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Research Article

Concentration-Dependent Plasma Protein Binding of Flurbiprofen in the Rat: An in Vivo Microdialysis Study

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Purpose. The in vivo plasma protein binding and pharmacokinetics of flurbiprofen were studied in awake, unrestrained rats using intravenous microdialysis sampling.
Methods. Flurbiprofen (20 mg/kg) was administered i.v. to 2 groups of 6 rats: in both groups sampling was carried out by microdialysis, but in the second group an addional 10 blood samples were withdrawn via a jugular cannula. In vitro and ex vivo (following i.v. administration of flurbiprofen 20 mg/kg to another group of 13 rats) plasma protein binding of the drug was determined by equilibrium dialysis.
Results. The area under the unbound plasma concentration-time profile of flurbiprofen (AUC_u), determined by microdialysis sampling, was somewhat smaller (−19%, $p = 0.066$) in the rats undergoing simultaneous serial blood sampling (2.21 ± 0.36 μg.h/ml) as compared to the rats undergoing microdialysis sampling only (2.73 ± 0.60 μg.h/ml). Comparison of total and unbound concentrations of flurbiprofen showed an in vivo plasma binding varying between 99.5% at low and 98.0% at high total flurbiprofen plasma concentrations. Plasma binding of flurbiprofen determined in vitro over the same concentration range was higher (99.5-99.9%) but also concentration-dependent. Plasma binding of flurbiprofen determined ex vivo, on the other hand, corresponded well with the in vivo binding.
Conclusions. Monitoring the fraction of drug unbound in blood of an individual rat throughout a pharmacokinetic experiment has now become possible by using simultaneous sampling of blood and intravenous microdialysates.

KEY WORDS: flurbiprofen pharmacokinetics; intravenous microdialysis; plasma protein binding.

INTRODUCTION

The reversible binding of a drug to plasma proteins is an important determinant of its pharmacokinetic and pharmacodynamic characteristics (1). For certain drugs saturable plasma protein binding may occur at therapeutic concentrations and is a well known cause of dose-dependent pharmacokinetics (2). Total concentrations of drug in plasma are most frequently measured when carrying out pharmacokinetic studies and the obtained values are converted to unbound concentrations using the unbound fraction in plasma (fu) estimated by one of several in vitro techniques, such as equilibrium dialysis or ultrafiltration (3). This approach has the serious limitation that fu values estimated in vitro using blank plasma spiked with the drug may be different from the in vivo value. This drawback may be (partially) overcome by determining fu ex vivo. Estimation of fu ex vivo at each sampling time during a pharmacokinetic experiment allows the estimation of unbound plasma concentrations and is very useful to accurately describe the unbound plasma concentration-time profile of the drug in question (4). However, this approach is not practical in small laboratory animals because of their limited blood volume.

Microdialysis is a sampling technique which has recently gained wide acceptance to measure in vivo extracellular concentrations of both endogenous (e.g. neurotransmitters) and exogenous substances (e.g. drugs) (5-7). Intravenous microdialysis sampling allows the on-line determination of unbound concentrations of drugs in blood and other tissues of small laboratory animals and man with an extremely high temporal resolution in comparison to the classical blood sampling techniques (6-8). Since no liquid is removed from the laboratory animal during microdialysis sampling, the only factor limiting the number of data points in the unbound plasma concentration-time profile when carrying out on-line microdialysis is the run time of the analytical method.

The 2-arylpropionic acid (2-APA) nonsteroidal antiinflammatory drugs are highly bound to plasma albumin (9) and concentration-dependent plasma protein binding has been reported in man for ibuprofen (10), naproxen (11), and to a lesser extent flurbiprofen (12). Information on the plasma protein binding of many of these agents in the rat is rather limited. The present study describes the pharmacokinetics of unbound flurbiprofen in the rat using intravenous microdialysis sampling. In a number of rats serial blood sampling was carried out simultaneous to continuous intrave-

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nous microdialysis sampling. The results of these experiments allowed us to estimate in vivo B_{max} and K_d, parameters characterizing the albumin-flurbiprofen interaction.

