Validation of Different Microdialysis Methods for the Determination of Unbound Steady-State Concentrations of Theophylline in Blood and Brain Tissue

Per Sjöberg,[1,3] Ing-Marie Olofsson,[1] and Thomas Lundqvist[2]

Received February 18, 1992; accepted May 19, 1992

Three microdialysis methods, the "tritium" method, the "point-of-no-net-flux" method, and a method using the low perfusion rate of 0.1 μl/min, were compared with respect to their ability to generate estimates of unbound steady-state concentrations (Cu_{ss}) of the antiasthmatic drug theophylline in blood and brain tissue in anesthetized rats. Concomitantly, the influence of the perfusion flow rate on the estimated extracellular Cu_{ss} obtained with the point-of-no-net-flux method was investigated. Theophylline was administered as a rapid intravenous bolus dose followed by constant intravenous infusion. Changes in perfusion flow rate from 2.0 to 0.75 μl/min and, finally, to 0.25 μl/min, using the point-of-no-net-flux method, had no significant effect on the estimated Cu_{ss} of theophylline in blood and striatum. This observation, particularly in the case of brain tissue, is not consistent with the theory that the process of dialysis drains a significant amount of substance from the immediate vicinity of the dialysis probe. Similar estimates of Cu_{ss} in blood as well as in brain tissue were obtained with all three methods. Their accuracy in estimating Cu_{ss} in blood was further strengthened by observations of unbound fractions similar to those reported in the literature. Furthermore, all three methods gave striatum/blood ratios at steady state of approximately 0.5, indicating that there is active transport of theophylline from brain tissue. It is concluded that the tritium method, when validated, can be used to study the time course of unbound drug concentrations in blood and tissues.

KEY WORDS: microdialysis; methods; theophylline; pharmacokinetics; rats.

INTRODUCTION

Information on the time course of unbound drug concentrations in body tissues is essential when elucidating mechanisms of drug disposition as well as concentration–response relationships. Among the experimental methods available for the study of unbound concentrations, *in vivo* microdialysis appears to possess particular potential. Under ideal conditions, microdialysis allows the continuous monitoring of unbound drug concentrations in the extracellular space of tissues not otherwise readily available for study.

A problem encountered in pharmacokinetic studies with *in vivo* microdialysis is the difficulty of obtaining accurate estimates of unbound extracellular concentrations simultaneously with good time resolution. The latter can generally be achieved only by using perfusion flow rates yielding recovery values over the dialysis membrane *in vivo* considerably below 100%. Thus, if good time resolution is wanted, the low recovery has to be compensated for by a correction factor which brings the dialysate concentration as close to the unbound tissue concentration as possible.

Recently, complex mathematical models have been developed which relate dialysate concentrations to tissue extracellular concentrations at various perfusion flow rates (1–3). Although these models account for the transport of substances through the tissue and probe membrane (1,2), and also for transport across the microvasculature and metabolism (3), they are based upon a number of nonvalidated assumptions. A simple and practical solution to the problem of obtaining a correction factor has been suggested by Alexander and co-workers (4). Their method employs tritiated water (^3H_2O) as an internal standard for the determination of the efficiency of the dialysis probe *in vivo*. Unfortunately, there is no information on the usefulness of this method in pharmacokinetic investigations.

The objective of the present study was to investigate whether the "tritium" method could be used to obtain accurate estimates of extracellular concentrations of the antiasthmatic drug theophylline in blood and brain tissue in rats. The tritium method was validated by comparing the estimated unbound steady-state concentration in blood and brain tissue and the unbound fraction in blood with equivalent data generated by experiments with a perfusion flow rate of 0.1 μl/min ("low-perfusion rate" method) and by the "point-of-no-net-flux" method (5,6). The influence of perfusion flow rate on the performance of the latter method was also investigated. Steady-state conditions of theophylline were obtained by a rapid intravenous bolus dose followed by constant intravenous infusion.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (ALAB, Sweden) weighing between 260 and 300 g were used throughout the experiments. They were acclimatized to laboratory conditions for at least 1 week prior to each experiment.

Chemicals

Theophylline and β-hydroxypropyltheophylline (internal standard) were obtained from Byk, Gulden Lomberg Chem Fabrik GmbH, Germany, and tritiated water (^3H_2O) was from Amersham Sweden AB, Sweden. All solvents and reagents were of analytical grade.

