential decrease in the drug. Analyses using the KaleiGraph® software indicated these data fit a 2-compartment model. Constants for regression and other parameters of the 2-compartment equation were calculated for rabbits R5, R6 and R7 (Table 1). Other parameters of the pharmacokinetics calculated from the constants of the previous equation are given in Table 2.

In the second experiment, following a continuous intravascular perfusion of morphine hydrochloride into rabbits R9 to R11, concentrations of morphine were determined for each of the samples taken following sacrifice of the rabbits. These results are presented in Table 3. While the concentrations of morphine differed between organs, there was an overall similarity between concentrations for any given organ among the three rabbits sampled.

Discussion

Insects and other arthropods can be valuable tools in death investigations. Determination of the species of flies infesting a corpse and analyses of their developmental stages can provide information concerning the postmortem interval, movement of the body following death and presence of antemortem injuries. The use of insects for qualitative assessment of drugs present in a decomposing body was first reported in 1980, when, due to extensive decomposition, adequate tissue samples could not be obtained for toxicological analyses (1). This resulted in sampling of larvae of the Calliphoridae *Cochliomyia macellaria* for analysis and demonstration of the presence of phenobarbital. Today, the use of larvae as alternative

specimens for toxicological analyses in cases of decomposed remains where no tissues suitable for analyses are present is well documented; however, there is still a need for further studies in this area. In a recent review, Goff and Lord (12) point out the advantages and pitfalls of this type of work. Most workers have found no correlation between the drug concentrations for the larvae and the tissues on which the larvae have fed while others have observed significant correlations between concentrations in liver tissues and those in the larvae (5,13,14). At the present time, extrapolation of blood or tissue drug concentration from larval drug concentration is not practically possible. It appears that the larva's metabolism plays a major role in determining the concentration of the drug in the larva (14) and this may vary with the stage of development of the larva. In addition there are also the initial differences related to the differences in the concentrations of the drug in various tissues on which the larva fed. In the same manner, determination of drug concentrations from analyses of the puparia or predatory species, such as the beetles, presents even greater potential difficulties due to the process of bioaccumulation (12,15). These problems have resulted in our attempt to formulate a new experimental approach using a standardized animal model.

We have selected morphine as our initial drug for study as heroin addiction is a significant public health problem in France and other countries and deaths for heroin overdoses are frequent. Morphine was used in our experiments because its elimination pharmacokinetics is not different after heroin or morphine perfusion and we were interested only in establishing a model for entomotoxicological studies and not in mimicking heroin overdoses. We anticipate applying this technique to other substances of abuse in future studies. The domestic rabbit was selected for the animal model for several reasons. Aside from being readily available, it is easy to place a catheter in the main artery of the ear for the continuous perfusion of the drug. The perfusion bottle must be placed 1.5 m above the animal during the perfusion process. Below this height, the pressure of the perfusion is not sufficient to overcome the rabbit's blood pressure. Another factor is that rabbits are large enough that a single animal can supply all of the tissues needed to rear a sufficient quantity of larvae needed for each toxicological study. More than 400 larvae can easily be reared from a rabbit weighing 3 kg. Toxicological analyses were performed using RIA methodology as this technique is rapid, reliable and allows for inexpensive testing of a large number of samples with a high sensitivity and specificity. Other toxicological methods were not used as morphine was the only substance administered to the rabbits.

The results of the first experiment show that the kinetics of morphine in the rabbits can be described by a two compartment model, with an initial short distribution phase, followed by a longer elimination phase. This step was essential to calculation of the pharmacokinetic parameters for our second experiment elaborating the model. The half-lives of both distribution and elimination phases were used to estimate the duration of the perfusion to reach a blood level plateau required. Based on the pharmacokinetic data in the two compartment model, this plateau is reached after five times the half-life of the elimination phase (16). A half-life of approximately 20 min (t1/2 β) indicated that the blood level plateau would be reached in 100 min. The perfusion was continued for 80 min after this plateau was reached to insure a good diffusion of morphine into all the tissues. Another goal of our study was to obtain concentrations of morphine in organs of the rabbits which were similar to those encountered in fatal human heroin overdoses (17). We point out that the Baselt and Cravey references is a compilation of toxicology data that includes tissue concentrations that

are total morphine (morphine plus morphine glucuronide). The RIA assay that we used measures only free morphine (18) but nobody knows the possible glucuronic acid conjugation of the morphine in insects. The rate of the perfusion was calculated from this pharmacokinetic equation: Css = Ko/Cl, where Css is the expected blood concentration and Ko is the rate of flow needed. Based on the results from Table 3, administration of 2 mg/kg/h over a 3 h period resulted in concentrations similar to those encountered in fatal heroin overdoses in human cases.

