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Coupling of microdialysis with capillary electrophoresis: a new approach to the study of drug transfer between two compartments of the body in freely moving rats

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ABSTRACT

The feasibility of *in vivo* microdialysis and capillary electrophoresis for pharmacokinetic studies was tested. Microdialysis probes were inserted in the jugular vein and brain of rats. After an intraperitoneal injection of phenobarbital, microdialysis was performed in the brain and the blood simultaneously in each rat under freely moving conditions. Capillary electrophoresis with ultraviolet absorption was used to measure phenobarbital in blood and brain dialysates. The time course of phenobarbital in the blood and in the extracellular space of the brain was followed. The results demonstrated that microdialysis can be used for pharmacokinetic studies in freely moving animals. Capillary electrophoresis has the potential to improve the time resolution of microdialysis. Other advantages of microdialysis and capillary electrophoresis for pharmacokinetic studies are discussed.

INTRODUCTION

The goal of pharmacokinetic studies is to determine the fate of a foreign substance, such as a medicament, in mammalian organisms. Pharmacokinetic studies are often referred to as ADME studies (absorption, distribution, metabolism, elimination). It is essential for these studies to sample the most relevant compartments of the body and determine the concentration of the drug and its metabolites in those parts as a function of time. Ideally the organism should suffer minimal disturbances during the sampling procedure, but this condition is very often difficult to fulfil. For instance, to study the transfer of drugs through the blood–brain barrier (BBB), the animal is killed in order to measure the total fraction of the drug in the blood and in the brain (post-mortem studies). In this last case, a kinetic study of BBB crossing of a drug cannot be done with the same animal under physiological conditions.

Recent developments of *in vivo* microdialysis and capillary electrophoresis look very promising for pharmacokinetic studies, specially to study the time course of the transfer of a given drug between two compartments. *In vivo* microdialysis allows the collection of chemical substances in the body fluids, such as the extracellular liquid of

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numerous organs, blood and cerebrospinal fluid, with minimal disturbance of the homeostasis of the animal. During microdialysis, a probe fitted with a semipermeable membrane is placed in the tissue or fluid to be dialysed and, depending on their concentration gradient and size, molecules pass through the pores of the dialysis membrane and are swept along by physiological perfusion fluid pushed inside the probe. The dialysates are then collected and analysed by an appropriate analytical technique [1–6].

In recent years, *in vivo* microdialysis has been used successfully to monitor changes in the levels of endogenous substances in the extracellular space of the brain of freely moving rats. Recently, this technique has been applied in pharmacokinetic studies and several results have been already published on the BBB crossing of drugs [7–9]. However, all the studies on the transfer of drugs through the BBB were performed with anaesthetized animals. Very recently, a comparative microdialysis study of the drug concentrations in blood in both anaesthetized and awake rats demonstrated that anaesthetics may interfere with drug pharmacokinetics by inhibition of metabolic enzymes in the liver [10]. Moreover, a long-term kinetic study of a drug cannot be done in anaesthetized animals. In this paper, we report the use of a flexible microdialysis probe [11] with a low molecular weight cut-off (MWCO) membrane placed in a vein and a rigid one inserted in the brain of the same animal. The time course of the concentration of the free fraction of a drug (unbound to proteins and the active one) in the blood and in the brain of a freely moving rat could be followed.

After intraperitoneal (i.p.) injection of phenobarbital into rats, the concentrations of this drug in blood and brain dialysates were determined by capillary electrophoresis (CE) with UV detection. General reviews on this growing analytical technique have already been published [12,13]. The main advantages of CE are high resolution, rapidity of analysis (1–5 min is common), high mass sensitivity (femtomole limits can easily be reached) and low volumes injected (a few nanolitres out of 1-μl samples can be analysed). This last advantage allows fast concentration changes of molecules in dialysates to be followed.

EXPERIMENTAL

Chemicals

$CaCl_2$, KCl and NaCl (Sigma, St. Louis, MO, USA) were used to prepare two different Ringer solutions. $Na_2B_4O_7$ and NaH_2PO_4 (Sigma) were used for the preparation of the buffers for the analysis by CE. Sodium dodecyl sulphate (SDS) was obtained from Bio-Rad Labs. (Richmond, CA, USA), mesityl oxide from Aldrich (Milwaukee, WI, USA), phenobarbital from Specia (Paris, France) and ketamine from Parke Davis (Substantia Division, Paris, France).