In Vitro Microdialysis

All *in vivo* microdialysis experiments were preceded by *in vitro* studies. In experiments where the "tritium" method was used the relative recovery of theophylline over the probe membrane was determined. In experiments with the

[1] Medical Products Agency, Uppsala, Sweden.
[2] Department of Biopharmaceutics and Pharmacokinetics, Uppsala University, Uppsala, Sweden.
[3] To whom correspondence should be addressed at Division of Pharmacology and Toxicology, Medical Products Agency, Box 26, S-751 03, Uppsala, Sweden.

0724-8741/92/1200-1592$06.50/0

"low-perfusion rate" method and with the "point-of-no-net-flux" method, the adequate function of the dialysis probe was established.

Recovery experiments *in vitro* were carried out by placing the dialysis probes (membrane length, 3 mm; CMA Microdialysis AB, Sweden) in test tubes containing Hepes–Ringer solution (147 m*M* Na^+, 4 m*M* K^+, 2.3 m*M* Ca^+, and 155.6 m*M* Cl^-, pH 6.0), theophylline (10 μg/ml), and ^3H_2O (0.25 μCi/mL). The tubes were placed in a heated water bath (37°C), where the solution was under constant stirring. The dialysis probes were perfused with Hepes–Ringer solution or artificial cerebrospinal fluid (126.5 m*M* NaCl, 27.5 m*M* $NaHCO_3$, 2.4 m*M* KCl, 0.5 m*M* KH_2PO_4, 1.1 m*M* $CaCl_2$, 0.85 m*M* $MgCl_2$, 0.5 m*M* Na_2SO_4, 5.9 m*M* glucose, pH 7.4) at a flow rate of 2.0 μl/min for 50 min by the use of a CMA/140 microinfusion pump (CMA Microdialysis AB, Sweden). Six 20-μl fractions were collected from each probe. Fractions 2, 4, and 6 were analyzed for their content of theophylline by HPLC, while the other fractions were analyzed for their content of ^3H_2O. The relative recovery (Rel rec) of theophylline was calculated according to Eq. (1).

$$\text{Rel rec (\%)} = C_{\text{dialysate}}/C_{\text{Hepes-Ringer}} \quad (1)$$

Separate experiments were performed to investigate the influence of low perfusion rates on the *in vitro* recovery of theophylline from Hepes–Ringer solution. The perfusion flow rates studied were 2.0, 0.2, and 0.1 μl/min and three probes were used at each flow rate.

In Vivo Microdialysis

Implantation of Dialysis Probes. Two days prior to the experiment, the rats were anesthetized with ether and cannulas were inserted into the right jugular vein and the right carotid artery. On the day of the experiment, the animal was anesthetized with urethane (2 g/kg s.c.) and a dialysis probe was inserted into the left jugular vein via a guide cannula (7). The animal was then placed in a Kopf stereotaxic instrument and another dialysis probe was implanted into the striatum (coordinates: anteriorly +0.2 mm, laterally −2.6 mm, and dorsally −7.5 mm relative to the bregma). The coordinates were derived from *The Rat Brain* stereotaxic atlas (8). The probes placed in the jugular vein and striatum were continuously perfused with Hepes–Ringer or artificial CSF solution, respectively, at a flow rate of 2.0 μl/min.

Tritium Method. Six experiments were conducted with this method. Approximately 15 min after implantation of the striatum probe, the animals were given an i.v. bolus dose of ^3H_2O (0.1 mCi in 0.1 ml of Hepes–Ringer solution). Perfusate samples (20-μl fractions) were collected during 50 min and analyzed for their content of ^3H_2O (see below). In the middle of the sampling interval between the last two fractions, a blood sample was collected from the carotid cannula for determination of the plasma ^3H_2O content.

Theophylline was then administered as a bolus dose of 5.7 mg/kg, followed by constant-rate infusion of 1.6 mg/kg/hr for 2 hr. This dosage regimen aimed at obtaining a plasma concentration of total (bound + unbound) theophylline of 10 μg/ml. Perfusate samples of 20 μl were collected for up to 2.5 hr after stopping the infusion. The samples were analyzed immediately for theophylline content by HPLC as described below. Every fifth perfusate sample and a simultaneously collected blood sample were used for determination of ^3H_2O, thus allowing the estimation of relative probe efficiency *in vivo* (Rel $\text{eff}_{in\ vivo}$) (4).

$$\text{Rel eff}_{in\ vivo} = \frac{(^3H_2O_{\text{brain dial}}/^3H_2O_{\text{plasma}})}{\text{Rel rec } ^3H_2O_{in\ vitro}\ (\%)} \quad \text{or} \quad \frac{(^3H_2O_{\text{blood dial}}/^3H_2O_{\text{plasma}})}{\text{Rel rec } ^3H_2O_{in\ vitro}\ (\%)} \quad (2)$$

