Validation of Subcutaneous Microdialysis Sampling for Pharmacokinetic Studies of Flurbiprofen in the Rat

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ABSTRACT: The objective of this study was to validate subcutaneous (sc) microdialysis sampling to study flurbiprofen pharmacokinetics and plasma protein binding in the awake freely moving rat. A linear microdialysis probe was manufactured using a Hemophane[®] hollow fiber which was tested *in vitro* and *in vivo* for the recovery of flurbiprofen and naproxen used as retrodialysis marker. Flurbiprofen was administered intraperitoneally and intravenously at a dose of 20 mg/kg in rats. In both cases, conventional blood sampling and sc microdialysis sampling were simultaneously performed. The microdialysates were analyzed on-line by high-pressure liquid chromatography. Naproxen, which was shown to have a similar *in vivo* loss by retrodialysis as flurbiprofen ($71.5 \pm 0.9\%$ and $71.0 \pm 0.8\%$ respectively, $n = 3$), was used to continuously monitor probe recovery. Concentration-dependent protein binding of flurbiprofen was demonstrated *in vivo* based on experiments with a simultaneous sc microdialysis and blood sampling. Values of unbound fraction were similar to those reported previously by intravenous microdialysis sampling, demonstrating that the sc unbound concentrations are very similar to those in the central compartment. There was no significant difference among pharmacokinetic parameters (AUC, CL, $t_{1/2z}$, Vd) for total or unbound flurbiprofen determined after intraperitoneal and intravenous administration. Subcutaneous microdialysis is a simple yet powerful tool to study the pharmacokinetics and the *in vivo* plasma protein binding of flurbiprofen in the awake unrestrained rat. © 2001 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 90:1897–1906, 2001

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INTRODUCTION

The principle of the microdialysis technique is based on the passive diffusion of compounds down a concentration gradient across the semipermeable membrane of a dialysis fiber. In function of the direction of the concentration gradient across the fiber, the microdialysis technique may be used not only to monitor the extracellular concentra-

tions of exogenous or endogenous compounds but may also be used to deliver drugs to a specific region of the body. Important advantages of microdialysis are: 1. increased sampling frequency to characterize the concentration–time profile with a discriminating resolution, 2. reduction of the number of experimental animals, 3. because the dialysis fiber prevents diffusion of proteins, samples are sufficiently purified for further analysis, and 4. possibility to study pharmacokinetics in awake freely moving animals in almost real time by on-line analysis of microdialysates by using various techniques. Originally developed and applied in brain

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research,¹ microdialysis has become during the last decade a common sampling method in conventional pharmacokinetic studies. Microdialysis sampling is a powerful tool to continuously monitor the extracellular unbound drug concentrations in different "compartments" of the body. Indeed, microdialysis probes can be implanted in virtually any body organ or tissue.²⁻⁵

Only a few articles are published describing the application of subcutaneous (sc) microdialysis sampling to study the pharmacokinetics of drugs in rats^{6,7} or in humans.^{8,9} Subcutaneous tissue is an attractive sampling site for several reasons: 1. this tissue is relatively uniform, 2. the extracellular fluid is in constant flux with the systemic circulation, and 3. the implantation of the probe in sc tissue is easy.⁷ Despite these interesting characteristics, no data are reported in the literature regarding the use of sc microdialysis sampling to study the pharmacokinetics of drugs with a very high plasma protein binding in the awake freely moving rat.

The purpose of the present study was to validate the sc microdialysis sampling technique to investigate the pharmacokinetics of a highly plasma protein bound drug such as flurbiprofen. First, the recovery of a simple linear microdialysis probe was determined *in vitro* and *in vivo* by using naproxen as retrodialysis marker.^{10,11} Subsequently, the ability of sc microdialysis sampling to measure unbound concentrations of flurbiprofen was demonstrated by studying the pharmacokinetics of this drug after intraperitoneal (ip) or intravenous (iv) administration with simultaneous conventional blood sampling. Finally, based on the results of the *in vivo* experiments with simultaneous microdialysis and blood sampling, concentration-dependent plasma binding of flurbiprofen was demonstrated.

EXPERIMENTAL

Materials

Flurbiprofen and naproxen were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were high-pressure liquid chromatography (HPLC) grade and all other chemicals used were AR grade.