Microdialysis experiment

Subjects. Male Sprague–Dawley rats (weighing 250–300 g) were individually housed with food and water *ad libitum*. Room temperature was kept at 22 ± 2°C and the dark–light cycle was 12–12 h.

Microdialysis probes. Two types of microdialysis probes were used (Fig. 1), a flexible one for blood dialysis and a rigid concentric one for brain dialysis [5] (Europhor Instruments, Toulouse, France). The cellulose dialysis membrane used for those two probes had a 6000 dalton MWCO and an outside diameter of 235 μm.

Surgery. The rat was anaesthetized with an intraperitoneal injection of ketamine (120 mg/kg). Three skin incisions were made: one at the scarpa triangle, another at the neck between the gonion and the collar bone and the third on the head in the anteroposterior direction between bregma and lambda. A femoral catheter, made of a segment of silastic tubing 28 cm long, was inserted in the femoral vein and tied with unresorbable suture. A flexible microdialysis probe (Europhor Instruments) (Fig. 1), with a membrane length of 2.5 cm and 6000 dalton MWCO, was introduced into the jugular vein and attached to it with surgical thread. The femoral catheter was passed under the skin up to the cervical incision. The inlet metallic tube of the catheter and the inlet and outlet tubes of the flexible probe were then pulled

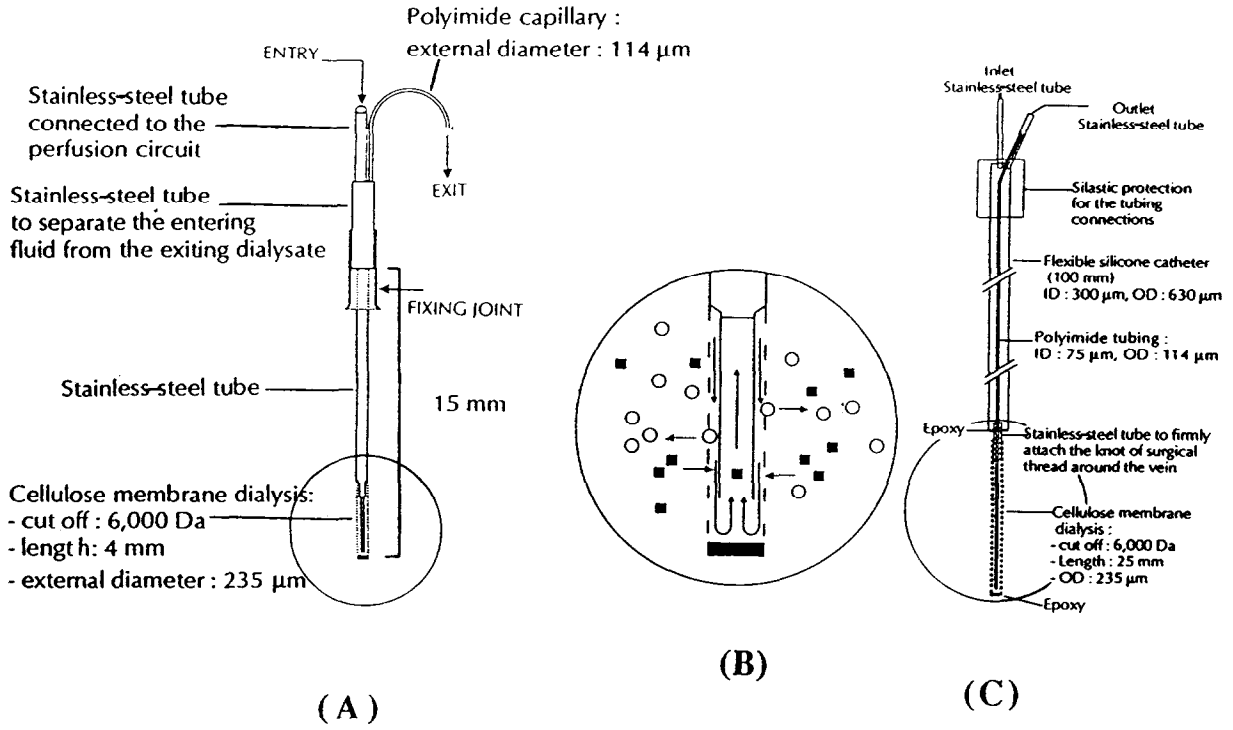

Fig. 1. Schematic diagrams of (A) the rigid concentric microdialysis probe and (C) the flexible microdialysis probe. (B) When the perfusion liquid arrives at the dialysis membrane, the chemicals of the extracellular fluid (■) diffuse through the dialysis membrane owing to the concentration gradient. It is also possible to inject drugs (○) into the extracellular fluid using the same concentration gradient phenomenom.

