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Determination of in vivo steady-state unbound drug concentration in the brain interstitial fluid by microdialysis

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Summary

The in vivo unbound concentrations of [^{3}H]water, caffeine and aminopyrine in the hippocampal interstitial fluid were directly determined by means of a brain microdialysis technique. The steady-state unbound concentration in the interstitial fluid (ISF) was determined from the dialysate concentration and the in vivo permeability rate constants of these compounds which were extrapolated from the in vitro permeability rate constant and from the effective dialysis coefficient of a reference compound such as antipyrine. The values of the unbound concentrations of [^{3}H]water, caffeine and aminopyrine obtained by extrapolation were very close to those predicted from the plasma concentrations based on the assumption that the unbound concentration in the tissue interstitial fluid is the same as that in the plasma. Moreover, the damage due to implantation of transcranial-type microdialysis cannula was evaluated using [^{14}C]sucrose as an extracellular marker. The average ISF/plasma ratios of [^{14}C]sucrose estimated for the initial 30 min dialysis were 7% at 1 h and 3.7% at 48 h after implantation of the cannula, respectively, which correspond well with that (2.0–6.1%) estimated from the blood-brain barrier permeability rate constant reported previously. Therefore, we conclude that the microdialysis technique using the reference method for determination of the unbound concentration of ISF is a useful procedure for the reliable evaluation of the in vivo unbound concentration in brain ISF and that the blood-brain barrier is not significantly damaged by cannula implantation.

Introduction

The newly developed microdialysis technique is rapidly becoming a very useful tool in neurochemical, biochemical and pharmacological research areas (Ungerstedt, 1984; Benveniste, 1989; Benveniste and Huttemeier, 1990). Recently, using this microdialysis technique with a transcranial-type cannula, we proposed a novel extrapolation method for determination of the true unbound concentration in tissue interstitial fluid (ISF) from the concentration in the dialysate sample (Deguchi et al., 1991). This technique, designated here as the 'reference method', has been demonstrated to be useful and to provide

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reliable data for muscle, liver and lung (Deguchi et al., 1992).

In contrast to the above tissues, the unbound concentration of drug in brain ISF is regulated by plasma protein binding, transport across the blood-brain barrier, exchange between ISF space and brain parenchymal cells and metabolism in brain parenchymal tissue. Moreover, recently reported experimental evidence, such as the enhanced dissociation of drugs and hormones from the protein-bound complex in brain capillaries (protein-mediated transport) (Pardridge and Landaw, 1984, 1985, 1987; Terasaki et al., 1986; Terasaki and Pardridge, 1987), existence of a carrier-mediated transport system for acidic and basic drugs (Kang et al., 1990b,c; Terasaki et al., 1991a), and the asymmetric transport properties of the blood-brain barrier (Cornford et al., 1985), may suggest that in vivo unbound concentration in the brain ISF is not readily estimated from the unbound plasma concentration determined in vitro. Recent studies have measured the unbound concentration in brain ISF in vivo using a brain microdialysis technique (Dubey et al., 1989).

Therefore, the primary purpose of this study was to demonstrate whether the reference method with antipyrine employed as a reference compound is suitable for determination of the in vivo unbound concentration in brain ISF using the three model substrates, [^{3}H]water, caffeine and aminopyrine, of almost equally high permeability across the blood-brain barrier (Greig, 1989). Moreover, with the aid of [^{14}C]sucrose as an extracellular marker, we evaluated damage to the blood-brain barrier as a result of the implantation of a transcranial-type microdialysis cannula, which might represent a critical problem in brain microdialysis.

Materials and Methods

Animals

Adult male Wistar rats weighing 250–300 g were purchased from Sankyo Laboratory Co. (Toyama, Japan). They had free access to food and water.

Materials

[^{3}H]Water (5 mCi/ml) was purchased from Amersham International Ltd (Bucks, U.K.). [^{14}C]Sucrose (5.0 mCi/mmol) was obtained from New England Nuclear (Boston, U.S.A.). Caffeine anhydride, aminopyrine and antipyrine were supplied by Wako Pure Chemical Industries Ltd (Osaka, Japan). β-Hydroxyethyltheophylline was obtained from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Other chemicals were of analytical grade and used without further purification.

