Gisela Skopp,[1] *Rainer Lutz,*[1] *Lucia Pötsch,*[2] *Beate Ganßmann,*[1] *Klaus Klinder,*[1] *Achim Schmidt,*[1] *Rolf Aderjan,*[1] *and Rainer Mattern*[1]

An In Vitro Experiment for Postmortem Vascular Permeation. The Passage of Morphine and Morphine Glucuronides Across a Vascular Wall

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ABSTRACT: A venous blood sample taken at autopsy cannot be considered to represent the antemortem blood concentration of a particular substance. Autolytic processes cause disintegration and increasing permeability of the physiological and anatomical barriers such as vascular walls and lead to changes in substance concentrations. In the present study, the experimental design represents an in vitro postmortem simulation of a drug substance crossing a venous wall. The postmortem behavior of morphine, morphine-3- and morphine-6-glucuronide was investigated. A Chien-Valia-diffusion chamber with a patch of inferior vena cava as diffusion barrier was used. For optimal simulation of postmortem events, vein sampling was restricted to selected autopsy cases. Parameters for the analysis of diffusion across the vascular tissue were dependence on time, temperature, and initial substance concentrations. The penetration behavior simulating venous efflux and influx of the substances was studied by different orientation of the venous wall in the experiments. Rhodamine B was used as a model substance to visualize the binding to the tissue and the passage across the venous wall. The permeation of morphine, morphine-3- and morphine-6-glucuronide across a vein tissue was found to be mainly dependent on the disintegration of the vascular wall and on the postmortem time period as well as on concentration gradients. From the data of this preliminary in vitro study, it can be concluded that a lag time for transvascular diffusion exists postmortem. However, it could be demonstrated, that adsorption to and penetration into the vascular tissue may alter intraluminal blood concentrations even at an early stage of the postmortem time period.

KEYWORDS: forensic science, forensic toxicology, postmortem redistribution, morphine, morphine glucuronides, Rhodamine B, Chien-Valia-diffusion chamber, vascular permeability, postmortem diffusion

The interpretation of postmortem toxicological analyses raises two fundamental questions concerning the fatal dose and the survival time. Although in recent years, pharmacokinetic data and equations have been used to give an answer, most toxicologists have relied upon published case reports and data from clinical studies. The first question to be answered, however, is: What happens to a blood drug concentration during the period from death to blood sampling at autopsy? The interpretation of the analytical results from postmortem specimens is further complicated because the magnitude of postmortem redistribution varies with sampling site and time.

The most recognized examples of the dependence of postmortem blood concentration on the collection site and on the time period of the postmortem interval were reported by Vorphal and Aderjan (1,2). These authors investigated antemortem and postmortem digoxin blood levels, concluding from the results that distributive changes might start before death (2). In 1981 Bandt concluded from nine cases involving tricyclic antidepressants that postmortem blood-drug concentrations changed with the time period since death and the sampling site (3). Site dependence of postmortem drug concentrations has been established for several drugs including methamphetamine (4), methadone (5), cimetidine (6), paracetamol and propoxyphene (coproxamol) (7), diphenhydramine and codeine (8), barbiturates (9) and cocaine (10). A great variability in both the magnitude and the direction of drug concentrations has been observed. A review was given by Prouty and Anderson in 1990 (11).

The phenomenon of blood sampling site dependence of measured drug concentrations is not restricted to the postmortem interval because it had been reported for several compounds after dosing in living humans and animals (12). However, the differences were insufficient to account for the considerable differences in postmortem blood drug concentrations from different sampling sites.

There are only a few investigations on temporal influences on postmortem drug concentrations compared with studies on site dependence. The time dependence of postmortem substance concentration at the same sampling site should be due to hypostasis and increasing permeability of all tissues including the vascular system (13). Immediately after death, hypostasis occurs by the settling of blood and plasma to the most dependent parts of the body. As the length of time of ischemia increases, the potentials of the biological membranes break down and cellular enzymes leak into the extracellular space resulting in tissue damage. Thus substance permeation into and across the vascular wall should cause changes in substance concentration.