under the skin of the neck until reaching out to the skull. Both the inguinal and cervical incisions were sutured. The rat was then mounted on a stereotaxic instrument to insert a guide shaft aimed at the striatum. The guide shaft was made of a 10-mm-long segment of 21-gauge stainless-steel tubing. The stereotaxic coordinates [14] were $A = +9$ mm (from the interaural line), $L = +3$ mm (from the midsaggital suture) and $V = -4$ mm (from the surface of the skull). The guide shaft, the inlet tube of the catheter and the inlet and outlet tubes of the flexible probe were cemented on the skull of the rat with an anchor to connect the animal to a dual- or a triple-swivel joint.

Microdialysis procedure. Seven days after surgery, the rat was placed in the experimental cage. The inlet tubes of the flexible probe and of a rigid microdialysis probe (Europhor Instruments) [5] (Fig. 1) were connected, via the dual-swivel joint, to two syringe pumps (see Fig. 2). The inlet tube of the femoral catheter was connected to the perfusion circuit when *in vivo* recoveries of the probe were to be determined. In these experiments the rat had to be perfused with phenobarbital until reaching a steady-state concentration. The rigid probe was inserted into the guide shaft and the perfusions were started. The perfusion flow-rate was 1.5 µl/min of a Ringer solution (146 m*M* NaCl, 3.9 m*M* KCl, 1.2 m*M* $CaCl_2$) for brain perfusion and 1.5 µl/min of a different Ringer solution (140 m*M* NaCl, 3.5 m*M* KCl, 2.4 m*M* $CaCl_2$) for blood perfusion. After a 1-h stabilization period, an intraperitoneal injection of 40 mg/kg phenobarbital was made and brain and blood dialysates were collected every 10 min.

Analysis of the dialysates by CE with UV detection

Apparatus. The dialysates were analysed by micellar electrokinetic capillary chromatography

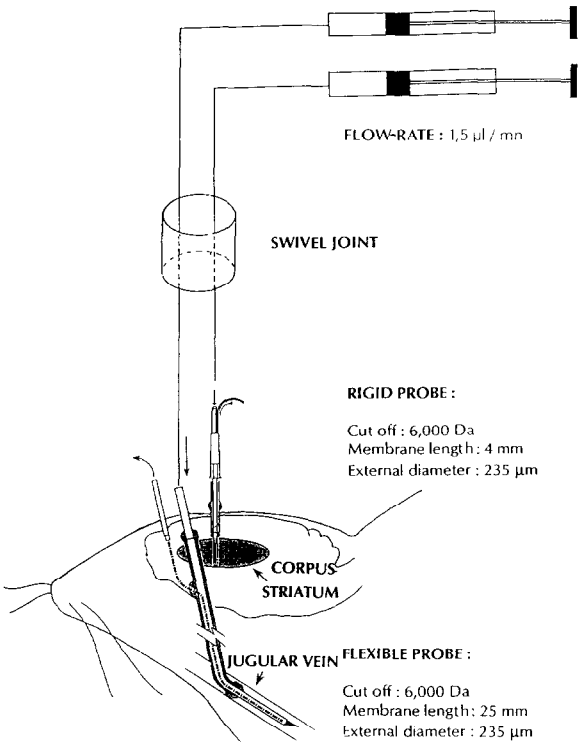

Fig. 2. Rat in which a flexible microdialysis probe has been implanted in its jugular vein and a rigid microdialysis probe in its brain corpus striatum. The characteristics of the probes and the connections of the inlet part of each probe to a dual-swivel joint are shown. The use of a dual-swivel joint allows the animal to move freely without twisting the perfusion catheters.