Microdialysis cannula

The transcranial-type of microdialysis cannula was prepared as described previously (Kang et al., 1990a; Deguchi et al., 1991, 1992; Terasaki et al., 1991b) using Cuprophan hollow-fiber (i.d., 0.2 mm; wall thickness, 11 μm; MW cut-off, 12500; Lento H.F., Organon Technica Corp., OK, U.S.A.) and stainless-steel tubing (o.d., 0.2 mm; MT Giken, Tokyo, Japan).

In vitro microdialysis

In vitro microdialysis studies were carried out in Ringer-Hepes buffer (RHB: 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 10 mM Hepes; pH 7.4) solutions containing [^{3}H]water (0.15 μCi/ml), caffeine (50 μg/ml), aminopyrine (100 μg/ml), antipyrine (100 μg/ml) and [^{14}C]sucrose (0.25 μCi/ml) at 37 °C, in order to determine the in vitro permeability rate constant (PA_{vitro}) of the substances. Details of this method have been described previously (Deguchi et al., 1991). The dialysis flow rates were 2.5, 5, 10, 25 and 50 μl/min for [^{3}H]water and antipyrine, 2.5, 10 and 50 μl/min for caffeine and aminopyrine, and 10 μl/min for [^{14}C]sucrose, with control by means of a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, U.S.A.). The dialysate solution was collected three times at each flow rate. 10 or 100 μl of the reservoir medium was sampled at the midpoint of the time intervals during each dialysate sample collection.

In vivo brain microdialysis

Brain microdialysis was carried out according to the same method as described previously (Kang et al., 1990a; Terasaki et al., 1991b). Briefly, the

microdialysis cannula was implanted horizontally in the hippocampus of the rat. The subjects' heads were held in a stereotaxic frame throughout the experiment. The same microdialysis cannula was used in the in vitro experiment.

For the study of [^{3}H]water, a bolus dose (200 μCi/kg) was administered intravenously to rats via a femoral vein at 1 h after the implantation of the transcranial-type dialysis cannula. From 20 min after a bolus injection, the dialysate samples were collected at flow rates of the order of 10, 50, 2.5, 25 and 5 μl/min during the appropriate time intervals. The dialysate sample was collected three times at each flow rate. To flush the dead volume of the cannula and to attain steady-state conditions, the dialysate sample was not collected during an appropriate interval when the dialysis flow rate was changed. The blood was withdrawn through the femoral arterial cannula at the midpoint of each dialysate collection period.

For studying aminopyrine, at 1 h after implantation of the microdialysis cannula, bolus doses of aminopyrine (90 mg/kg) and antipyrine (75 mg/kg) were administered intravenously via a catheter, followed by the immediate infusion of aminopyrine and antipyrine at constant rates of 660 and 150 μg/min per kg, respectively, using an infusion pump. From 20 min after the start of infusion, dialysate samples were collected three times at flow rates of the order of 2, 50 and 10 μl/min and three times at each flow rate. Blood was withdrawn at 5, 27.5, 57.5, 77.5 and 110 min after the start of infusion.

In the case of caffeine, the respective bolus doses of caffeine and antipyrine were 28 and 75 mg/kg, with the corresponding infusion doses of caffeine and antipyrine being 128 and 150 μg/min per kg.

In addition to the above experiments, antipyrine was independently examined. Antipyrine was administered intravenously via a catheter at a dose of 75 mg/kg, and followed by intravenous infusion at a dose of 150 μg/min per kg.