As shown in Table 3 and previously published data, concentrations of morphine vary with the organ studied. Concentrations are similar in lung, muscle, kidney, liver and heart but appear higher in the spleen and vitreous, and lower in fat tissue. Previous published experimental studies have dealt with concentrations corresponding to lethal dosages for their animal models (10,11). These values are quite different from those encountered in humans, as rabbits are particularly resistant to morphine intoxication. The model produced from this study allows for closer approximation of clinical situations and may prove to be of greater value in future studies.

Conclusion

These studies have resulted in an experimental animal model providing controlled blood and tissue levels of morphine. These tissues can be used as a substrate for rearing of fly larvae in entomological studies. This model allows for known and reproducible concentrations of drugs in the rabbit viscera, and the expected concentrations in the various organs can be easily calculated to approximate those normally encountered in fatal overdoses of heroin in humans.

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ERRATA/CORRECTIONS

We have identified a number of instances in which the authors of work published in the Journal of Forensic Sciences have miscited papers originally published in the Journal of the Forensic Science Society as having been published in the Journal of Forensic Sciences.

The known instances of this error for volume 44 of the Journal of Forensic Sciences are detailed/corrected below. We have not checked other volumes for similar errors. The Journal of Forensic Sciences regrets these errors.

Since 1995 (Volume 35), the Journal of the Forensic Science Society has been published under the title "Science and Justice."

The editors of both journals take this opportunity to remind authors of the necessity for ensuring the accuracy of the references they cite in manuscripts submitted for publication. The Instructions for Authors of both journals make it clear that accuracy of reference citation is the responsibility of authors, and good scholarship demands attention to this matter.

A. R. W. Forrest
Editor, Science and Justice

R. E. Gaensslen
Editor, Journal of Forensic Sciences

The journal citation in reference 7 in Foreman LA, Smith AFM, Evett IW. Bayesian validation of a quadriplex STR profiling system for identification purposes. should read: J Forensic Sci Soc 1992;32:5–14.

The journal citation in reference 5 in Bourel B, Hedouin V, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Effects of morphine in decomposing bodies on the development of Lucila sericata (Diptera: Calliphoridae). should read: J Forensic Sci Soc 1991;31:469–72.

The journal citation in reference 8 in Hedouin V, Bourel B, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Determination of drug levels in larvae of Lucila sericata (Diptera: Calliphoridae) reared on rabbit carcasses containing morphine. should read: J Forensic Sci Soc 1994;34:95–7.

The journal citation in reference 15 in Hedouin V, Bourel B, Martin-Bouyer L, Becart A, Tournel G, Deveaux M, Gosset D. Morphine perfused rabbits: A tool for experiments in forensic entomotoxicology. should read: J Forensic Sci Soc 1991;31:469–72.

The journal citation in reference 10 in McDermott SD, Willis SM, McCullough JP. The evidential value of paint. Part II. A Bayesian approach. should read: J Forensic Sci Soc 1992;32:333–48.

The journal citations in references 4 and 5 in Infante F, Dominguez E, Trujillo D, Luna A. Metal contamination in illicit samples of heroin. should read for 4: J Forensic Sci Soc 1979;19:203–9. and for 5: J Forensic Sci Soc 1980;20:177–81. [in reference 5 only the volume number is miscited]. And in both references, the lead author's name is "Joyce JR."

The journal citation in reference 1 in Savolainen P, Lundeberg J. Forensic evidence based on mtDNA from dog and wolf hairs. should read: J Forensic Sci Soc 1988;28:335–9.

The journal citation in reference 1 in Kupfer DM, Chaturvedi AK, Canfield DV, Roe BA. PCR-based identification of postmortem microbial contaminants—A preliminary study. should read: J Forensic Sci Soc 1968;8:73–6.

In every instance cited above, future citations of the J Forensic Sci papers containing the errors should contain the following: [published erratum appears in J Forensic Sci 2001 Jan;46(1)] immediately following the article title and before the journal citation, in accordance with the Uniform Requirements for the Submission of Manuscripts to Biomedical Journals style.