(MECC) [15] in a Fast Impact automatic analyser equipped with an UV detector and fast scanning capabilities (Europhor Instruments). The fused-silica capillary was 44 cm (34 cm to the detector) × 50 µm I.D. × 360 µm O.D. (Polymicro Technologies, Phoenix, AZ, USA). The capillary was rinsed with 1 *M* NaOH and with the separation buffer between injections.

Electrophoretic conditions. MECC separations were performed in 25 m*M* $Na_2B_4O_7$–50 m*M* NaH_2PO_4–50 m*M* SDS buffer (pH 7) using an applied voltage of 19 kV and a current of about 60 µA. Mesityl oxide was used as an internal standard; it is a neutral molecule that migrates with the electroosmotic flow. A 4-µl sample of dialysate was mixed with 2 µl of 5.1 m*M* mesityl oxide) and placed in a microvial. Injection volumes were 19 nl (5-s injection by vacuum). The fast scanning function of the detector was used to obtain the UV spectrum of the drug and integration was performed at 200 nm. The operating temperature was regulated at 25 ± 0.1°C.

Calibration of the probes. Microdialysis probes never recover 100% of the analyte in the surrounding medium. Therefore, in order to know the real concentration of the analyte in the extracellular compartment, the probes have to be accurately calibrated. Once the percentage recovery of the probe for a particular analyte is known, the real concentration of the analyte in the extracellular compartment is calculated by multiplying the concentration obtained in the dialysate by the recovery value. Two major recovery calibration methods, *in vitro* and *in vivo*, were used.

In vitro calibration of the probes. For the *in vitro* recovery determination of the probes for phenobarbital, we prepared a solution of phenobarbital at 1 mg/ml in Ringer solution. The composition of the Ringer solution was the same as that described for brain microdialysis when rigid microdialysis probes were tested, or that described for blood microdialysis when flexible microdialysis probes were tested (see *Microdialysis procedure*). The solution of phenobarbital was placed in a beaker at room temperature (24°C) and gently stirred. The microdialysis membrane of the probe to be tested was then placed in the phenobarbital solution and perfused at 1.5 µl/min with the same Ringer solution as used to dilute phenobarbital. The dialysates were collected for 10 min and then the concentrations of phenobarbital in the dialysates and in the beaker were measured by CZE under the same conditions as described before. The experiment was performed with three flexible and three rigid microdialysis probes.

In vivo calibration of the probes. In order to measure the *in vivo* microdialysis probe recovery for a particular compound, the best method is to insert a probe in the brain or the blood and then to add to the perfusion liquid of the probe various concentrations of the compound and to measure the concentration of the compound in the dialysate. The concentration in the dialysate minus the concentration in the perfusion fluid is

plotted (ordinate) against the concentration in the perfusion fluid (abscissa). The series of points thus obtained are fitted to a straight line by the least-squares method. The point of intersection of this line with the abcissa corresponds to the concentration of the compound in the extracellular compartment (see Fig. 3). This method was initially developed to determine glucose concentrations in the intercellular water space of human subcutaneous tissue [16] and has been succesfully used to measure the recovery of other endogenous compounds such as dopamine [17]. However, with an exogenous compound, a steady-state concentration of this compound in the organism of the animal has to be reached. The method consists in continuously injecting the drug into the blood until a steady-state concentration is reached. In this study, we used the results of Engasser *et al.* [18] on the pharmacokinetic determination of phenobarbital in rats to calculate the dose of phenobarbital to be infused into the femoral vein and the time of infusion required to reach a steady-state concentration of phenobarbital in the blood and in the brain.

The inlet metallic part of the femoral catheter was connected to a syringe filled with phenobarbital dissolved in Ringer solution. Phenobarbital was infused at a rate of 0.034 mg/kg · min and 24 h after the beginning of the infusion of phenobarbital blood and brain dialysates were collected