Portions of the plasma obtained from the blood samples were stored at −20°C for later analyses of plasma concentrations of total theophylline. The unbound concentration of theophylline (C_u) was calculated as

$$C_u = C_{\text{dialysate}}/(\text{Rel rec} \times \text{Rel eff}_{in\ vivo}) \quad (3)$$

Low-Perfusion Rate Method. Four separate *in vivo* experiments were performed with the low perfusion flow rate of 0.1 μl/min. The animals were prepared as described above and the same dosage regimen of theophylline was used. After attainment of steady state (1 hr after the start of the infusion), the probes were perfused for 80 min prior to sampling. Owing to the very low perfusion rate, this was necessary for equilibrium between the concentration at the end of the dialysis tubing and the concentration within the dialysis probe. The dialysate was then collected for 2.5 hr and analyzed immediately. At the start, in the middle, and at the end of the collection period, blood samples were withdrawn from the carotid cannula and the plasma was analyzed for total theophylline concentration. Since it was assumed that the low perfusion flow rate of 0.1 μl/min would result in an equilibrium between the extracellular concentration in blood/striatum and the concentration of dialysate, the unbound concentration was obtained through

$$C_u = C_{\text{dialysate}} \quad (4)$$

whereas the fraction unbound was calculated as

$$f_u = C_u/C_{\text{plasma}} \quad (5)$$

In this equation, C_{plasma} is the mean plasma concentration of samples taken at the start, middle, and end of the dialysis experiment.

Point-of-No-Net-Flux Method. In this method theophylline is added to the perfusion fluid at different concentrations, some higher and some lower than the concentration anticipated to be present in the extracellular space. Cannulas and dialysis probes were implanted as described above. Three separate experiments with different perfusion rates were conducted using the same dosage regimen of theophylline as described above. After steady state was reached (1 hr) and six 20-μl fractions had been obtained, the two dialysis probes were perfused at 2.0, 0.75, or 0.25 μl/min with Hepes–Ringer or artificial CSF fortified with theophylline. Four concentrations were used at each flow rate: 5, 8, 11, and 14 μg/ml for the jugular vein probe and 2, 5, 8, and 11 μg/ml for the striatum probe. Two samples of 5 μl each were collected at every concentration. To ascertain that steady-state levels of theophylline had been maintained during the course of the experiment and to allow calculation of the unbound fraction, four blood samples were drawn during the

course of the experiment for analysis of total theophylline concentration.

For assessment of unbound theophylline at each perfusion flow rate, the difference between the $C_{dialysate}$ and the concentration in the perfusion medium (C_{in}) was calculated.

$$\Delta C = C_{dialysate} - C_{in} \quad (6)$$

Linear regression analysis of the plot of C_{in} against ΔC was used to determine the intercept with the X axis (the point of no net flux), i.e., the estimated unbound concentration. The slope of the regression line, which according to Lönnroth and co-workers (5) gives the *in vivo* recovery, was also obtained.

Analysis of Theophylline

All samples (plasma extracts, dialysates, and theophylline in buffer) were analyzed with a Waters liquid chromatographic system (Model 501 pump, Model 481 UV detector) and a Nova-Pak C_{18} Radial-Pak column. The plasma samples were prepared as described previously (9), while dialysates and theophylline in buffer were assayed without prior treatment. Theophylline and the internal standard, β-hydroxypropyltheophylline, were eluted with 13% acetonitrile in 7 m*M* phosphoric acid at a flow rate of 0.8 ml/min. UV absorption was monitored at 272 nm. The calibration curves (made by the addition of known amounts of theophylline to the plasma, buffer, and dialysate samples) were linear in the range of the obtained experimental values. The coefficient of variation for the injection volume of 16 μl was 0.8% at a theophylline concentration in the plasma samples of 10 μg/ml and 2.5% at a concentration of 0.5 μg/ml in the dialysates and buffer samples. For the injection volume of 4 μl (dialysate samples from the experiments with the point-of-no-net-flux and low-perfusion rate methods), the coefficient of variation was 1.3%.

Determination of Radioactivity

The amount of ^3H_2O in the dialysates, plasma, and buffer solutions was determined by dissolving samples of 15 μl in 4 ml of scintillation fluid (Instagel: 0.5 *M* HCl, 9:1, v/v) and counting the radioactivity in a Packard Tricarb Model B2450 liquid scintillation spectrometer.

Pharmacokinetic Calculations

Pharmacokinetic parameters were calculated using standard noncompartmental techniques.

Statistics

One-way ANOVA and Students' t test were used to detect possible differences in the pharmacokinetic parameters obtained with the different microdialysis methods. The results of the point-of-no-net-flux method experiments were analyzed by two-way ANOVA to detect the possible influence of perfusion flow rate on the estimated unbound concentration.