Koren and Klein investigated postmortem redistribution of morphine with time in the heart blood of rats as a model to try to predict postmortem drug release in humans (14). Morphine concentrations rose from the time of death up to 24 h with no additional increase at 96 h suggesting a substantial temporal redistribution of morphine after death.

[1]Institute of Forensic Medicine, Ruprecht-Karls-University, Voßstr. 2, 69115 Heidelberg, Germany

[2]Institute of Forensic Medicine, Johannes-Gutenberg-University, Am Pulverturm 3, 55131 Mainz, Germany

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As for heroin-related death, the situation will be more complex especially when regarding heroin metabolism. Heroin is rapidly deacetylated in whole blood to 6-acetylmorphine, which is further hydrolyzed to morphine. Glucuronidation of morphine occurs with a lag time of about 6 min at the 3- and 6- carbons (15). Morphine-6-glucuronide exhibits significant analgesic activity and respiratory depression (16,17). In pharmacokinetic studies after morphine dosing, the ratios of the molar concentrations of morphine-3- and morphine-6-glucuronide to the molar concentration of morphine were found to be dependent on time but not on the dose taken (15). Thus the interpretation of postmortem morphine and morphine glucuronide findings seems not to be complicated by the fact that concentration in overdoses overlap concentrations from formerly frequent dosing. Moreover, the ratio of the molar concentrations of morphine and its glucuronides was suggested to be a simple means for assessing survival time in heroin-related death (18).

To investigate the permeation of morphine, morphine-3- and morphine-6-glucuronide through a vascular wall without the need for an animal model, a Chien-Valia-diffusion chamber (19) was used with a vein sample from the inferior vena cava as a membrane clamped between the connecting flange surfaces of the bichamber. The changes of substance flux of morphine, morphine-3-glucuronide and morphine-6-glucuronide were reported, depending on time, temperature, initial concentration, and direction of solute flow. To prove the applicability of the chamber, preliminary experiments were carried out using Rhodamine B as a permeant.

Material and Methods

Experimental Design

Chien-Valia-Diffusion Chamber—The diffusion chambers were made by a local glass-blower. They consisted of two identical, L-shaped half cells comprising horizontal cylinders, 0.9 cm in diameter with vertical, stoppered sampling ports, and a diffusion area of 0.63 cm^2. A vein patch was used as diffusion membrane separating the two compartments. The diffusion area was placed in a vertical position by wrapping the half cells with Parafilm® . One of the chambers defined as donor compartment was filled either with Rhodamine B solution or fresh spiked blood samples. The other compartment filled with phosphate buffered saline (PBS, pH 7.4) was defined as acceptor compartment. To study postmortem redistribution, the efflux from a vein as well as the influx into a vein was simulated by orientation of the endothelial area, either towards the donor or towards the acceptor chamber. To simulate postmortem conditions, the donor and acceptor solutions were not agitated except for a few times prior to sampling. Sampling of chamber fluid was effected through the vertical ports at given times.

Preparation of Vein Samples from Inferior Vena Cava—Postmortem vein samples obtained from eight autopsy cases were used as diffusion membranes. It was assured that time of death had occurred within 24 to 60 h prior to autopsy and that the bodies had been stored at 4°C within 6 h after exitus. Exclusion criteria were an age over 50, infectious diseases, and intoxications. The dissection of the inferior vena cava was carefully performed to avoid artificial damage. The tissue was rinsed with physiological saline solution and briefly blotted between filter papers. From the inferior vena cava six areas (approx. 1.9 cm in diameter) were punched out and clamped between the connecting flange surfaces of the diffusion chambers. The investigations were started immediately according to the experimental design and run for about 120 h. All tests were run in duplicate and values given are mean values.

Rhodamine B—Preliminary experiments were performed using Rhodamine B to check the tightness and to test the applicability of the Chien-Valia-diffusion chambers. From the results of the test series investigating intra- and inter-vein variability in permeation behavior, the selection criteria for vein sampling in the opiate studies were established. Briefly, the tests performed were: The donor compartment was supplied with 5 mL of a Rhodamine solution (0.1 mg/mL) and the acceptor compartment with 5 mL PBS. At given time intervals, a 500 μL sample was taken out of the vertical port (acceptor) and directly analyzed by UV spectrophotometry. The volume taken was always returned. Moreover, the model substance allowed visualization of the diffusion process across the venous wall and the substance binding to the diffusion membrane. To calculate the amount of Rhodamine B in the tissue used as diffusion area, an additional Rhodamine B experiment was performed taking samples from both the acceptor and the donor compartments. After the experiments had been stopped, the vein samples were examined in daylight and in UV light (365 nm) for vertical diffusion of Rhodamine B out of the apparent diffusion zone.