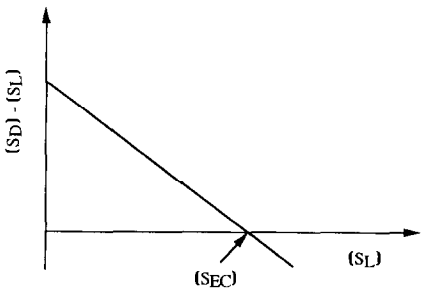

Fig. 3. Difference in concentration of the compound determined in the dialysate ($[S_D]$) and in the perfusion liquid which enters the probe ($[S_L]$) as a function of the concentration of this compound in the perfusion liquid of the probe. Point zero, the point at which $[S_D] - [S_L] = 0$, corresponds to the concentration of the compound in the perfusion fluid that matches the concentration of the compound in the extracellular space or in the blood ($[S_{EC}]$).

for 10 min and the concentration of the drug was measured by CE. This value was taken as the concentration of phenobarbital in dialysates when no phenobarbital was added to the perfusion fluid, and corresponded to the first point on the curve. We then took into account the *in vitro* recovery value to calculate a concentration of phenobarbital close to the true extracelllular brian or blood concentration. This concentration was added to the perfusion fluid. After 10 min, the concentration of phenobarbital in the dialysate was measured and a second point was determined. We then calculated by extrapolation the concentration in the perfusion fluid that produces the same concentration as in the dialysate. This value should represent the true concentration of phenobarbital in the extracellular fluid of the brain or in the blood.

RESULTS

Capillary electrophoretic analysis of phenobarbital

As we used microdialysis membranes with a 6000 dalton MWCO, large molecules such as proteins could not enter the microdialysis probes and the microdialysates were injected directly into the CE instrument without chemical pretreatment. The electropherogram presented in Fig. 4 at 200 nm or from 200 to 300 nm (in order to obtain qualitative information on the molecules from the UV spectrum) shows that mesityl oxide and phenobarbital, in the complex matrix of a dialysate, are well resolved and that the separation is achieved in less than 8 min.

Linearity of the measurements was determined using standard solutions of phenobarbital. The calibration graphs for phenobarbital in Ringer's solution were linear from 0.002 to 0.01 mg/ml (r^2 = 0.983) and from 0.01 to 0.05 mg/ml (r^2 = 0.986). The lowest concentration measured (0.002 mg/ml) corresponds to 38 pg of phenobarbital injected into the capillary. This value was close to the limit of detection.

The phenobarbital/internal standard peak-area ratio values obtained for brain dialysates were in the range of concentration between 0.002 and 0.01 mg/ml. For blood dialysates the ratios