RESULTS

In the *in vitro* microdialysis experiments conducted with a perfusion rate of 2.0 μl/min, the relative recoveries of theophylline over the dialysis membrane were 24–51% (mean value, 33 ± 5%). At the lower perfusion rates of 0.2 and 0.1 μl/min, the mean relative recovery was 97 ± 1.0 and 98 ± 0.8%, respectively.

The *in vivo* recovery of ^3H_2O, as determined by the ratio of the ^3H_2O concentration in the dialysate from the probe placed in the jugular vein and the plasma ^3H_2O concentration, varied considerably whereas the *in vivo* recovery of ^3H_2O determined by the probes placed in the striatum showed little variation (Table I). On average, the Rel $eff_{in\ vivo}$ of the probes placed in the jugular vein was 65%, while the corresponding figure for the probes placed in the striatum was 44%. In most experiments, there was a decrease in relative probe efficiency over time, but in a few cases, the opposite effect was observed. An example of the relationship between relative probe efficiency and duration of sampling is given in Fig. 1.

Experiments using the perfusion flow rate of 2.0 μl/min revealed that steady-state concentrations of unbound theophylline in blood and striatum were reached within 1 hr after the start of the infusion (Fig. 2). This information was used when designing the experiments carried out with the low perfusion flow rate or different theophylline concentrations in the perfusion medium (point-of-no-net-flux method). The elimination half-life of unbound theophylline in blood was 2.4 ± 1.2 hr. In individual animals, the ratio of the

Table I. *In Vivo* Recovery of ^3H_2O[a] and Relative Probe Efficiency[b] at the Perfusion Flow Rate of 2.0 μl/min for Dialysis Probes Placed in the Jugular Vein and Striatum of Male Rats

Expt. no.	*In vivo* recovery of ^3H_2O (%)				Relative probe efficiency			
	Blood		Striatum		Blood		Striatum	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
1	31.6	26.4–40.0	24.5	23.1–26.4	0.49	0.41–0.62	0.41	0.39–0.45
2	41.9	37.2–46.9	26.0	23.6–28.9	0.67	0.60–0.75	0.43	0.39–0.48
3	46.6	39.0–52.6	29.2	25.3–31.4	0.87	0.73–0.99	0.48	0.42–0.52
4	35.7	25.7–44.6	31.7	30.2–33.6	0.48	0.35–0.61	0.43	0.41–0.46
5	55.2	46.9–65.8	29.1	26.6–31.3	0.74	0.63–0.89	0.41	0.38–0.44
6	44.4	42.3–47.1	29.0	27.3–30.9	0.62	0.59–0.66	0.48	0.46–0.52

[a] ^3H_2O in dialysate from blood (or brain)/^3H_2O in plasma.
[b] For definition see Eq. (2).

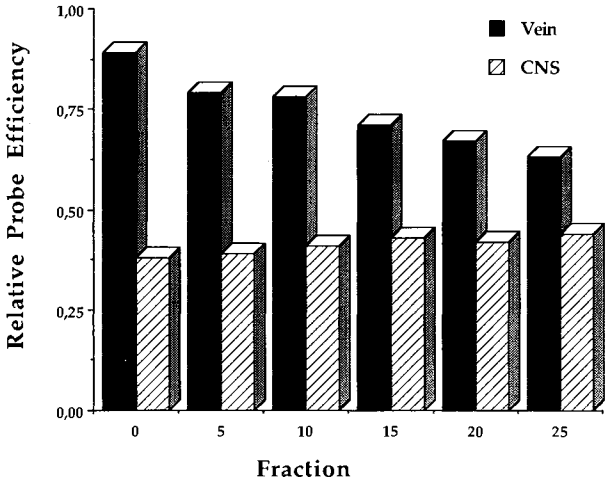

Fig. 1. Relative probe ^3H_2O efficiency *in vivo* as a function of dialysis time in a male rat; for definition of relative probe efficiency see Eq. (2).