Morphine and Morphine Glucuronides Studies—For these studies fresh blood samples obtained from volunteers were used and spiked with morphine, morphine-3- or morphine-6-glucuronide to yield final concentrations of 1, 2 or 5 mg/mL prior to any cava dissection and chamber preparation, and all experiments started within 1 h. Venous wall patches from the same individual were used in the particular investigation. The pH values of the blood samples in the donor compartments were determined at the beginning and at the end of the test. The following experiments were performed to study the influence on substrate diffusion of: a) Temperature, the investigations were performed at 4°C as well as at 20°C, b) The initial concentration, in the donor chamber, various concentrations of morphine, morphine-3- and morphine-6-glucuronide (1 mg/L, 2 mg/L, 5 mg/L resp.) were used, and c) Venous efflux or influx, for efflux conditions, the endothelium of the venous wall faced the donor chamber, for influx simulation, the adventitia faced the donor compartment. At given time intervals, 75 μL samples were taken out of the vertical ports and analyzed by HPLC.

Chemicals and Reagents

All reagents were of HPLC or analytical grade. Morphine hydrochloride trihydrate (fw 375.8, CAS 6055-06-07) was purchased from Merck (Darmstadt, Germany), morphine-3-ß-D-glucuronide (fw 461.5, CAS 20290-09-9), morphine-6-ß-D-glucuronide dihydrate (fw 497.5, CAS 20290-10-2) Rhodamine B (N,N,N′,N′-tetraethylrhodamine hydrochloride, fw 479.0, CAS 81-88-9) and phosphate buffered saline (PBS, pH 7.4 at 25°C) were from Sigma (München, Germany).

Triethylammonium phosphate buffer (TEAP, 1M) was obtained from Fluka (Buchs, Switzerland) and diluted in a ratio of 1:40 with double distilled water before use. A Rhodamine B solution was prepared using PBS (0.1 mg/mL).

Instrumentation

The Rhodamine B concentration was determined on a Perkin Elmer 550 SE UV/VIS spectrophotometer (Überlingen, Germany) by means of its absorbance at 543 nm. All values were corrected for the absorbance of a PBS blank. The same stock solution was

used for preparation of calibration standards and for the investigations. The determination of the Rhodamine B concentration was linear between 0.0015 and 0.05 mg/mL with a correlation coefficient of >0.999. The precision of the assay was characterized by an intra-assay CV of 2.4% (n = 5) and an inter-assay CV of 2.5% (n = 5) for the medium concentration range (0.0125 mg/mL).

HPLC analysis was performed with a Hewlett Packard 1050 series LC pump, a Shimadzu fluorescence detector and a Spectra Physics SP 4290 integrator. Samples were eluted from an Et 280/4 Nucleosil 100 C18 reverse phase column (Macherey & Nagel, Düren, Germany) with diluted TEAP as the mobile phase. Fifty μL of the aqueous phase were directly injected. For detection, the excitation wavelength was 220 nm, and emission was recorded at 340 nm. All values were corrected for the particular chamber blank. Standard solutions were prepared from the stock solutions which were used for the experiments. The simultaneous determination of the concentrations of morphine and of its glucuronides was linear between 0.015 and 5 mg/L with a correlation coefficient of >0.996. The precision of the assay was characterized by an intra-assay coefficient of variation of 2.9, 4.4, and 2.9% (n = 5) and an inter-assay coefficient of variation 5.7, 6.1, and 7.2% (n = 5) for morphine, morphine-3- and morphine-6-glucuronide (0.5 mg/L for each analyte).