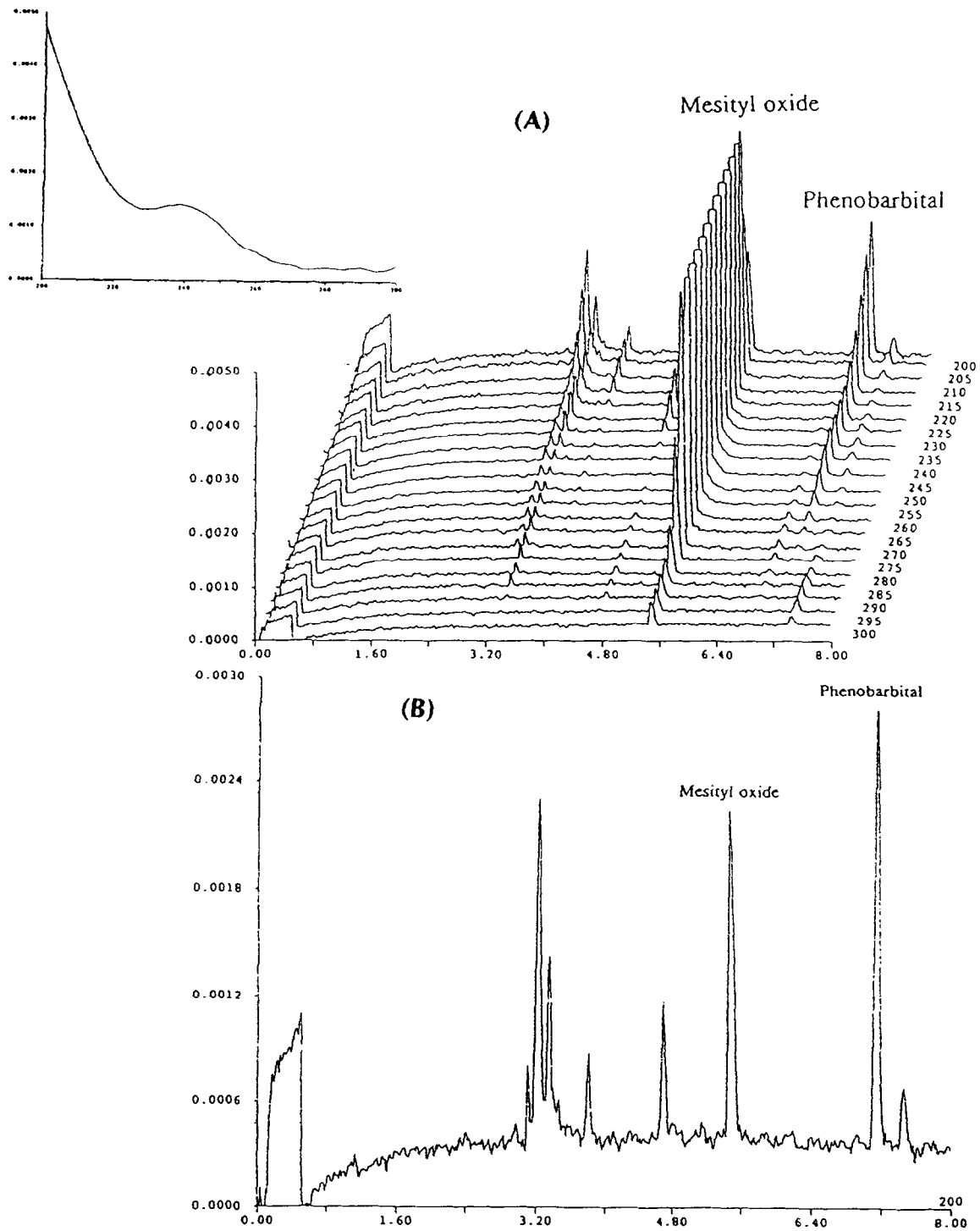

Fig. 4. (A) Three-dimensional electropherogram of a rat blood dialysate 10 min after i.p. injection of 40 mg/kg phenobarbital. A 4-μl volume of dialysate (collected during the 10 min following the i.p. injection) was mixed with 2 μl of 5.1 m*M* mesityl oxide (internal standard). The inset shows the UV spectrum of phenobarbital identified in the dialysate. (B) The same electropherogram at 200 nm. This wavelength was used for the integration calculations.

were between 0.01 and 0.05 mg/ml. However, in order to determine the true concentrations of phenobarbital in the extracellular space of the brain and in the blood, it was necesary to calculate the recovery of the microdialysis probes.

Determination of the in vitro and in vivo recoveries of the microdialysis probes (flexible and rigid) for phenobarbital

Hsiao *et al.* [19] demonstrated that an *in vitro* probe extraction value cannot be extrapolated to the *in vivo* extraction value. Their study was performed with acetaminophen and they measured the concentration of this drug in the dialysate after having perfused the probe with the drug in order to calculate the recovery of extraction. In this study, we compared the *in vitro* and *in vivo* recoveries of flexible and rigid microdialysis probes by using another method. We calculated that only 12 ± 4% (n = 3) of 1 mg/ml phenobarbital concentration was found in the dialysates of the rigid microdialysis probes compared with 80 ± 10% (n = 3) for flexible microdialysis probes (Table I).

TABLE I

IN VITRO AND *IN VIVO* RECOVERY VALUES FOR FLEXIBLE MICRODIALYSIS PROBES (MEMBRANE LENGTH 25 mm) AND FOR RIGID MICRODIALYSIS PROBES (MEMBRANE LENGTH 4 mm)

The MWCO was 6000 dalton for both probes.

