Microdialysis: An Alternative for *in Vitro* and *in Vivo* Protein Binding Studies

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The aim of the present study was to compare the performance of conventional equilibrium dialysis method with a microdialysis method in studying drug protein binding. The two methods were assessed by comparing the measured mean unbound drug fraction in different plasma species *in vitro* in plasma of four different species and at two concentrations of the non-indolic melatonin analog S 20098. For the microdialysis study, the unbound drug fraction was calculated after correction for membrane recovery. Plasma protein binding of S 20098 ranged from 75 to 95%. In humans, rabbits and rats (10 ng/ml), equal unbound percentages were found between equilibrium dialysis and microdialysis. Microdialysis gave slightly but significantly higher values in rat (2000 ng/ml), and in monkey plasma independent of the drug concentration. Microdialysis was also performed *in vivo* in freely moving rats under steady-state conditions, yielding similar unbound fraction values (26.0 ± 0.9%) to those obtained using microdialysis probes in rat plasma *in vitro* (24.4 ± 1.6%). These results support the use of *in vivo* microdialysis in pharmacokinetic studies in freely moving animals.

KEY WORDS: microdialysis; protein binding; equilibrium dialysis; *in vitro-in vivo* binding.

INTRODUCTION

Unbound drug concentration is considered to be a major determinant for pharmacokinetic and pharmacological responses to drugs in the body. Studies of drug binding to proteins are typically performed using equilibrium dialysis, ultrafiltration and ultracentrifugation (1–4). However, the classical equilibrium dialysis method suffers from some restrictions (1,2,4), including excessive dialysis time, possibly causing drug and/or protein degradation and allowing bacterial growth; nonspecific binding of the drug to the dialysis membrane; volume shift from the buffer to the protein cell.

The microdialysis technique uses the same basic principle and consists of a membrane permeable to water and small solutes which is continuously flushed on one side with a physiological solution. The dialysates are collected and analysed chemically, and they reflect the composition of the extracellular fluid. Consequently, microdialysis (5–9) is useful for studying the pharmacokinetics of a drug (10), in different tissues after a primary probe calibration step. Some *in vitro* experiments have already shown a good agreement in the evaluation of unbound fraction performed by equilibrium dialysis or ultrafiltration compared to microdialysis (11–14). One *in vivo* study (15) has been performed on anesthetized rats for the determination of the unbound fraction of theophylline. The purpose of the present study was to compare *in vitro* microdialysis with an equilibrium dialysis method in studying the protein binding of the non-indolic melatonin analog S 20098 in plasma from different species with different binding characteristics. Further, *in vivo* microdialysis was performed in freely moving rats to estimate the binding of S 20098 to plasma proteins under physiological conditions.

MATERIAL AND METHODS

Chemicals

S 20098 was a new non-indolic melatonin analog and was supplied by Servier France. All solvents and reagents were of analytical grade.

Microdialysis probe

Flexible microdialysis probes (concentric type) consisted of a tubular membrane (cellulose hollow fiber: 25 mm length, molecular-weight cut off 6000 daltons, outside diameter 235 μm, Europhor Instruments, Toulouse, France) were used. Probes were linked to a syringe infusion pump (EP 122 Europhor, Toulouse, France) and were perfused at a flow rate of 1 μl/min.

Determination of recovery

Relative recovery was estimated from plasma using the Point-of-no-net-flux method, first *in vitro* with a known concentration of S 20098 at 37°C as the surrounding medium. Second, the same experiment was performed *in vivo* in freely moving rats under steady-state conditions. The dialysis probe was perfused with Ringer's solution (Cin) containing varying concentrations of the drug. Taking into account the dead volume and the time to reach a stable dialysate concentration, we have determined the appropriate time to start collection and then drawn three 20 μl consecutive samples (Cout). The difference between Cin − Cout = Dc was calculated and plotted versus Cin. By linear least square regression, the slope of the regression line was calculated and expressed the recovery (16,17). The intercept with abscissa gave the situation where there was no net transport of drug across the dialysis membrane.