Results

Rhodamine B Testing

In all series using Rhodamine B as a permeant, the initial concentration gradient for the dye was directed from the inside of the vascular wall to the outside. In some cases, there was a faintly visible coloration of the PBS solution in the acceptor compartment only 2 h after the experiment had been started. In vein sampled according to the selection criteria, a virtually linear increase in Rhodamine B concentration could be observed in the acceptor compartment after 20 h. After 80 h, the velocity expressed in terms of substance concentration change per unit time always had decreased in the experiments. As a consequence, a characteristic sigmoid curve as shown in Fig. 1 resulted. The intra-vein variability as well as the inter-vein variability was rather small for the vein samples according to the criteria as mentioned above.

The amount of Rhodamine B in the membrane was calculated from the concentration differences monitoring the donor and the acceptor compartment. It was found that about 50% of the Rhodamine B molecules had been absorbed by the vascular wall within 24 h after the experiment was started. As time proceeded, Rhodamine B concentration in the venous wall decreased, resulting in an increase of concentration in the acceptor compartment. This observation may be a result of autolytic deterioration of the membrane.

After the experiments had been stopped, the vein samples were examined in daylight and by means of a UV lamp. The apparent diffusion zone was highly fluorescent whereas the outlying ring of the vein disk showed no dyeing.

Morphine and Morphine Glucuronides Testing

Influence of Storage Temperature—The stability of morphine glucuronides has been shown in many previous studies (20), and hydrolysis did not seem to be a critical point for testing. The time course of morphine, morphine-3-glucuronide and morphine-6-glucuronide concentrations in the acceptor chambers plots exhibited the same profile as was observed for Rhodamine B. The initial mass transfer through the vascular wall was found to be slightly slower in the experiments performed at 4°C than at ambient temperature. The results differed by at most 33% during the first 40 h. Then, the differences became smaller and after 50 h, the progress curves for the vein samples stored at 4°C or at ambient temperature were nearly identical until the experiments had been stopped (Fig. 2).

Influence of the Initial Substance Concentration—For all substances investigated, the lag time was significantly shorter for an initial concentration of 5 mg/L compared with a starting concentration of 2 or 1 mg/L (Fig. 3). A more rapid rise of the linear part for the time versus concentration plot was observed for the higher initial concentration. A distinct plateau was not observed for all vein samples but the substance concentration per unit time decreased markedly after 80 to 100 h.

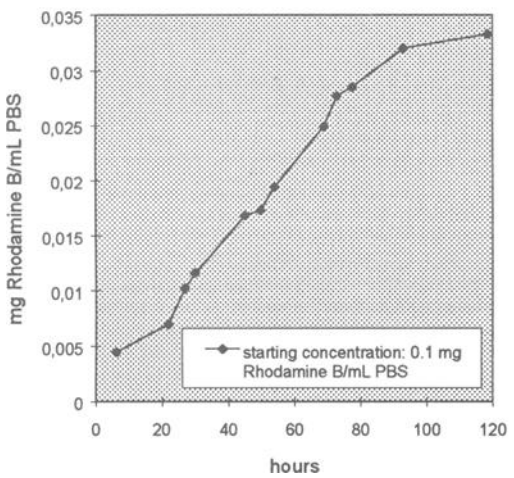

FIG. 1—*Example for the time course of Rhodamine B concentration (starting concentration: 0.1 mg/mL) passing through a venous wall. Efflux simulation.*

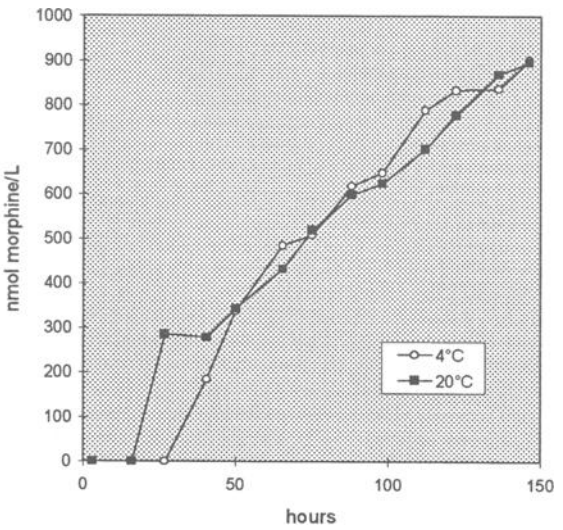

FIG. 2—*Time course of morphine concentration in the acceptor compartment. Temperature dependence (4°C, 20°C). Initial concentration: 3107 nmol/L. Efflux simulation.*