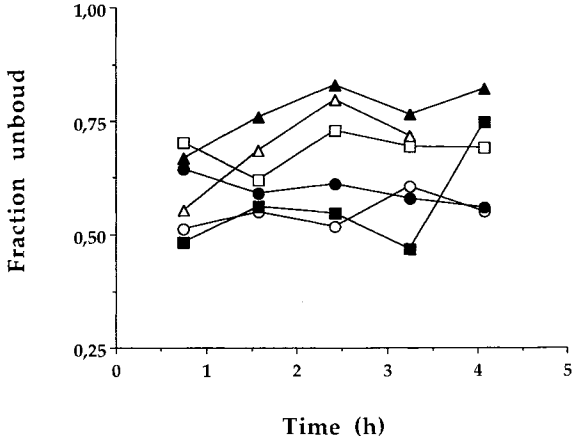

Fig. 3. Unbound fraction (f_u) of theophylline in the blood of six male rats as a function of dialysis time; each rat is represented by a different symbol. The unbound concentrations were determined according to the tritium method.

unbound concentration in striatum to that in blood was virtually constant from the second sampling interval onward. The coefficient of variation for this ratio, calculated for 21 measurements in each of six animals, was 4.4%.

In the experiments conducted with the tritium method and a perfusion flow rate of 2.0 μl/min, the mean unbound fraction of theophylline in blood was 0.64 ± 0.10. The relationship between the unbound fraction and the duration of dialysis in the different experiments is shown in Fig. 3. Although differences in the estimated unbound fraction over time were seen in some animals, no overall tendency toward either an increase or a decrease was recorded.

Table II shows the following parameters at steady state: the unbound concentrations (Cu_{ss}) in blood and striatum, the ratio of unbound theophylline in striatum/blood, and the unbound fraction in blood as obtained with the three methods. The values presented from experiments with the point-of-no-net-flux method were obtained at the perfusion rate of 2.0 μl/min. There was no statistically significant difference among the values obtained with the three microdialysis methods. However, Cu_{ss} in striatum as obtained with the tritium method was close to significantly different from the value obtained with the point-of-no-net-flux method, the lower and upper confidence limits being −0.0019 and 0.1919, respectively.

The experiments with the point-of-no-net-flux method were also designed to investigate the possible influence of perfusion rate on estimated unbound concentration in blood and striatum at steady state. The varying perfusion flow rates of 0.25, 0.75, and 2.0 μl/min did not influence the estimated Cu_{ss} in blood and striatum (Table III). Figure 4 gives the plot of ΔC vs C_{in} for the different flow rates in one animal.

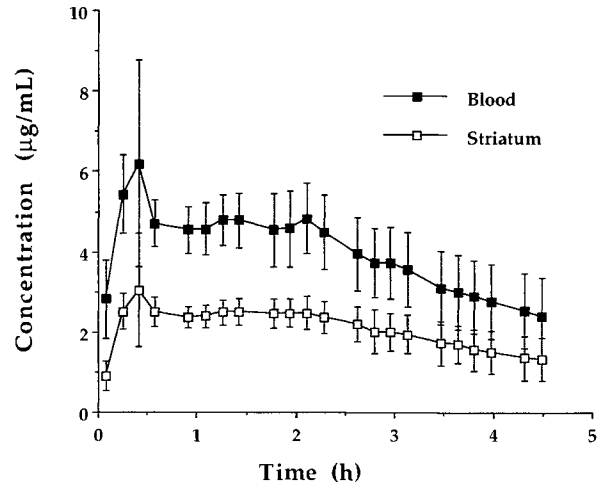

Fig. 2. Concentration–time profiles of unbound theophylline in blood and striatum of male rats as determined by the tritium method. Mean values ± SD; $n = 6$.

DISCUSSION

This comparative microdialysis study in rats demonstrated that estimated unbound theophylline concentrations in blood and brain tissue at steady state did not differ among the "tritium," the "point-of-no-net-flux," and the "low-perfusion rate" methods. The accuracy of all methods in estimating unbound concentrations of theophylline in blood was further strengthened by the observation of unbound fractions in plasma of the same magnitude as those reported in the literature (10–14).

With one exception (15), the tritium method, as described by Alexander and co-workers (4), does not seem to have been used for the estimation of unbound drug concentrations during *in vivo* microdialysis. Furthermore, there appears to have been no attempt to validate whether this particular method estimates actual unbound drug concentrations in blood and other tissues. The method, which rests on the assumption that the compound of interest has similar diffusion characteristics in tissues as tritiated water, could ideally be used to estimate actual drug concentrations during non-steady-state conditions. Also, if an experimental design similar to ours is used, the tritium method compensates for fluctuations in probe performance over time. In the present study, where probe efficiency was calculated from dialysate and blood samples collected every fiftieth minute during the

Table II. Pharmacokinetic Parameters at Steady-State as Determined by the Tritium, Point-of-No-Net-Flux, and Low-Perfusion Rate Methods[a]