METHODS

Drugs and Reagents

Flurbiprofen and naproxen were purchased from Sigma Chemical Co. (St. Louis, Missouri). Solvents were of HPLC grade and all other chemicals used were AR grade.

Animals

Male Wistar rats, weighing between 260 and 320 g were obtained from the Animal Breeding Facilities of the Faculty of Medicine. The animals were housed in an environmentally controlled room at 20-22 °C with a 12-hour light/dark cycle. Food (type A04, U.A.R., Epinay-sur-Orge, France) and tap water were provided ad libitum. All experimental procedures in rats were approved by the University Animal Experimentation Ethics Committee.

In Vivo Microdialysis Sampling

Full details of the construction, surgical implantation and in vitro evaluation of the microdialysis probes are described elsewhere (8). During the surgical implantation of the microdialysis probe, two jugular vein cannulae were also placed, one to administer the drug and the other to withdraw blood samples.

Approximately 24 hours following the surgical implantation of the microdialysis probe, a 20 mg/kg flurbiprofen dose (dissolved in polyethylene glycol 400, 10 mg/ml) was injected i.v. via one of the jugular vein cannulae. The dialysate samples were collected directly into a 10 µl PEEK^R injection loop of a 10-port valve (Valco Instruments Co. Inc., Houston, Texas) and automatically injected every 10 minutes onto a high-performance liquid chromatography (HPLC) system. Microdialysis sampling was carried out during 6 to 8 hours following drug administration. In one group of rats (n = 6) serial blood samples were withdrawn together with microdialysis sampling, whereas in the other group (n = 6) only microdialysis sampling was carried out. In the simultaneous blood sampling studies, blood (250 µl) was collected in heparinized tubes via the second jugular vein cannula at the following times: 0 (blank), and 5, 15, 30 min, 1, 2, 3, 4, 5, and 6 hours following drug administration. Blood samples were immediately centrifuged and the plasma stored at −20 °C until analysis.

In Vitro and ex Vivo Plasma Protein Binding of Flurbiprofen

The unbound fraction of flurbiprofen in plasma was determined in vitro by equilibrium dialysis (Dianorm, Münich, Germany). Blank blood was collected in heparinized tubes (Microtainer, Beckton & Dickinson, Rutherford, New Jersey) by orbital sinus sampling and immediately centrifuged to separate the plasma. Plasma thus obtained from several rats was pooled and subsequently spiked with flurbiprofen to obtain the following concentrations: 0, 5, 10, 20, 40, 60, 80, and 100 µg/ml. These plasma samples were dialyzed in duplicate for 4 hours at 37 °C against isotonic phosphate buffer (pH 7.4) in 1 ml teflon dialysis cells using semipermeable membranes with a 5000 MW cut-off (Spectrapor 2, Spectrum Medical Industries, Los Angeles, California). Plasma albumin concentrations were determined before and after dialysis by the bromcresol dye binding method (Sigma Diagnostics, St. Louis, Missouri) and employed to correct the fraction unbound for volume shifts (13). Flurbiprofen concentrations in plasma and buffer were determined by HPLC as described below. The fraction unbound (fu) was calculated as follows: $fu = [flur]_{bu}/[flur]_{pl}$, where $[flur]_{bu}$ and $[flur]_{pl}$ stand for the flurbiprofen concentrations at equilibrium in the buffer and plasma compartments, respectively.

To determine the ex vivo plasma protein binding of flurbiprofen, flurbiprofen (20 mg/kg) was administered i.v. to 13 rats via a jugular cannula (implanted the day before). Five ml blood was withdrawn in heparinized tubes via the second jugular cannula at the following times after drug administration: 15 and 30 min, 1, 3 and 6 hours. Each rat was only sampled once. Each plasma sample thus obtained was dialyzed in duplicate and the fu estimated as described above.