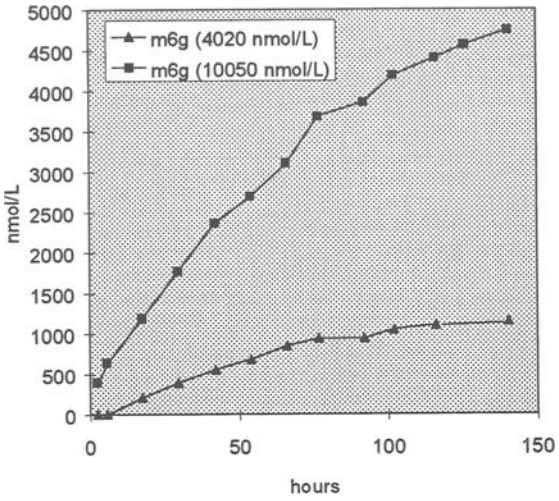

FIG. 3—*Time course of morphine-6-glucuronide (m6g) concentration in the acceptor compartment. Dependence on starting concentration (4020 nmol/L, 10050 nmol/L). Efflux simulation.*

Influence of the Direction of Substance Flow Simulating Venous Efflux Versus Venous Influx—In Fig. 4, the concentration profiles of morphine-6-glucuronide in the acceptor compartments with the gradient from inside to outside (upper curve) and the gradient from outside to inside (lower curve) of the vascular wall is shown. As time went on, substance flow from the luminal side of the vessel to its outside was markedly increased when compared with the mass transport from the outside to the inside of the vascular wall. The final differences of solute crossing the vascular wall in either direction was about 40% of the starting solution for all substances under investigation. At the end of the experiments, the pH values in the blood samples had slightly increased up to pH 7.6 to 7.8.

Influence of the Molecular Structure—The permeability profiles for morphine, morphine-6- and morphine-3-glucuronide are shown in Fig. 5, exhibiting very similar curve characteristics for all three substances with a virtually linear increase ranging from 20 to 80 h. For the glucuronides, the molar concentrations in the acceptor compartment at given times tended to be higher compared with the molar concentration of morphine.

Discussion

This study represents preliminary data of the in vitro concentration profiles of morphine, morphine-6- and morphine-3-glucuronide passing through a vascular wall clamped between the flange surfaces of a Chien-Valia-diffusion chamber. The in vitro model was used to simulate postmortem permeation of drug substances through the vein wall.

Diffusion systems have been developed for the assessment of drug release from topical formulations and transmembrane permeation. The L-bichamber was designed to separate the drug donor vehicle and the receptor solvent by a diffusion rate-limiting membrane. The adequate stirring of the two cylinders of the cell halves was not a critical point in our experiments as they were used as static cells. As a consequence, the development of localized drug concentrations and diffusion boundary layers at the membrane surfaces must be taken into account. Therefore, the cells were carefully shaken before a sample was taken from the chamber fluid.

The preliminary experiments using Rhodamine B as a permeant showed the tightness and applicability of the Chien-Valia-diffusion chamber. Moreover, small diffusion boundary layers were observed only at the beginning of the experiment in accordance with a calculated value of 0.05 mm for the theoretical diffusion boundary layer thickness for the Valia-Chien cell (21).

From a practical point of view, the inferior vena cava proved to be a suitable vein specimen concerning size and handling. The inferior vena cava is a large vein with a relatively thin intima, the elastica interna being discontinuous and largely fenestrated, the media containing a few layers of muscle fibers with extreme thinning in some regions. The adventitia represents the greatest part of the vascular wall. Thick bundles of longitudinally orientated