Parameter	Method for determination of unbound drug		
	Tritium[b] (n = 6)	Point of no net flux[c] (n = 3)	Low perfusion rate[d] (n = 4)
Cu_{ss} striatum (μg/ml)	2.5 ± 0.3	4.3 ± 0.6	3.5 ± 0.7
Cu_{ss} blood (μg/ml)	5.1 ± 0.6	6.8 ± 0.5	6.4 ± 1.2
Ratio striatum/blood	0.53 ± 0.07	0.63 ± 0.05	0.54 ± 0.04
Unbound clearance (L/kg × hr)	0.35 ± 0.06	0.24 ± 0.02	0.26 ± 0.05
Fraction unbound	0.64 ± 0.10	0.65 ± 0.07	0.64 ± 0.04

[a] Mean values ± SD are given.
[b] Perfusion flow rate, 2.0 μl/min.
[c] Values given are those obtained at the perfusion rate of 2.0 μl/min.
[d] Perfusion flow rate, 0.1 μl/min.

in vivo dialysis, the probe efficiency varied over time (Table I and Fig. 1). This variation was more pronounced for the probe placed in the jugular vein. It seems possible that this observation is related to the environment in the vein where the dialysis probe is constantly exposed to circulating blood. The occurrence of significant time-dependent changes in *in vivo* probe performance suggests that this parameter should be monitored during the course of microdialysis experiments, particularly if dialysis is carried out in blood. The observation that the relative probe efficiency was higher for the probe placed in the jugular vein than for the probe placed in the striatum (Table I), is consistent with the slower diffusion process caused by the tortuous extracellular space and the lower volume fraction in brain tissue (1,16).

Although the point-of-no-net-flux method was introduced some years ago (5), its application in pharmacokinetic investigations has not been compared to that of other methods. The *in vitro* performance of the method has been studied (17), and in a recent study on endogenous extracellular dopamine concentrations in the nucleus accumbens of the rat (18), the method was evaluated and found to be more useful than the "extrapolation-to-zero-flow" method as described by Jacobsson and co-workers (19).

Since it has been shown that microdialysis, by drainage, may lower the concentration of a substance outside the dialysis probe (1) and since such an effect presumably is dependent on the perfusion flow rate, it was of interest to study the influence of the perfusion flow rate on the estimated extracellular concentrations of theophylline when using the point-of-no-net-flux method. The results obtained at the investigated flow rates of 0.25, 0.75, and 2.0 μl/min showed no significant effect of flow rate on estimated unbound concentrations in blood and brain tissue (Table III). Although only three animals were studied, the study had adequate statistical power (80%) to allow a 7% (blood) and 9% (striatum) difference to be detected. The absence of an effect of flow rate on estimated extracellular concentrations, particularly in the case of brain tissue, is probably related to the high steady-state concentrations of theophylline and to the fact that the diffusion of theophylline in brain tissue is faster than the drainage via dialysis. Since the perfusion flow rate did not affect the estimated extracellular concentration, the use

Table III. Unbound Steady-State Concentrations of Theophylline in Striatum and Blood of Three Rats (A, B, and C) as Determined by the Point-of-No-Net-Flux Method; the Influence of Different Flow Rates is Shown[a]

Flow rate (μl/min)	Cu_{ss} (μg/ml) in striatum/blood		
	A	B	C
2.0	4.53/6.72	3.60/6.29	4.63/7.26
0.75	4.08/6.69	4.05/6.22	4.49/7.35
0.25	4.89/7.62	4.44/6.59	4.99/7.22

[a] The infusion rate was 1.6 mg/kg/hr; for other experimental conditions and calculations, see Materials and Methods.

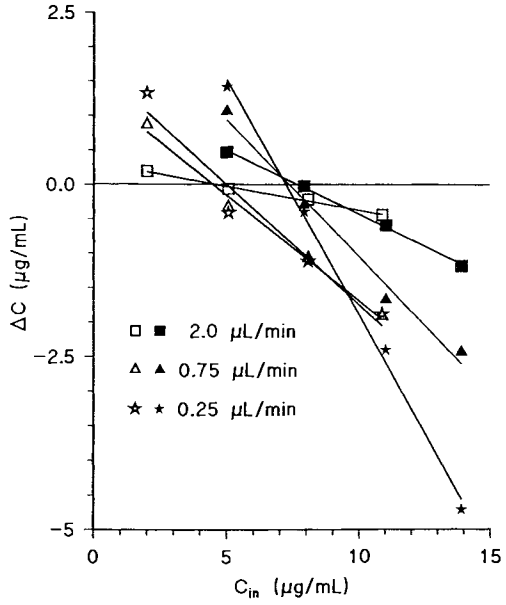

Fig. 4. Plot of theophylline concentration lost to or gained from the tissue (ΔC) against different concentrations of theophylline in the perfusion medium (C_{in}) at different perfusion flow rates. Data from one rat are shown; each point represents the mean concentration of two dialysate fractions. The intercept with the X axis is the point of no net flux or estimated unbound concentration. Filled and open symbols represent data from blood and striatum, respectively.

of the higher flow rate is advocated. With a higher flow rate, more data can be obtained within a given time period, which increases the accuracy of the determination.