Analytical Methods

On-line analysis of microdialysate samples was accomplished by HPLC as described elsewhere (8). Flurbiprofen concentrations in plasma samples (simultaneous blood sampling and intravenous microdialysis experiments, ex vivo plasma protein binding studies) were determined using the same HPLC equipment and conditions as described for the on-line analysis of microdialysate samples (8). To 100 µl of plasma were added 900 µl of methanol containing 3 µg naproxen/ml (internal standard). The mixture was vortexed and centrifuged and an aliquot of the supernatant was injected manually on the HPLC system using a 10 µl sample loop. Aliquots of buffer after completion of equilibrium dialysis were directly injected onto the HPLC system. Flurbiprofen microdialysate concentrations (C_μ) were converted to unbound concentrations (C_u) as follows:

$$C_u = \frac{C_\mu}{L_{retro}}$$

where L_{retro} is the in vivo loss of flurbiprofen by retrodialysis determined just before i.v. administration of flurbiprofen. During intravenous microdialysis sampling the probe was perfused with a physiologic phosphate buffer containing naproxen (250 ng/ml) and the retrodialysis of this marker was used to monitor the integrity of the probe during the pharmacokinetic experiment (8).

Data Analysis

Plasma and microdialysate flurbiprofen concentration-time profiles were fitted to a biexponential equation by nonlinear least squares curve fitting using the program COMSTAT (14). The following pharmacokinetic parameters were then obtained from the best-fit coefficients and exponents: terminal elimination half-life ($t_{1/2}$), MRT, CL, and V_{ss}. The fraction of flurbiprofen unbound in plasma (fu) as a function of total flurbiprofen plasma concentration was calculated as the ratio of the bi-exponential equations describing the unbound concentrations (microdialysis sampling) and plasma concentrations (serial blood sampling) of flurbiprofen as a function of time.

Based on unbound concentrations determined by microdialysis and total plasma concentrations the binding of flurbiprofen was analyzed in terms of one site and two site models using the nonlinear least squares regression program COMSTAT (14):

$$C_b = \frac{B_{max}\, C_u}{K_d + C_u} + a\, C_u \qquad \text{one site model}$$

$$C_b = \frac{B_{max,1}\, C_u}{K_{d,1} + C_u} + \frac{B_{max,2}\, C_u}{K_{d,2} + C_u} + a\, C_u \qquad \text{two site model}$$

where C_b and C_u are the bound and unbound concentrations of flurbiprofen respectively, B_{max} is the maximum binding site concentration and K_d is the equilibrium dissociation constant. Binding constants for the first and second site are indicated by 1 and 2. Non-saturable binding is shown as a linear function of C_u with slope a. The most appropriate model (one site or two site model, with or without non-saturable binding) was determined using the goodness-of-fit criteria described by Boxenbaum et al. (15).

Unless otherwise stated, values in the text and tables are expressed as means ± SD. Comparisons between groups were performed by the Student t test. A p-value of 0.05 or less was considered significant.

RESULTS

The pharmacokinetics of flurbiprofen were studied in two groups of rats following a single dose (20 mg/kg) i.v. administration. In rats of the first group, besides microdialysis sampling, a total of 10 blood samples was withdrawn at regular intervals following administration of the drug (simultaneous blood sampling experiments). In the second group only microdialysis sampling was carried out (control experiments). Figure 1 shows the average total blood concentrations of flurbiprofen in the blood sampling group and the average unbound concentrations of flurbiprofen determined by intravenous microdialysis sampling in both study groups. There was a small but consistent decrease of approximately 19% (p = 0.066) in the AUC_u of flurbiprofen when blood samples were taken simultaneously as compared to microdialysis sampling alone. Table I summarizes the pharmacokinetic parameters of total and unbound flurbiprofen determined by microdialysis sampling. The systemic clearance of unbound flurbiprofen was significantly faster when blood samples were simultaneously withdrawn: 45.2 ml/min as compared to 35.6 ml/min for the microdialysis only experiment. Vu_{ss} and $t½_z$ were not affected by simultaneous blood sampling. Interestingly, the terminal half-life of flurbiprofen was somewhat longer (p = 0.064) when considering total plasma concentrations (4.5 ± 1.5 h) as compared to unbound concentrations of flurbiprofen (2.8 ± 1.4 h).