Hairless male rats (Iffa Credo, Saint-Germain, France), weighing between 280 and 350 g, were used. Food (type A04; U.A.R., Epinay-sur-Orge, France) and tap water were provided *ad libitum*.

Surgical Procedure

Rats were anesthetized with a mixture of 4 mg/kg droperidol and 0.08 mg/kg fentanyl (Thalamonal[®]; Janssen Pharmaceutica, Beerse, Belgium). Cannulae of silastic tubing [0.94 mm outside diameter (o.d.), 0.51 mm inside diameter (i.d.); Dow Corning, Valbonne, France] were implanted in the right and left jugular vein using standard surgical procedures. The animals were allowed to recover from the surgery overnight. The described experimental procedures in the rats were approved by the University Animal Experimentation Ethics Committee.

Microdialysis Probe

The linear microdialysis probes were manufactured using a Hemophane[®] dialysis fiber (210 μ m i.d.; Gambro AB, Lund, Sweden) with a molecular weight cut-off of 5000 Da. The fiber (20 mm length) was glued at both ends to a piece of silicone tubing (0.012 in. i.d., 0.025 in. o.d.; Specialty Manufacturing Inc., Saginaw, MI) using B-210 cyanoacrylate glue (3M, Brussels, Belgium).

In Vitro and *In Vivo* Evaluation of the Recovery of the Probe

To characterize the dialysis efficiency of the probes, relative recoveries for flurbiprofen and naproxen (retrodialysis marker) were determined *in vitro*. The probes were placed in glass beakers at 37°C containing different concentrations of flurbiprofen or naproxen and perfused with an isotonic phosphate buffer pH 7.4 [phosphate buffered saline (PBS) = Na₂HPO₄ 3.191 g/L; NaH₂PO₄ 0.775 g/L, NaCl 5.58 g/L] at 2 μ L/min. Analyte concentrations were measured in the dialysate and expressed as a percentage of the concentration in the surrounding medium.

In vivo recovery was determined by the retrodialysis method or "internal reference technique"^{12,13} which consists of adding a retrodialysis marker to the perfusate and measuring the rate of delivery of this compound. This *in vivo* calibration method relies on the assumption that the recovery of the analyte is quantitatively the same as the delivery of the retrodialysis marker. After implantation of the probe in sc tissue, the probe was perfused at a flow rate of 2 μ L/min with PBS pH 7.4 containing flurbiprofen (250 ng/mL) or naproxen (250 ng/mL). Analyte concentrations

were measured in the microdialysate samples (C_{out}) every 10 min for 8 h. The recovery was determined from the ratio of the concentration lost to the initial concentration in the perfusate (C_{in}):

$$\text{Recovery}_{\text{in vivo}} = [(C_{\text{in}} - C_{\text{out}})/C_{\text{in}}] \times 100$$

Implantation of the Microdialysis Probe in the Rat and Microdialysis System

The probe implantation was performed as follows. The animals were anesthetized with a mixture of 4 mg/kg droperidol and 0.08 mg/kg fentanyl injected subcutaneously. The skin of the dorsal region was punctured horizontally by a 20-gauge iv plastic cannula. The steel mandrel was removed and the microdialysis probe was inserted subsequently. The plastic cannula was withdrawn, leaving the probe in the sc tissue. The probe was sutured to the skin to secure it. Then the rat was placed in a freely awake moving system of microdialysis (CMA/120, Stockholm, Sweden) for the duration of the experiment. The rat had free access to food and water during the microdialysis sampling period.

The probe inlet and outlet were connected to a dual channel swivel (Instech Laboratories Inc., Plymouth, PA) using PEEK[®] tubing (0.65 mm o.d. $\times$ 0.12 mm i.d.; CMA). The inlet of the swivel was then connected to a syringe pump (CMA/100) while the outlet was linked to an on-line injector (CMA/160). The probe was perfused with PBS pH 7.4 containing naproxen (250 ng/mL) as retrodialysis marker at a flow of 2 μ L/min during the pharmacokinetic experiment.

Probe Depth Measurement

To evaluate the sc implantation procedure, the probe depth was measured by 20 MHz ultrasound scanning using a Dermascan-C instrument (Philips; Philips Research Laboratories, Eindhoven, Holland). Five probes were implanted in rats and the depth measurement was performed at the half distance of the probe under the skin.