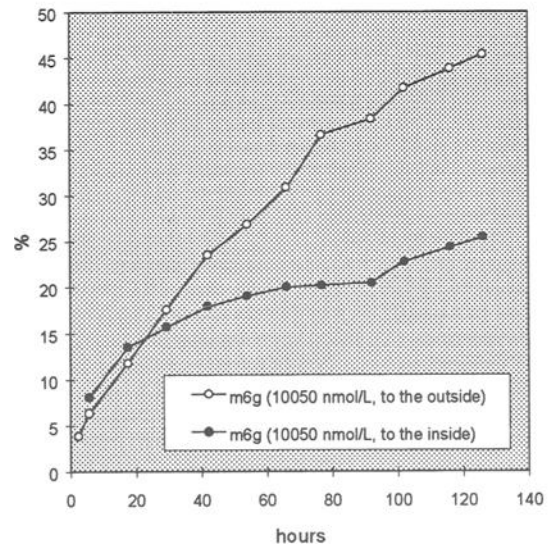

FIG. 4—*Concentration profile for morphine-6-glucuronide (m6g, initial concentration: 10050 nmol/L) simulating venous efflux versus venous influx, concentration in acceptor compartment as a percentage of initial concentration in donor compartment.*

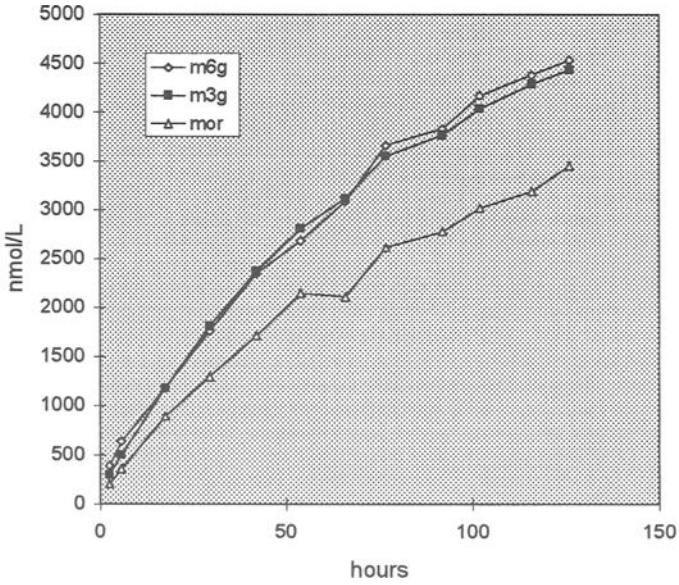

FIG. 5—*Time course of concentration for morphine (mor), morphine-6-glucuronide (m6g) and morphine-3-glucuronide (m3g). Initial concentration: morphine, morphine-3- and morphine-6-glucuronide: 10,000 nmol/L. Efflux simulation.*

elastic and collagen fibers were found associated with numerous smooth muscle fibers. Little is known about the permeability characteristics of the endothelium. Sinapius observed in 1958 that only 6 h after death had occurred, the intima may become detached (22). From this finding and from histological investigations (23), it can be concluded that the endothelial barrier of the vein samples used was at least partly damaged or even absent. As time passed, deterioration at the ultrastructural level was assumed to become more serious. These changes may strongly influence the permeation characteristics resulting in a flattening instead of the formation of a plateau of the hours versus concentration plots for some vein specimens.

The penetration of a substance through a cell membrane or through cellular layers depends on size, shape, charge, and partitioning behavior. Passive mechanisms of substance transport are filtration, free diffusion, solvent drag, and the Gibbs-Donnan-effect. These mechanisms are governed by hydrostatic and osmotic pressure, a concentration or an electrochemical gradient. In our experiments, the levels of chamber fluid in both cell halves were not quite the same throughout the duration of the experiments except for the Rhodamine B experiments. Small alterations of hydrostatic and osmotic pressure in the acceptor compartment were accepted in view of the fact that the increasing tissue disintegration affected the results more severely. Because the vein patch was held in a vertical position, there were no gravitational effects on the vascular wall. The chambers were filled with whole blood or an isotonic PBS solution of a pH value of 7.4. This arrangement allowed study of effects exclusively due to the increasing permeability of the venous wall. Temporal changes of pH value were small, the difference not exceeding 0.4. From the experimental conditions, it can be concluded that the principal driving force was the concentration gradient and free diffusion the underlying mechanism. Postmortem diffusion across vascular walls must be considered to be more extensive in a corpse than demonstrated by the in vitro investigations. Autolytic processes in the blood fluid as well as in the soft tissues in contact with the vascular walls and changes in the pH milieu will probably promote the diffusion phenomena.