Table I. Pharmacokinetic Parameters of Unbound (Upper Panel) and Total Flurbiprofen in Rats: Data Obtained by Microdialysis with and Without Simultaneous Blood Sampling (SBS)

Parameter	With SBS (n = 6)	Without SBS (n = 6)	p-value[a]
AUCu (μg · h/ml)	2.21 ± 0.36	2.73 ± 0.50	0.066
CLu (ml/min)	45.2 ± 6.4	35.6 ± 5.9	0.022
Vuss (L)	7.09 ± 3.15	6.18 ± 1.76	NS
$t½_z$ (h)	2.8 ± 1.4	3.6 ± 2.1	NS
MRT (h)	2.6 ± 1.1	3.1 ± 1.6	NS
AUC (μg · h/ml)	328.9 ± 85.1		
CL (ml/min)	0.32 ± 0.10		
Vss (ml)	101.9 ± 12.2		
$t½_z$ (h)	4.5 ± 1.4		
MRT (h)	5.8 ± 1.7		

[a] Unpaired t-test.

The ratio of unbound to total blood concentrations of flurbiprofen, both obtained during the experiments with simultaneous microdialysis and blood sampling, was not constant but increased with increasing total flurbiprofen blood concentration (fig. 2). This concentration-dependent plasma binding of flurbiprofen was also demonstrated in vitro using equilibrium dialysis: the unbound fraction of flurbiprofen in plasma was approximately 0.001 at a total concentration of 5 μg/ml and gradually increased to reach a value of approximately 0.005 at a total concentration of 100 μg/ml. However, plasma binding of flurbiprofen as determined in vitro was

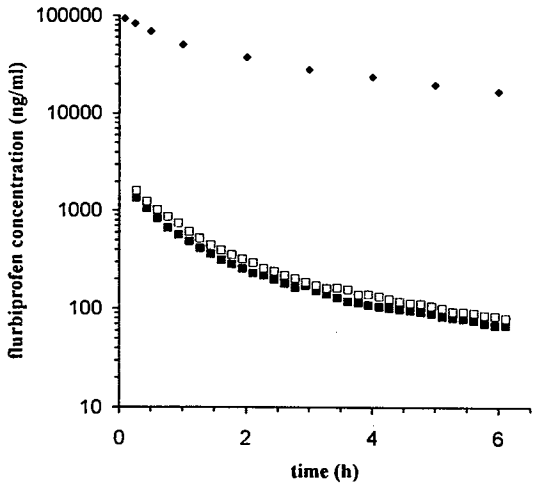

Fig. 1. Semi-log presentation of the concentration-time profiles of total flurbiprofen in plasma (♦) and unbound flurbiprofen in microdialysate samples (□: microdialysis sampling only; ■: microdialysis and simultaneous blood sampling). Each point represents the mean concentration in 6 rats.

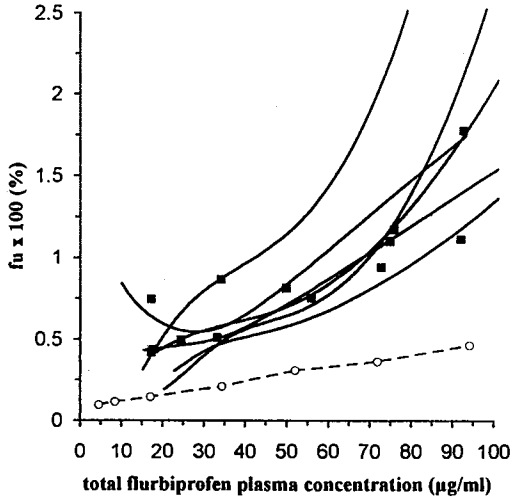

Fig. 2. Individual curves showing fu as a function of total flurbiprofen plasma concentrations were obtained by taking the ratio of unbound concentrations in microdialysate to total plasma concentrations of flurbiprofen in the 6 rats undergoing simultaneous intravenous microdialysis and serial blood sampling. The unbound plasma fraction of flurbiprofen determined in vitro is also shown (○----○). fu was also determined ex vivo in plasma obtained in 13 rats (■) at different times following I.V. administration of flurbiprofen.

higher than the binding derived from the simultaneous microdialysis and blood sampling experiments. Ex vivo plasma binding studies on the other hand were entirely consistent with the binding curves obtained by simultaneous microdialysis and blood sampling experiments (fig. 2).