	Recovery (%)	
	Rigid probe (n = 3)	Flexible probe (n = 3)
In vitro	12 ± 4	80 ± 10
In vivo	10 ± 5	75 ± 10

According to the *in vivo* recoveries obtained for three flexible and three rigid microdialysis probes in three different rats we calculated the concentrations of phenobarbital after i.p. injection of 40 mg/kg of this drug into three other rats. The plots of the concentrations of phenobarbital in the extracellular space of the striatum and in the blood as a function of time are presented in Fig. 5. We observed in blood first a large

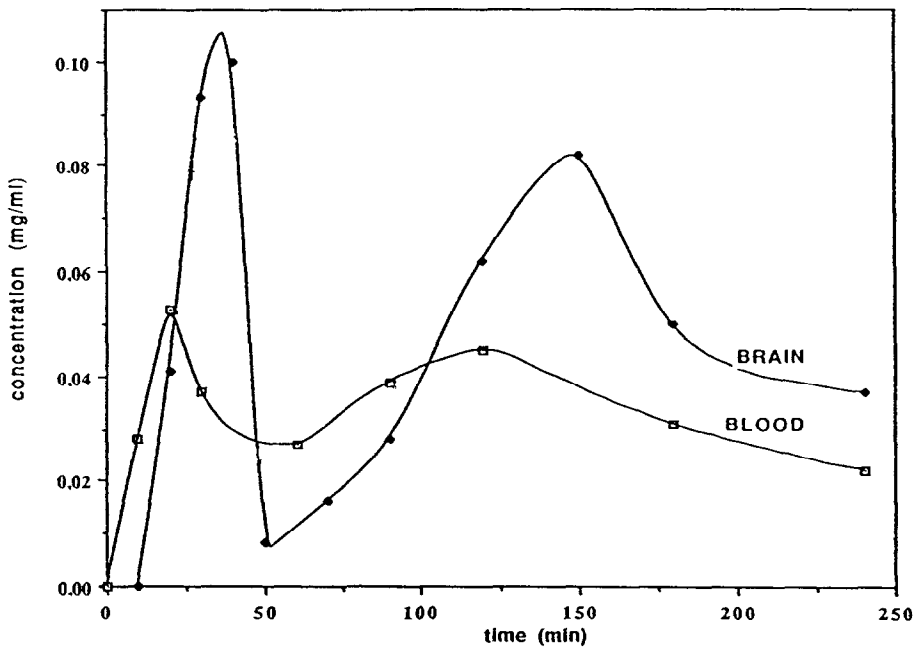

Fig. 5. Phenobarbital concentrations in the extracellular liquid of the striatum and the blood after an i.p. injection (40 mg/kg). The points represent mean values from three rats.

plasmatic peak followed by a flatier peak 100 min later. The same phenomenon was found in brain dialysates with a lag time of 15 min for the first and 30 min for the second peak. These lag times may be due to the BBB diffusion limitation processes. Even when the differences in concentrations calculated were large from one rat to another, the plots always had the same profile.

DISCUSSION

The great difference in recovery between the flexible and rigid microdialysis probes (80% *in vitro* and 75% *in vivo* for the flexible probe, against 12% *in vitro* and 10% *in vivo* for the rigid probe, Table I) is due to the differences in length of the dialysis membrane (25 mm for the flexible probe and 4 mm for the rigid probe). The surface for dialysis is 15.7 mm^2 for the flexible microdialysis probe and 2.5 mm^2 for the rigid probe. Interestingly, even though the *in vitro* and *in vivo* recovery experiments were not done with the same probe, we found no large differences in the *in vitro* and *in vivo* recoveries for the same type of probe. In fact, the *in vivo versus in vitro* recovery was 2 and 5% for the rigid and the flexible probe, respectively. However, we did find variations in the recoveries under the two conditions from one probe to another. This may explain, in part, why we obtained high standard deviations for the concentration values from the three rats studied. Moreover, the variations in the responses from one rat to another added to this dispersion. These variations could result from the intraperitoneal route of administration. More studies with more rats and with the calculation of the *in vivo* recovery for each probe are required in order to establish the exact concentrations of phenobarbital in the physiological fluids. Nevertheless, the profile of the concentration *versus* time plots was identical from one rat to another.

Both in the blood and in the brain, we observed a first peak followed by another, flatter peak about 100 min later. The observation of these two peaks may have different explanations. First, they could represent the phenobarbital and one of its metabolites. This seems unlikely. The retention time of the molecule analysed did not change and moreover we obtained the same UV spectrum. Second, it could be possible that the phenobarbital precipitates in the peritoneum and then it could be delivered into the general vascular circulation at different rates in time. Finally, after intravenous injection of phenobarbital into the rat, Engasser *et al.* [18] found the existence of a second peak of phenobarbital in the blood and in the brain 1 h after the injection of the drug. They explained part of this phenomenom by the existence of an enterohepatic cycle for this drug in rats. In the present study, we also found two peaks of phenobarbital in the blood and in the brain, but the lag time between the two peaks was 100 min. This difference of 40 min between our results and those of Engasser *et al.* [18] may be due to the fact that they did not measure the phenobarbital concentration between 1 and 2 h after the intravenous administration of the drug. It seems reasonable to propose, like Engasser *et al.* [18], that the two peaks we found may correspond in part to an enterohepatic cycle of phenobarbital in the rat.