In Vitro Plasma Protein Binding of Flurbiprofen

The unbound fraction of flurbiprofen in plasma was determined *in vitro* by equilibrium dialysis (Dianorm, München, Germany). Blank blood was collected in heparinized tubes by cardiac puncture and immediately centrifuged. Plasma of different

rats was pooled and subsequently spiked with flurbiprofen to obtain the following concentrations: 0, 5, 10, 20, 40, and 60 μ g/mL. These plasma samples were dialyzed in duplicate for 4 h at 37°C against PBS pH 7.4 in 1 mL Teflon dialysis cells using semipermeable membranes with a 6000–8000 MW cut-off (Spectra/Por[®] 1; Spectrum Laboratories Inc., Los Angeles, CA). Plasma albumin concentrations were determined before and after dialysis by the bromocresol dye binding method (Sigma Diagnostics) and used to correct the unbound fraction for volume shifts.¹⁴ Flurbiprofen concentrations in plasma and buffer were determined by HPLC with fluorescence detection. The unbound fraction (f_u) was calculated as follows: $f_u = [\text{flu}]_{\text{bu}}/[\text{flu}]_{\text{pl}}$, where $[\text{flu}]_{\text{bu}}$ and $[\text{flu}]_{\text{pl}}$ are the flurbiprofen concentrations at equilibrium in the buffer and plasma compartments, respectively.

On-line Analysis of Microdialysate Samples by HPLC

The chromatographic system consisted of a high-pressure liquid chromatographic pump (model 420; Kontron Instruments, Milan, Italy), an on-line injector with a 10-port injection valve (CMA/160), a programmable fluorescence detector (SpectraSystem FL2000; Spectra-Physics, San Jose, CA), and a Varian model 4290 integrator (Varian Instruments, Walnut Creek, CA). The microdialysate samples were collected directly in the 10.5 μ L PEEK injection loop (Bioanalytical Systems, Inc., Congleton, Cheshire, UK) and automatically injected every 10 min onto a C₁₈ Nucleosil column (100 $\times$ 4 mm, 5 μ ; Macherey-Nagel, Düren, Germany). The mobile phase consisted of phosphate buffer 50 mM (pH 3.0) and acetonitrile (54:46, v/v) and was delivered at 0.850 mL/min. The eluate was monitored using the following excitation (ex) and emission (em) wavelengths: 262 nm (ex) and 356 nm (em) from 0–4 min (naproxen), and 258 nm (ex) and 310 nm (em) from 4–10 min (flurbiprofen). The lower limit of detection and the lower limit of quantification were respectively 2.5 and 7 ng/mL.

HPLC Assay of Plasma Samples

Concentrations of flurbiprofen in plasma were determined by using an HPLC method.^{15,16} To 50 μ L of plasma was added 1400 μ L of methanol containing 3 μ g naproxen/mL (internal standard). After vortexing, the mixture was centrifuged, and

50 μ L of supernatant was injected on the HPLC system using a 10 μ L sample loop. The HPLC system consisted of an HPLC pump (model 501, Waters Associates, Inc., Milford, MA), an autosampler (Gilson model 231, Villers-le-Bel, France) equipped with a 500 μ L syringe, a UV detector (model 481; Waters Associates), and an LDC/Milton Roy CI-10B integrator (LDC Milton Roy, Riviera Beach, FL). The HPLC column and the chromatographic conditions were the same as described for the on-line analysis of microdialysate samples. The eluate was monitored at 254 nm. The lower limit of detection and the lower limit of quantification were respectively 0.1 and 0.25 μ g/mL.

Pharmacokinetic Studies

To evaluate the ability of sc microdialysis to measure the unbound concentrations of flurbiprofen, a first study after ip administration (20 mg/kg) with simultaneous sc microdialysis and serial blood sampling was performed in four rats. A second study by sc microdialysis sampling with simultaneous serial blood sampling after iv bolus (20 mg/kg) was performed in five rats.

In all pharmacokinetic studies, after implantation of the probe and before the administration of flurbiprofen, a period of 90–120 min was required to stabilize the skin blood flow which may have been perturbed by the insertion of the steel mandrel during the implantation procedure.¹⁷ After ip and iv administration of flurbiprofen, the microdialysates and blood samples were collected for a total duration of 6 to 8 h. Blood samples (250 μ L) were collected in syringes containing heparin at the following times: 0 (blank), 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min. Blood samples were immediately centrifuged and the plasma was stored at -20°C until analysis.