Based on the results of the in vivo study, i.e. simultaneously determined unbound (derived from i.v. microdialysate samples) and total plasma concentrations of flurbiprofen, the plasma protein binding of flurbiprofen was evaluated. The necessity to take non-saturable binding of flurbiprofen into account was assessed by comparing fits with and without the nonspecific binding components i.e. a C_u. In all cases the one site binding model was sufficient to fit the data. For 5 of the 6 rats inclusion of the nonspecific binding component into the one site model significantly improved the fit (Fig. 3). The binding parameters B_{max} and K_d for the interaction of flurbiprofen with plasma proteins are summarized in Table II.

Table II. Binding Parameter Estimates for Flurbiprofen in Rat Plasma Using a One Site Binding Model Including a Linear Non-Saturable Term

	B_{max} (μg/ml)		K_d (μg/ml)		*a*	
Rat	Value	S.E.[a]	Value	S.E.	Value	S.E.
1	44.8	10.0	0.145	0.071	16.8	4.9
2	46.7	8.3	0.110	0.043	41.4	5.8
3	62.5	16.0	0.129	0.074	15.7	8.0
4	56.9	9.2	0.181	0.056	22.7	4.0
5	31.8	8.6	0.082	0.047	44.6	6.6
6	72.3	34.9	0.517	0.244	17.2	15.2

[a] S.E.: standard error of the parameter estimate.

DISCUSSION

In vivo microdialysis sampling allows the measurement of unbound drug concentrations in blood and other body fluids and tissues with a high temporal resolution. By simultaneous blood sampling and intravenous microdialysis sampling information is obtained regarding the in vivo plasma protein binding of the compound under study. However, since classical blood sampling techniques in the rat lead to a significant loss of blood over a relatively short period of time, the question arises whether the actual blood sampling has an effect on the plasma binding and the pharmacokinetic behavior of the compound under investigation. Results of the present study on the pharmacokinetics of flurbiprofen in rats showed that serial blood sampling can indeed influence the blood (plasma) concentrations of the drug and thus its pharmacokinetic behavior. Hulse et al. (16) demonstrated that repetitive blood sampling in rats resulted in a decrease in hematocrit and plasma albumin levels and an increase in plasma free fatty acid levels. As a result, the unbound plasma fraction of dicumarol, a highly protein-bound drug, was significantly increased after multiple blood sampling. In view of these observations it is not surprising that simultaneous blood sampling lowered flurbiprofen concentrations. The effect, however, was rather limited (19%). This is in contrast with the findings of Telting-Diaz et al. (17), who demonstrated that microdialysate concentrations of theophylline were approximately 60% higher when serial blood samples were simultaneously withdrawn. Possible explanations for this rather unexpected observation were not given by the authors.

It is well recognized that the unbound fraction of drugs in plasma as determined by in vitro techniques such as equilibrium dialysis and ultrafiltration, is not necessarily similar to the in vivo unbound fraction. When performing in vitro plasma protein binding experiments, blank plasma is spiked with the drug to yield (a) certain total drug concentration(s) at which the unbound fraction is then determined. Unfortunately, this approach may not only introduce errors inherent to the in vitro technique used (3), but also neglects the potential competition between metabolites and the parent com-