It is generally assumed that *in vivo* dialysis with very low perfusion rates results in recovery values approaching 100% (16,20). The present results of mean recovery values for theophylline *in vitro* of 33, 97, and 98%, at flow rates of 2.0, 0.2, and 0.1 μl/min, respectively, are in accordance with this theory. Moreover, the similarity between the results obtained with the low-perfusion rate method and those with the point-of-no-net-flux method suggests close to 100% *in vivo* recovery of theophylline at the perfusion flow rate of 0.1 μl/min. Interestingly, it has been suggested that estimates of *in vivo* recoveries can be obtained from data generated by the point-of-no-net-flux method; the slope of the regression line equals the *in vivo* recovery (5). By this method, the perfusion flow rates in our study, 2.0, 0.75, and 0.25 μl/min, gave mean *in vivo* recoveries ranging from 13 to 68 and from 7 to 35% for dialysis probes placed in the jugular vein and striatum, respectively. If these figures are accurate, they indicate that the *in vivo* recovery from the probe placed in striatum is 50% lower than the recovery from the probe placed in the jugular vein. More importantly, the data indicate that the *in vivo* recovery at the flow rate of 0.1 μl/min is far from 100%; this relates particularly to the probe placed in the striatum. This observation does not agree with the consistent results obtained with the low-perfusion rate method and the point-of-no-net-flux method. It is tempting to speculate that the *in vivo* recoveries as calculated from the point-of-no-net-flux data are erroneously low. Recent findings that active processes such as uptake and release may influence the slope of the regression line, and hence the estimated *in vivo* recovery, without affecting the extracellular concentration (21) support this assumption. Although our results and those of Justice (21) question the relevance of the *in vivo* recovery figures generated by the point-of-no-net-flux method, more data are needed to determine their accuracy.

In vivo microdialysis makes testing of the free drug hypothesis possible, i.e., whether the unbound blood concentration at steady state is equal to the unbound extracellular concentration. In this study, all three methods of estimating extracellular concentrations gave striatum/blood ratios of unbound theophylline that were somewhat higher than 0.5 (Table II). An even higher striatum/blood ratio, but still below unity, was recently reported by Ståhle and co-workers (22). A ratio below unity suggests that there is a barrier for the uptake of theophylline in the CNS or an efficient transport system from the CNS. Alternatively, theophylline is extensively metabolized in the brain. The rapid attainment of a stable ratio between the unbound concentration in striatum and that in blood (Fig. 2) suggests free transport of theophylline from the blood to the extracellular space of the striatum. Further, information on theophylline biotransformation in brain tissue (10) does not suggest that the observed ratio of unbound concentration in striatum/blood is due to tissue specific metabolism. It also appears unlikely that bulk flow, which in CSF has been estimated to 2–2.5 μl/min (23,24), could clear theophylline from the brain extracellular space to any significant extent. A more plausible explanation is that theophylline is transported out of the CNS by an active transport system such as that described for other xanthine derivatives (25). Studies examining this possibility are presently in progress.

In conclusion, this study has shown that there are several acceptable microdialysis methods for the determination of steady-state concentrations of unbound theophylline in blood and brain tissue. When investigating steady-state kinetics, the point-of-no-net-flux method could be the method of choice since this method does not need prior validation. Validation is a prerequisite for both the low-perfusion rate method and the tritium method. When investigating the time course of unbound drug concentrations in blood and tissues, the tritium method could be applied.

ACKNOWLEDGMENT

The technical assistance of Eva Arlander is gratefully acknowledged.