Data Analysis

Pharmacokinetic parameters were determined by the so-called "noncompartmental" approach. AUC values were calculated using the linear trapezoidal rule from 0 to t (last microdialysate or blood sampling time) with extrapolation to infinity (plasma concentration at time t divided by slope λ_z). Terminal dialysate or plasma half-time ($t_{1/2z}$) was calculated as $0.693/\lambda_z$ where λ_z is estimated by linear regression of the terminal log-linear

phase of the dialysate or plasma concentration–time curve. Bioavailability (F) was determined from the ratio of the AUC value after ip administration to the AUC value after iv bolus. Clearance (CL) and the apparent volume of distribution (V_d) were calculated with standard pharmacokinetic equations.¹⁸

Based on unbound concentrations determined *in vivo* by microdialysis and blood sampling experiments, the binding of flurbiprofen was analyzed in terms of a one binding site model with or without a linear non-saturable term using the nonlinear least squares regression program WinNonlin 1.5 (Pharsight Corporation, London, U.K.):

$$C_b = \frac{B_{\max} \cdot C_u}{K_d + C_u} + a \cdot C_u \quad \text{one binding site model with a non-saturable binding}$$

$$C_b = \frac{B_{\max} \cdot C_u}{K_d + C_u} \quad \text{one binding site model without a non-saturable binding}$$

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. Non-saturable binding is shown as a linear function of C_u with slope a . The most appropriate model (one site with or without non-saturable binding) was determined using the goodness-of-fit criteria described by Boxenbaum et al.¹⁹

Data were validated by using the Dixon test. Values in the text, Figures, and Tables are expressed as mean $\pm$ SEM. Mean values of pharmacokinetic parameters were compared by the Student t test, except for $t_{\max}$ where the Mann-Whitney nonparametric test was used. A p value ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

On-line Analysis of Microdialysate Samples by HPLC

By preventing the diffusion of proteins into the dialysate, microdialysis provides relatively purified samples free from proteins and cellular matter and which therefore do not require further sample preparation. Thus, chromatograms of microdialysate samples injected directly into the HPLC system contained no interfering sub-

stances. Retention times are short (3.0 min for naproxen and 4.7 min for flurbiprofen) allowing on-line injection at 10 min intervals (data not shown).

In Vitro Calibration of the Microdialysis Probe

A microdialysis probe with a simple linear design was developed with a dialysis fiber being derived from an artificial kidney. The length of the membrane was selected in order to obtain a high recovery of the analyte while allowing an easy implantation. To characterize the dialysis efficiency of the probes and to validate the choice of naproxen as internal standard for the retrodialysis method, *in vitro* relative recovery of flurbiprofen and naproxen was estimated. Mean *in vitro* recoveries were $74.0 \pm 1.1\%$ for flurbiprofen (with four probes) and $76.9 \pm 0.4\%$ for naproxen (with three probes) (Table 1). *In vitro* recoveries of naproxen and flurbiprofen were significantly different ($p = 0.02$). The linear relationship ($R^2 = 0.998$) between the concentration in a surrounding solution and in the dialysate over a wide concentration range for both analytes shows that the diffusion through the membrane is independent of the flurbiprofen or naproxen concentration (Fig. 1).

Probe Depth Measurement

The evaluation of the implantation process was performed by measuring the mean probe depth. The mean probe depth determined by ultrasound imaging was 1.8 ± 0.1 mm ($n = 5$) (between 1.6 and 2.1 mm), demonstrating the reproducibility of the implantation procedure.

This method confirmed the sc implantation of the probe just below the smooth skeletal muscle layer which separates the hypoderm from the sc tissue.

Figure 1. *In vitro* calibration of the microdialysis probe for flurbiprofen and naproxen (insert). Analyte concentrations were measured in the dialysate and in the surrounding medium.