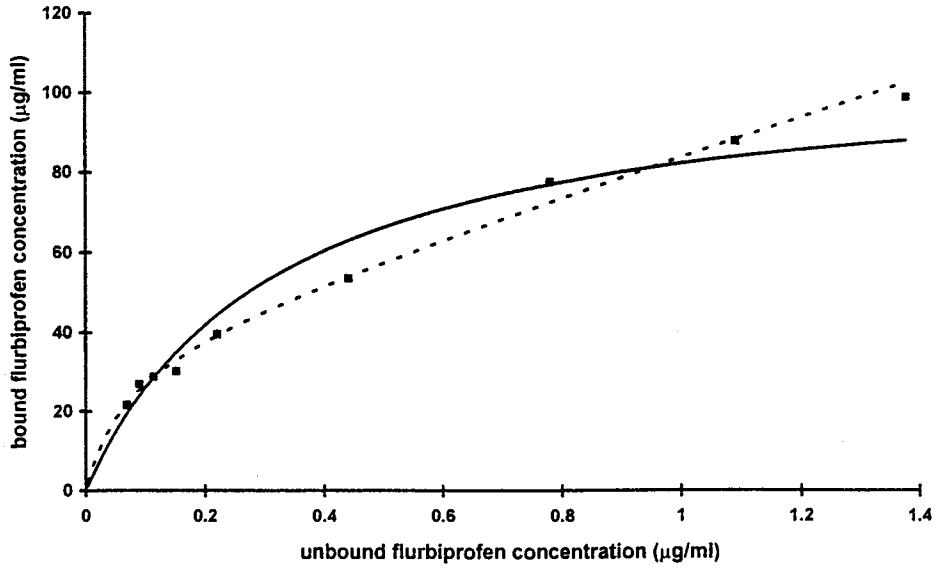

Fig. 3. Binding of flurbiprofen to rat plasma obtained in vivo using simultaneous serial blood sampling and intravenous microdialysis sampling is plotted as bound vs. unbound concentration. Symbols (■) represent experimental data points; the lines are based on two different fits, i.e. one binding site model with (- - - -) and without (—) a non-saturable binding.

pound for the binding proteins. Consequently, ex vivo determination of the plasma protein binding of a drug may offer a distinct advantage over the in vitro approach. This has now been confirmed for flurbiprofen using intravenous microdialysis sampling: results from the ex vivo plasma protein binding studies are entirely consistent with the in vivo results obtained from the pharmacokinetic studies using microdialysis and simultaneous blood sampling. In vitro values for the unbound fraction of flurbiprofen in plasma, on the contrary, systematically underestimated the unbound fraction in vivo. A factor which may have contributed to the difference observed between the in vitro and ex vivo/in vivo plasma binding of flurbiprofen is the fact that the plasma used for the in vitro plasma binding experiments was obtained from rats who had not undergone any surgery, whereas both the ex vivo and in vivo binding data were obtained from animals one day following a surgical intervention. However, unlike α_1-acid glycoprotein plasma concentrations which have been shown to increase significantly in rats following a surgical intervention, concentrations of albumin, to which flurbiprofen is bound in plasma, were not affected (18).

Our results show that the plasma protein binding of flurbiprofen in rats is concentration-dependent, even following administration of a single i.v. bolus dose of 20 mg/kg. Although concentration-dependent plasma binding of flurbiprofen was demonstrated in man (12), it had never been shown before in rats. As a matter of fact, very little information is available on the plasma protein binding of flurbiprofen in this animal species. In one of the early reports on the pharmacokinetics of flurbiprofen, plasma binding in several species including rat was found to be "more than 99.5%" (19). More recent pharmacokinetic studies of flurbiprofen in rats, even those using stereospecific assays, do not measure plasma protein binding (20,21). The concentration-dependent plasma binding of flurbiprofen may explain why the terminal plasma half-life is longer when considering total (4.4 ± 1.6 h) as compared to unbound flurbiprofen concentrations (2.8 ± 1.0 h). A similar effect of concentration-dependent plasma protein binding on the apparent half-life was demonstrated for disopyramide (22). Plasma protein binding may also play an important role in the stereospecific pharmacokinetics of flurbiprofen in the rat, especially since interactions between the R- and S-enantiomer at the level of plasma protein binding may be possible (20), as has been shown in man (23). Intravenous microdialysis sampling offers the opportunity to study the effect of enantioselective plasma protein binding and competition of the R- and S-enantiomers for common plasma binding sites on the stereospecific pharmacokinetics of 2-arylpropionic acids in rats by direct in vivo determination of the binding parameters B_{max} and K_d. We are currently developing the analytical methodology to perform in vivo microdialysis sampling with on-line stereospecific HPLC analysis of (R)- and (S)-flurbiprofen to directly investigate, using the approach presented here, the competition between the flurbiprofen enantiomers for the same binding sites on plasma proteins.

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