REFERENCES

1. H. Benveniste, A. J. Hansen, and N. S. Ottosen. Determination of brain interstitial concentrations by microdialysis. *J. Neurochem.* 52:1741–1750 (1989).
2. P. M. Bungay, P. F. Morrison, and R. L. Dedrick. Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. *Life Sci.* 46:105–119 (1990).
3. P. F. Morrison, P. M. Bungay, J. K. Hsiao, B. A. Ball, I. N. Mefford, and R. L. Dedrick. Quantitative microdialysis: Analysis of transients and application to pharmacokinetics in brain. *J. Neurochem.* 57:103–119 (1991).
4. G. M. Alexander, J. R. Grothusen, and R. J. Schwartzman. Flow dependent changes in the effective surface area of microdialysis probes. *Life Sci.* 43:595–601 (1988).
5. P. Lönnroth, P.-A. Jansson, and U. Smith. A microdialysis method allowing characterization of intercellular water space in humans. *Am. J. Physiol.* 253:E228–E231 (1987).
6. P. Lönnroth, P.-A. Jansson, B. B. Fredholm, and U. Smith. Microdialysis of intercellular adenosine concentration in subcutaneous tissue in humans. *Am. J. Physiol.* 256:E250–E255 (1989).
7. L. Hurd, J. Kehr, and U. Ungerstedt. In vivo microdialysis as a technique to monitor drug transport: Correlation of extracellular cocaine levels and dopamine overflow in the rat brain. *J. Neurochem.* 51:1314–1316 (1988).
8. G. Paxinos and C. Watson. *The Rat Brain in Stereotaxic Coordinates*, Academic Press, North Ryde, NSW, Australia, 1982.
9. K. C. van Horne and T. Good. Automated on-stream syringeless injection in HPLC. *Int. Clin. Prod. Rev.* Sept.:14–23 (1983).
10. I. M. Ramzan and G. Levy. Chronic theophylline administration has no apparent effect on theophylline concentrations required to produce seizures in rats. *Proc. Soc. Exp. Biol. Med.* 182:176–180 (1986).
11. L. Shum and W. J. Jusko. Theophylline tissue partitioning and volume of distribution in normal and dietary-induced obese rats. *Biopharm. Drug Disp.* 8:353–364 (1987).
12. M. Yasuhara and G. Levy. Caffeine as a potential risk factor for theophylline neurotoxicity. *J. Pharm. Sci.* 77:745–747 (1988).
13. L. Shum and W. J. Jusko. Effects of obesity and ancillary variables (dialysis time, drug, albumin, and fatty acid concentrations) on theophylline serum protein binding. *Biopharm. Drug Disp.* 10:549–562 (1989).
14. R. Aichartpichean, T. Hasegawa, M. Nadai, T. Kuzuya, and T. Nabeshima. Structure-pharmacokinetic relationships among the N^1,N^3-alkylxanthines in rats. *J. Pharm. Pharmacol.* 43:262–269 (1991).
15. C. Ingvast-Larsson. The use of an "internal standard" for control of the recovery in microdialysis. *Life. Sci./Pharmacol. Lett.* 49:73–78 (1991).
16. H. Benveniste and P. C. Hüttemeier. Microdialysis—theory and application. *Prog. Neurol.* 35:195–215 (1990).
17. L. Ståhle, S. Segersvärd, and U. Ungerstedt. A comparison

between three methods for estimation of extracellular concentrations of exogenous and endogenous compounds by microdialysis. *J. Pharmacol. Meth.* 25:41–52 (1991).

18. L. H. Parsons and J. B. Justice, Jr. The extracellular concentration and in vivo recovery of DA in the nucleus accumbens using microdialysis. *J. Neurochem.* 58:212–218 (1992).
19. I. Jacobson, M. Sandberg, and A. Hamberger. Mass transfer in brain dialysis devices—a new method for the estimation of extracellular amino acid concentrations. *J. Neurosci. Meth.* 15:263–268 (1985).
20. S. A. Wages, W. H. Church, and J. B. Justice, Jr. Sampling considerations for on-line microbore liquid chromatography of brain dialysate. *Anal. Chem.* 58:1649–1656 (1986).
21. J. B. Justice. Quantitative neurotransmitter measurement. In H. Rollema, B. Westerink, and W. J. Drijfhout (eds.), *Monitoring Molecules in Neuroscience,* Krips Repro, Meppel, The Netherlands, 1991, pp. 41–43.
22. L. Ståhle, S. Segersvärd, and U. Ungerstedt. Drug distribution studies with microdialysis II. Caffeine and theophylline in blood, brain and other tissues in rats. *Life Sci.* 49:1843–1852 (1991).
23. N. H. Bass and P. Lundborg. Drug infusion into the spinal subarachnoid spaces of unanesthetized rats during early postnatal life: A technique for the study of brain development. *Acta Pharmacol. Toxicol.* 32:294–303 (1973).
24. D. Burns, J. London, D. J. Brunswick, M. Pring, D. Garfinkel, J. L. Rabinowitz, and J. Mendels. A kinetic analysis of 5-hydroxyindoleacetic acid excretion from rat brain and CSF. *Biol. Psychiat.* 11:125–157 (1976).
25. R. Spector. Hypoxanthine transport and metabolism in the central nervous system. *J. Neurochem.* 50:969–978 (1988).