Binding experimental design

Protein binding was performed in triplicate at 37°C for equilibrium dialysis and microdialysis methods at two concentrations of S 20098: 10 and 2000 ng/ml. For *in vitro* experiments plasma from Wistar rat, Macaca fascicularis monkey, New Zealand rabbit and human were used. Plasma was extracted in Vacutainer EDTA K3 (15%) tubes (Becton Dickinson, Meylan, France) and frozen at −20°C.

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Equilibrium dialysis

1 ml plasma was dialysed against phosphate buffer (0.15 M), pH = 7.4. Dialysis membrane (Dialysis Diachema), molecular weight cut off 10 000 daltons were used. After one hour dialysis, samples were collected from both chambers.

In vitro microdialysis studies

Microdialysis probes were placed in a 20 ml plasma beaker. After the stabilization time previously evaluated, three 20 µl fractions were collected. Three corresponding aliquotes from the dialysis medium were drawn to estimate total plasma concentrations.

In vivo microdialysis study

Animals. Male Wistar rats (Dépré, France) weighing 300-400 gr were used throughout the experiments. They were maintained on standard laboratory rat diet on *ad lib* food and water.

Microdialysis conditions. The day prior to the experiment, rats were anesthetized with an intraperitoneal (150 mg/kg) injection of Imalgene 1000 (Rhône-Mèrieux, Lyon, France) and a cannula was inserted into the left jugular vein for intravenous administration of the drug. The left carotid artery was cannulated for blood collection. The microdialysis probe was implanted in the right jugular vein. Cannulas (polyethylene, PE EO 3401 Biotrol, France) were filled with heparin-saline solution. Twenty four hours later, awake animals were housed in the microdialysis cage. Probes were perfused with Ringer's solution. Rats were given an appropriate loading dose of S 20098 bolus I.V. followed by a constant infusion rate to maintain a steady-state concentration of 30 ng/ml. Microdialysis samples (20 µl) were collected from the beginning of the infusion by 20 minute periods. Steady-state free concentrations were confirmed in consecutive samples showing the same analytical response. Dialysate samples were analysed immediately by H.P.L.C. In order to verify that steady-state was reached and was maintained during the course of the experiment and in order to determine total plasma concentrations of S 20098, three blood samples (1, 3 and 6 hours after the beginning of the infusion) were drawn from the carotid cannula. After centrifugation, plasma samples were stored at −20°C until analysis. The unbound fraction of S 20098 was calculated according to the following equation:

$$\text{fu (\%)} = \frac{\text{dialysate concentration/relative recovery}}{\text{total plasma concentration}} \times 100$$

Chromatographic conditions

All sample concentrations were determined with an H.P.L.C. fluorimetric system consisting of an isocratic LC. Pump 250 Perkin-Elmer, a Rheodyne injection valve (Model 7125 with a 20 µl sample loop) and a Perkin-Elmer LC 240 fluorescence detector. The column was a 15 cm × 0.5 mm packed with 5 µm Spherisorb C18 phase. The excitation and emission wavelengths were 277 and 356 nm respectively. The mobile phase consisted of 30% acetonitrile, 70% phosphate buffer delivered at a flow rate of 1.2 ml/min. The analytical validation was assessed in a previous study. The detection response was tested using peak heights. It was linear both in the range from 0.2 to 10 ng/ml and from 5 to 1000 ng/ml resulting in correlation coefficients of 0.999. The detection limit, based on peak height vs. baseline noise ratio 2:1 was 0.1 ng/ml of S 20098 in Ringer's solution. The limit of quantification was 0.2 ng/ml. Precision values based on three replicate injections were 18.5% for 0.2 ng/ml and below 5.4% for the other concentrations.

Statistics

First order least square regression was used to calculate the slope and intercept values in the Point-of-no-net-flux method. Correlation between microdialysis and equilibrium dialysis data were performed and the intensity of the relation calculated. Unbound fractions obtained with microdialysis *in vitro* were compared to those obtained with equilibrium dialysis at each concentration for each plasma species by a Fisher analysis of variance and Newman-Keuls tests. *In vitro* and *in vivo* microdialysis estimations of protein binding were compared by a Fisher analysis of variance. The results are presented as means ± standard deviations for three measurements.