Simple diffusion does not demonstrate saturation kinetics, and a balancing of substance concentration was not always achieved within 120 h. Therefore, the concentration profiles were analyzed according to Fick's 2nd law of diffusion. The curves obtained were non-Fickian curve types. The passage through a tissue membrane involves two additional factors that are the friction between solute and layer and between solvent and layer. The thermodynamic approach according to the Kedem-Katchalsky equations seems more promising and takes differences in hydrostatic and osmotic pressure into account (24). Regional and postmortem variations in permeability of the vein samples used and the steadily increasing autolysis did not allow a current mathematical treatment of the results.

However, the results clearly demonstrated that a postmortem permeation process through a vascular wall depended on autolysis, temperature, initial substrate concentration, molecular structure, and on the orientation of solute flux. Differences in permeation behavior due to storage temperature were observed for a time period of about two days in the experiments. Later, the difference became smaller and was even negligible. This may be attributed to a loss of inter- and intracellular integrity for the vein investigated. Considering the time interval prior to autopsy, it can be concluded from the results that even under favorable conditions, postmortem redistribution might be slowed only briefly by keeping the body in a cold place.

A lag time was observed for all substances investigated. From the Rhodamine B experiments, it can be concluded that the first process to occur was diffusion of solute into the vascular wall. As soon as the cross-sectional area was saturated, the molecules moved forward into the acceptor compartment. At high initial substance concentration, saturation of the vein wall was achieved more quickly resulting in a shorter lag time. As the Rhodamine B concentration in the acceptor compartment increased, its concentration in the vascular wall decreased. At the point in time when the progress curve had reached a limiting value, a small concentration (ca. 6%) was still bound to the tissue. This could be possibly explained by drug binding sites in the vascular wall. In addition to diffusion across a vascular wall, drug binding dependent on the tissue affinity and on the time since death is assumed to influence the result of postmortem drug concentration of a blood sample taken from a large vein.

The higher concentrations in the acceptor compartment as measured by the solute flux directed from the inside to the outside of the blood vessel indicated that the vein wall exhibits a certain directional preference for the permeation process.

For the antidepressive agent amitriptyline, postmortem redistribution of the drug and of its metabolite tended to change in the same direction (11). Regarding lipid solubility and the pKa as essential factors to influence the permeation characteristic of a nonionized drug substance in a more lipophilic environment, a similar pattern could be expected for amitriptyline and nortriptyline but not for morphine and its glucuronides. Glucuronides are considered to be highly polar, hydrophilic conjugates. In 1991 Carrupt et al. concluded from force-field and quantum mechanical calculations that morphine-3-glucuronide and morphine-6-glucuronide can exist in two conformational forms, the extended conformers being highly hydrophilic forms and the folded conformers being more lipophilic (25). The permeation behavior observed suggests that the glucuronides mask a significant portion of their polar moieties to cross a vascular wall, which is a medium of relatively low polarity.

In heroin involved death, the use of drug metabolism data for the estimation of survival time was investigated by several working groups, based on the concentrations of total and free morphine or of 6-acetylmorphine in blood, brain regions, or in tissue (20,26–28). From pharmacokinetic data after morphine dosing (15), the defined time-dependence of the parent drug to metabolite ratios seemed to offer a simple means for assessing the time interval from administration of heroin to the time of death (18). The results of this study, however, indicate that besides an intra-individual variation in pharmacokinetic parameters, disintegration and increasing permeability of all biological tissues such as the venous walls may cause differences in postmortem redistribution of parent compound and glucuronides and should be considered restrictive factors for precisely assessing survival time based on parent drug to metabolite ratios.

Although the diffusion cell cannot exactly simulate the events that occur postmortem, and is restricted in application to large veins, it appears to be a useful tool to investigate the permeation characteristics of a substance through a vascular wall and to elucidate some of the mechanisms of postmortem redistribution—A step toward overcoming a phenomenon that has been called a toxicological nightmare (9).

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Additional information and reprint requests:
Dr. Gisela Skopp
Institute of Legal Medicine
Voss strasse 2
69115 Heidelberg, Germany