In Vivo Calibration of the Microdialysis Probe by the Retrodialysis Method

Because an *in vitro* determination of recovery may only provide a crude estimate of the behavior of a microdialysis probe, extrapolating such a value to an *in vivo* experiment is impossible because the nature of the tissue to be sampled and its interactions with the analyte affect the recovery.²⁰

A dialysis membrane of 20 mm length resulted in an *in vivo* loss by retrodialysis value of approximately 70% (Table 1). Loss by retrodialysis determined *in vivo* was slightly higher for

Table 1. *In Vitro* Recovery by Dialysis and *In Vivo* Loss by Retrodialysis of Flurbiprofen and Naproxen^a

	Recovery <i>In Vitro</i> (%)	Recovery <i>In Vivo</i> (Loss by Retrodialysis) (%)
Flurbiprofen	74.0 ± 1.1^b ($n = 4$)	71.5 ± 0.9^c ($n = 3$)
Naproxen	76.9 ± 0.4 ($n = 3$)	71.0 ± 0.8 ($n = 3$)

^aValues are the mean $\pm$ SEM.

^bSignificantly different ($p = 0.02$) from *in vitro* recovery of naproxen.

^cNot significantly different ($p = 0.67$) from *in vivo* recovery of naproxen.

flurbiprofen ($71.5 \pm 0.9\%$ with three probes) than naproxen ($71.0 \pm 0.8\%$ with three probes), but the difference was not significant ($p = 0.67$). A higher recovery could be obtained by increasing the length of the dialysis fiber. But, as reported by Yang et al.,³ it is preferable to have a recovery value between 50 and 70%, which permits determining the recovery with a higher precision and accuracy. When the recovery approaches 100%, concentration of the retrodialysis marker in the microdialysate becomes too small for accurate and reproducible HPLC analysis. Because no significant difference was demonstrated between the *in vivo* loss of naproxen and flurbiprofen, the *in vivo* retrodialysis data for naproxen were used to convert dialysate concentrations of flurbiprofen into unbound concentrations.

Microdialysis Sampling After ip Administration

To evaluate the potential of sc microdialysis sampling and to investigate the behavior of the probe in terms of dialysis efficiency during 8 h of experiment, a pharmacokinetic study with simultaneous sc microdialysis and blood sampling was performed. The results of this study demonstrate the applicability of the manufactured probes, implanted in the sc tissue of the dorsal region of the rat for the characterization of the pharmacokinetics of unbound flurbiprofen after ip administration of a single dose of 20 mg/kg. Figure 2 shows the average semi-logarithmic concentration–time profiles for total flurbiprofen in plasma and unbound flurbiprofen in the sc extracellular space in four rats. This graph clearly illustrates that sc microdialysis sampling allows the characterization of the unbound concentration–time curve with a discriminating resolution. The difference in scale between the unbound concentration and total concentration is easily understandable by the high protein binding of flurbiprofen ($> 99.5\%$ at low concentrations).

The *in vivo* loss of the retrodialysis marker during the pharmacokinetic studies was also used to monitor the integrity of the microdialysis probe. In the experiments after ip administration, *in vivo* loss of naproxen was $62.1 \pm 0.6\%$ ($n = 170$). The interindividual coefficient of variation was lower than 10% (7.4% for four different rats). These results demonstrate a relatively constant dialysis efficiency of the probes after implantation and during all the experiments (Fig. 2).

The unbound concentration of flurbiprofen in sc tissue versus time shows a short equilibration

Figure 2. Semi-logarithmic presentation of the concentration–time profiles of total flurbiprofen in plasma (●) and unbound flurbiprofen in microdialysate samples (▼) after ip injection at a dose of 20 mg/kg. Insert, loss (%) of naproxen by retrodialysis (■). Values are the mean $\pm$ SEM ($n = 4$).

phase with a $t_{\max}$ of 22.9 ± 2.3 min. The total plasma concentration–time curve is characterized by such a rapid absorption phase that the curve is very similar to those observed after iv injection. Pharmacokinetic parameters are summarized in Table 2.

Microdialysis Sampling After iv Administration

Figure 3 shows the mean semi-logarithmic concentration–time profiles for total flurbiprofen in plasma and unbound flurbiprofen in the sc extracellular space in five rats after iv administration (20 mg/kg) with simultaneous microdialysis and blood sampling. The unbound concentration–time curve shows a rapid equilibration phase with a $t_{\max}$ of 15.7 ± 1.1 min. The $t_{\max}$ determined after ip was significantly longer than the $t_{\max}$ observed after iv ($p = 0.043$, Mann-Whitney nonparametric test).