According to the results shown in Fig. 5, the concentration of the free fraction of phenobarbital in the extracellular space of the brain was about twice that in the blood. As we have used 6000 dalton MWCO dialysis membranes, we measured only the free fraction of the drug in the blood and in the extracellular space of the brain. The concentrations we found in blood were consistent with those found by Engasser *et al.* [18] with a 48% value for the free fraction of the drug that they calculated. However, we found much more drug (about twice as much) in the extracellular space of the brain than in the blood. It was difficult to compare this value with that reported by Engasser *et al.* [18] because they measured the intra- and extracellular concentration of phenobarbital after tissue homogenization and extraction of the drug, and they expressed their results by weight of cerebral tissue.

Hurd *et al.* [7] compared the concentrations of cocaine in the brain and in the blood by using a microdialysis probe in the brain and in the blood in anaesthetized rats. Like phenobarbital, co-

caine, is a very lipophilic drug, and Hurd *et al.* [7] found, like us, twice as much free drug in the brain as in the blood. Their conclusion regarding cocaine may be the same as for phenobarbital, *i.e.*, that the high degree of lipophilicity of the drug was responsible for the high degree of diffusion of the molecule through the BBB. Moreover, as the unbound fraction of phenobarbital was greater in the brain than in the systemic circulation, it is possible that enhanced dissociation of the drug from proteins occurs in the brain microvasculature. This suggests a protein-mediated transport of phenobarbital.

The results presented here show that *in vivo* kinetic studies of a physiological barrier crossing of drugs can be done by microdialysis to collect the drug in a freely moving rat. We have described a method for performing the *in vivo* calibration of a probe for an exogenous substance in awake animals. This parameter is essential in order to establish the true concentrations of the drug in the physiological fluids. This *in vivo* calibration study has already been carried out for other drugs but in anaesthetized animals by Stahle *et al.* [20]. In the present experiments, we used freely moving animals because anaesthetics can change the pharmacokinetic parameters of a given drug [10]. Moreover, the long-term kinetics of the distribution of a drug in its targeted organs cannot be studied in anaesthetized animal. There is another difference between Stahle *et al.*'s study [20] and ours: we used continuous intravenous infusion of the drug to reach a steady-state concentration in the blood and in the brain, whereas Stahle *et al.* [20] used a single injection of the drug. Continuous intravenous infusion, which takes into account the pharmacokinetic parameters of a drug, suppresses the variations of drug concentration with time. Therefore, it is a better method for *in vivo* probe calibration.

In the pharmacokinetic field, *in vivo* microdialysis is not limited to the study of the tissue distribution of a drug but also allows the metabolism of a drug to be followed by collecting the metabolites *in situ* without extracting this tissue [21]. It seems also that *in vivo* microdialysis can be used for studying the binding of drugs to proteins avoiding *in vitro* techniques [8].

By using more classical analytical techniques, such as classical high-performance liquid chromatography (HPLC), at least a few microlitres of dialysate are needed for measurements of drug concentrations. This means that samples have to be collected for at least 10 min in order to obtain a sufficient volume of sample for analysis. In contrast, HPLC with microcolumns requires 1 μl or less, and CE requires only nanolitre volumes. Therefore, the time resolution of microdialysis can be improved, and low flow-rates can be used. This should be especially true when using CE with an on-line injector. However, for low concentrations of a drug in biological samples, and for low recoveries of the probe, more sensitive methods for CE are required. Currently, laser-induced fluorescence detection coupled to CE has allowed the detection of picomolar to femtomolar concentrations of molecules in a few nanolitres [22]. The combination of microdialysis and CE with laser-induced fluorescence detection has great potential for pharmacokinetic studies of drugs that are very potent at very low doses.

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