RESULTS

Probe recovery

A stable dialysate concentration was reached within a 20 minutes perfusion period. Then, the first collection began 30 minutes after starting the probe perfusion. Mean recovery obtained from the slope of the regression line was 100.5 ± 2.6% and 91.3 ± 5.6% for *in vitro* and *in vivo* experiments respectively. Figure 1 shows the regression lines obtained in three freely moving rats to estimate *in vivo* probe recovery.

Binding experimental results

In vitro. The estimates of unbound fractions obtained with equilibrium dialysis and microdialysis are shown in Table I. Non specific adsorption determined during equilibrium dialysis method was 30%. The extent of plasma protein binding of the S 20098 was statistically different between each of the four studied species and ranged from 75 to 95%. Results

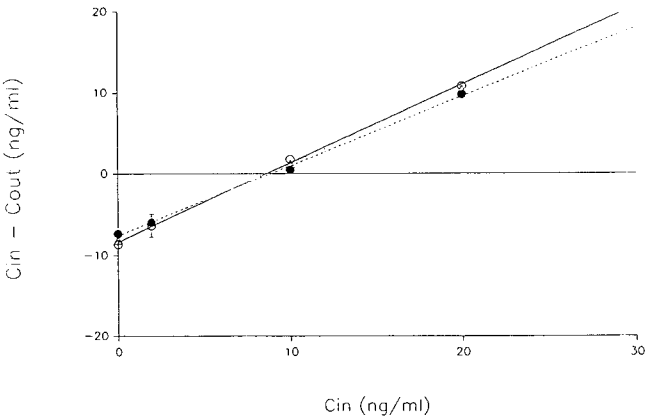

Figure 1: Probe recovery estimated *in vivo* by the Point-of-no-net-flux method applied to three freely moving rats.

Table I: *In vitro* estimation of unbound fractions of S 20098 by equilibrium dialysis and microdialysis in different plasma species. H: 2000 ng/ml, L: 10 ng/ml, n = 3.

	Equilibrium dialysis fu %	Microdialysis fu %
Rabbit H	14.8 ± 1.5	13.9 ± 0.8
Rabbit L	13.9 ± 1.1	12.5 ± 0.5
Rat H	18.7 ± 1.1**	24.4 ± 1.6*
Rat L	21.8 ± 0.4**	22.6 ± 0.7
Monkey H	21.4 ± 0.4**	29.7 ± 1.5*
Monkey L	25.2 ± 0.6**	28.2 ± 0.8*
Man H	5.8 ± 0.1	5.2 ± 0.1
Man L	5.5 ± 0.1	5.2 ± 0.1

*: $p < 0.05$ between methods
**: $p < 0.05$ between doses

obtained with two largely different values (10 and 2000 ng/ml) showed that S 20098 protein binding was not concentration dependent in human and rabbit plasma and showed a concentration dependent binding in rat and monkey plasma. A comparison of the microdialysis with equilibrium dialysis data is presented in Figure 2, the correlation factor was $r = 0.96$ ($p < 0.01$).

In vivo. Total plasma concentrations of S 20098 from carotid blood samples, dialysate concentrations and calculated unbound fractions are summarized in Table II. Mean unbound fraction values obtained from three freely moving rats were 26.0 ± 0.9%.

DISCUSSION

In order to determine the drug concentrations surrounding the dialysis membrane which essentially depend on mass transport processes, several methods have been developed to circumvent the differences in mass transport between media (16–21). In the Point-of-no-net-flux method used in this work, it was assumed that the direction of the diffusion across the microdialysis membrane follows the concentration gradient. This method is based on determining mass transport of the drug across the membrane as a function of perfusate concentration. The *in vitro* performance of this method has already been assessed (22) and more recently the method was evaluated *in vivo* (15,18).