The *in vivo* loss of the retrodialysis marker during the pharmacokinetic studies was also used to monitor the integrity of the microdialysis probe. In the experiments after iv administration, *in vivo* loss of naproxen was $63.4 \pm 0.5\%$ ($n = 188$). The interindividual coefficient of variation was lower than 5% (3.5% for five different rats). These results demonstrate a relatively constant dialysis

Table 2. Pharmacokinetic Parameters of Unbound Flurbiprofen and Total Flurbiprofen in Rats

Parameters		After iv (<i>n</i> = 5) Mean $\pm$ SEM	After ip (<i>n</i> = 4) Mean $\pm$ SEM
Unbound flurbiprofen	AUC _u ($\mu\text{g h mL}^{-1}$)	1.65 $\pm$ 0.17	1.48 $\pm$ 0.38
	CL _u (mL min^{-1})	56.7 $\pm$ 2.0	68.9 $\pm$ 13.9
	Vd _u ^b (L)	21.2 $\pm$ 4.2	25.3 $\pm$ 2.8
	<i>t</i> _{1/2z} (h)	3.8 $\pm$ 0.7	4.7 $\pm$ 0.8
	F _u ^a		0.897
Total flurbiprofen	AUC _u ($\mu\text{g h mL}^{-1}$)	293.6 $\pm$ 29.1	212.2 $\pm$ 33.4
	CL (mL min^{-1})	0.35 $\pm$ 0.02	0.36 $\pm$ 0.07
	Vd ^b (mL)	197.4 $\pm$ 13.8	181.8 $\pm$ 16.9
	<i>t</i> _{1/2z} (h)	5.8 $\pm$ 0.5	4.4 $\pm$ 0.5
	F		0.722

^aBioavailability determined from the ratio of AUC_u after ip administration to AUC_u after iv bolus.

^bVd/F for ip administration.

efficiency of the probes after implantation and during all the experiments (Fig. 3). No significant difference between *in vivo* loss of naproxen after ip and iv administration was observed (*p* = 0.089).

As reported previously by Evrard et al.,²¹ the terminal half-life of flurbiprofen was somewhat longer (*p* = 0.066; paired *t* test) when considering total flurbiprofen concentrations (5.8 $\pm$ 0.5 h) as compared with unbound concentrations of flurbiprofen (3.8 $\pm$ 0.7 h). The concentration-dependent

plasma binding of flurbiprofen may explain this difference. A similar effect of concentration-dependent plasma protein binding on the apparent half-life was demonstrated for disopyramide.²² However, after ip administration, this observation concerning the terminal half-life was not confirmed. The change in the unbound fraction which occurred around the total plasma concentration of 20 $\mu\text{g/mL}$ could explain the inflection point of the unbound concentration–time curve. The slope of the curve decreases when the unbound fraction decreases, demonstrating that the transfer and elimination processes occur as a function of the unbound drug.²³

Pharmacokinetic parameters calculated from sc microdialysis experiments are in good agreement with those determined by using iv microdialysis sampling.²¹ For one of the four rats after ip administration, the AUC value was markedly smaller (124.0 $\mu\text{g h mL}^{-1}$ versus 278.0, 245.9, and 200.1 $\mu\text{g h mL}^{-1}$) without being a statistical outlier. This may explain the relatively low value of 0.722 for the ip bioavailability, although there was no significant difference between pharmacokinetic parameters determined after ip and iv administration. Table 2 summarizes the pharmacokinetic parameters.

In Vivo Determination of the Protein Binding

Because only unbound drug molecules pass through the dialysis membrane, microdialysis was shown to be a powerful tool to study the protein binding of drugs *in vitro*^{24,25} and *in vivo*.^{26,27} An iv microdialysis study showed that plasma protein binding of flurbiprofen is concentration-dependent in the rat, even after

Figure 3. Semi-logarithmic presentation of the concentration–time profiles of total flurbiprofen in plasma (●) and unbound flurbiprofen in microdialysate samples (▼) after iv bolus at a dose of 20 mg/kg. Insert, loss (%) of naproxen by retrodialysis (■). Values are the mean $\pm$ SEM (*n* = 5).