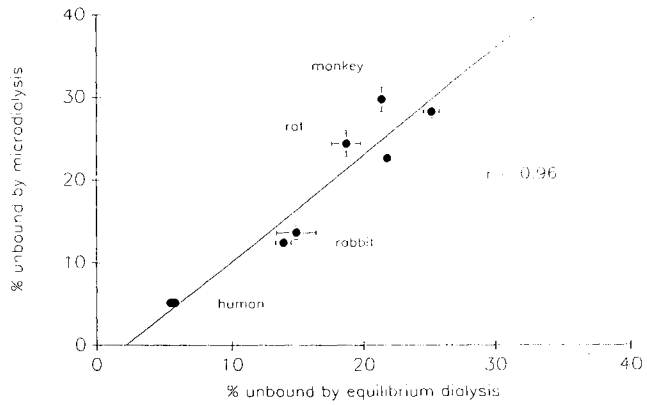

Figure 2: Correlation between equilibrium dialysis and microdialysis.

Table II: Unbound fractions, total concentrations from carotida blood collection, dialysate concentrations of S 20098 in three different freely moving rats under steady-state conditions, n = 3.

	C total ng/ml	C dialysat ng/ml	Recovery %	fu %
Exp. 1	34.2 ± 7.8	8.6 ± 0.4	96.7	26.0
Exp. 2	34.1 ± 6.2	7.3 ± 0.4	85.5	25.0
Exp. 3	32.8 ± 4.3	8.1 ± 0.2	91.6	26.9
Mean				26.0
S.D.				0.9
C.V. %				3.5

The error induced by the Point-of-no-net-flux method has been estimated by microdialysis in overloaded buffer. The concentration calculated by the Point-of-no-net-flux method did not differ by more than 10% from the concentration in buffer. The high recovery value (about 100%) observed can be compared to previous studies using low infusion rates and a large enough surface (23–25). However, the recovery obtained by this method may be influenced by the perfusion flow rate (15) or by the perfused concentrations (16).

The S 20098 showed significant binding to plasma protein in all species studied. The extent of protein binding ranged from 75 to 95% with statistically different values between species, as often observed during interspecies pharmacokinetic experiments. S 20098 unbound fractions obtained from equilibrium dialysis and microdialysis were the same *in vitro* for two species (humans and rabbits), equal for rat plasma (10 ng/ml), and slightly higher but statistically significant with microdialysis in rat (2000 ng/ml) and in monkey plasma independent of the concentrations of the drug. The differences between microdialysis and equilibrium dialysis could result from non-specific adsorption. Indeed, the non-specific adsorption observed with equilibrium dialysis (30%) might be an important factor of variability at low plasma binding (rat, monkey). This problem associated with equilibrium dialysis could be due to the large area of the membrane used 4.52 cm^2 for equilibrium dialysis against 15.7 mm^2 for microdialysis. Another disadvantage of the equilibrium dialysis method, due to the volume shift from the buffer to the protein cell, could partly explain the differences observed. Compared to equilibrium dialysis, microdialysis offers the advantage of unchanged volumes of the sample and drug concentrations. The binding equilibrium does not change during the experiment (25).

In conclusion, small differences between unbound fractions were obtained with equilibrium dialysis and microdialysis for two of the plasma studied, but the accuracy of microdialysis in estimating unbound concentrations of drug in blood was confirmed by the observation of the degree of binding of the same magnitude as those obtained by dialysis equilibrium. The good correlation of the microdialysis and the equilibrium dialysis data showed that there is no apparent bias in the estimation of S 20098 binding by microdialysis relative to equilibrium dialysis.

In a previous study, Sjöberg et al (15) concluded that microdialysis has become a useful tool in estimating unbound fraction of a drug in anesthetized rats. We compared

in vitro and *in vivo* experiments by using a microdialysis probe in awake animals. Equilibrium dialysis and microdialysis *in vitro* agreed well, and S 20098 microdialysis binding experiments *in vitro* and *in vivo* gave the same results, 24.4 ± 1.6 and 26.0 ± 0.9%, respectively.

The present data support the use of microdialysis in pharmacokinetic studies. For correctly determinating interstitial tissue concentrations *in vivo*, using correction factors other than *in vitro* recovery, the use of *in vivo* microdialysis, working at steady-state conditions, is valuable for estimating drug binding to plasma proteins in freely moving animals.

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