the administration of a single iv bolus dose of 20 mg/kg.²¹

The ratio of unbound to total plasma concentrations of flurbiprofen, obtained from the experiments with simultaneous sc microdialysis and blood sampling, was not constant but increased with increasing total flurbiprofen plasma concentration (Fig. 4). The percentage of the ratio of unbound to total plasma concentrations of flurbiprofen is relatively constant at total plasma concentrations lower than 20 µg/mL with values around 0.4%. The *in vivo* unbound fraction, expressed as percent, increases after a sigmoidal relationship when total plasma concentration approaches the concentration of the available binding sites to reach values between 1.0 and 1.5% at a total concentration of 50 µg/mL.²⁸ This concentration-dependent plasma binding was also demonstrated *in vitro* by using equilibrium dialysis. The unbound fraction of flurbiprofen, determined *in vitro*, was approximately 0.2% at a total concentration of 5 µg/mL and linearly increased to reach a value of approximately 0.8% at a total concentration of 60 µg/mL. The unbound fraction of flurbiprofen as determined *in vitro* was lower than the unbound fraction derived from the simultaneous microdialysis and blood sampling experiments. This difference is easily explained by errors inherent to the *in vitro* technique used²⁹ but also by the lack of potential competition

Figure 4. Unbound fraction of flurbiprofen as a function of total flurbiprofen plasma concentrations as determined by simultaneous microdialysis and serial blood sampling after the administration of 20 mg/kg flurbiprofen by iv bolus (five rats, $\diamond$) or ip (four rats, $\triangle$). The unbound fraction determined *in vitro* is also shown ($\bullet$). Values are the mean $\pm$ SEM.

Figure 5. Binding of flurbiprofen to rat plasma derived from the simultaneous microdialysis and blood sampling experiments is plotted as bound versus unbound concentration. Symbols (∇ , $\diamond$, $\triangle$, $\circ$, $\square$) represent experimental data points from five rats after iv bolus and from four rats after ip administration ($+$, $\blacklozenge$, $\blacktriangle$, $\blacktriangledown$). The lines are based on two different fits, i.e., one binding site model with (—) and without (---) a non-saturable binding term.

between metabolites and the parent compounds for the binding proteins. The binding isotherms from *in vivo* experiments are in good agreement with those reported by Evrard et al.²¹ For *in vivo* data, the best fit was obtained by using a one binding site model with a non-saturable term (Fig. 5). The binding parameters of flurbiprofen, $B_{\max}$, and K_d determined by nonlinear least square regression were respectively 20.2 ± 2.7 and 0.03 ± 0.01 µg/mL with a non-saturable term equal to 39.8 ± 5.4 ($n=9$). The concentration-dependent binding of flurbiprofen could also explain the observed non-parallelism between the total flurbiprofen plasma concentrations and the unbound flurbiprofen sc concentrations (Fig. 3). Our results demonstrate that the *in vivo* plasma protein binding of flurbiprofen can be determined by sc microdialysis sampling. Very similar f_u values were found in this study compared with the f_u values reported by Evrard et al.²¹ by using the same approach with iv microdialysis sampling. Evrard et al.²¹ fully validated iv microdialysis sampling with simultaneous blood sampling as a method to determine the unbound fraction of flurbiprofen in the circulation. Because sc implantation of the microdialysis probe in the rat is much easier than iv implantation, sc microdialysis sampling offers a much simpler approach to measure *in vivo*

unbound flurbiprofen concentrations and binding parameters.

CONCLUSIONS

In the field of pharmacokinetics, one of the advantages of microdialysis sampling is to provide concentration–time profiles with a high temporal resolution. Because it is relatively easily accessible, sc tissue is an attractive sampling site. Therefore, we developed an sc microdialysis sampling technique to study the pharmacokinetics of flurbiprofen following ip and iv administration. Based on experiments with a simultaneous microdialysis and blood sampling, concentration-dependent protein binding of flurbiprofen was demonstrated. Pharmacokinetic parameters and fu values calculated from sc microdialysis experiments are in good agreement with those determined by using the iv microdialysis technique.²¹ These results constitute indirect evidence that the unbound concentrations of flurbiprofen in the sc compartment are very similar to those in the central compartment. An important advantage of sc microdialysis compared with iv microdialysis is that the implantation of the probe is much more convenient and faster.

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