Evaluation of damage by implantation of the microdialysis cannula

The surgical procedure for implantation of the microdialysis cannula was the same as described above. At 1 and 48 h after implantation of the transcranial-type cannula, microdialysis was performed. The renal artery and vein were ligated in order to maintain a constant plasma level. [^{14}C]Sucrose (200 μCi/kg) was injected intravenously into rats via a femoral vein. Immediately, thereafter, dialysate samples were sequentially collected at a constant flow rate of 2.5 μl/min every 30 min. Blood was withdrawn through the femoral arterial catheter at 2, 5, 10, 15, 45, 75, 105 and 135 min after the bolus injection of [^{14}C]sucrose.

Analytical procedure

Radioactivities of [^{3}H]water and [^{14}C]sucrose in the dialysate, reservoir medium and plasma were counted using a liquid scintillation counter (LSC 700, Aloka Ltd, Tokyo, Japan).

Aminopyrine and antipyrine concentrations in the plasma and dialysate were simultaneously determined using a high-performance liquid chromatography (HPLC) method. The HPLC system was constructed with a pump (880-PU, Japan Spectroscopic Co. (Jasco), Tokyo, Japan), UV spectrophotometer (875-UV, Jasco) and an integrator (C-R4A, Shimadzu Corp., Kyoto, Japan). The extraction procedure and HPLC conditions except for the composition of the mobile phase were the same as those described previously (Deguchi et al., 1991). The mobile phase was composed of acetonitrile : 78 mM phosphate buffer (17.5 : 82.5, by vol.). Under the present analytical conditions, antipyrine, aminopyrine and phenacetin (an internal standard) were clearly separated with retention times of 9.4, 19.6 and 26.0 min, respectively.

For caffeine and antipyrine, the HPLC method was also used for simultaneous quantification by a modification of the method of Nakazawa et al. (1985). The HPLC system was identical to that described above. A 50 μl portion of the plasma sample, 10 μl of internal standard solution containing 250 μg/ml β-hydroxyethyltheophylline in RHB and 1.5 ml chloroform : isopropanol (75 : 25) were vigorously mixed for 10 min. After centrifugation at 1000 rpm for 10 min, the organic layer was evaporated to dryness at room temperature. The residue was reconstituted with a mobile phase of composition 0.005 M sodium acetate : meth-

anol : acetonitrile : tetrahydrofuran (92.5 : 3 : 2.8 : 1.7, by vol.) and a 20 μl aliquot was injected onto an HPLC column. The dialysate samples were injected directly, omitting the extraction and filtration steps. The HPLC conditions were as follows: flow rate, 1.0 ml/min; UV wavelength, 270 nm. Antipyrine, caffeine and β-hydroxyethyltheophylline peaks on chromatograms were completely separated, without any interference by peaks of caffeine metabolites, and had retention times of 9.8, 14.0 and 18.6 min, respectively.

The peak area was used for quantification. The concentration was determined from the calibration curve constructed via the same procedure as that for the respective samples.

Plasma unbound fraction

For the determination of the plasma unbound fractions of caffeine, aminopyrine and antipyrine, ultrafiltration was performed as described in a preceding study (Deguchi et al., 1992).

Extrapolation of brain ISF concentration

In this study, brain ISF concentrations were extrapolated from dialysate concentrations using two different methods. One was based on extrapolation, referred to as the reference method, the other being the conventional procedure (recovery method) using only the relative recovery determined during in vitro microdialysis.

Reference method This method has been described in detail previously (Deguchi et al., 1991) and its usefulness has been demonstrated in the cases of muscle, liver and lung tissues whose capillary permeabilities to small solutes are not restricted (Deguchi et al., 1992). This is based on the idea that the difference between the in vivo and in vitro microdialysis efficiencies is corrected by that of the reference compound such as antipyrine.

The in vitro permeability rate constants (PA_{vitro}) of a drug and a reference compound were determined according to the following equation (Deguchi et al., 1991):

$$CL_{vitro} = FC_d/C_r = F\{1 - \exp(-PA_{vitro}/F)\} \quad (1)$$

where CL_{vitro} is the in vitro dialysis clearance, C_d and C_r denote the dialysate and reservoir concentrations in the in vitro study, respectively, and F is the dialysis flow rate. The in vivo permeability rate constant (PA_{vivo}) was also determined from the following equation (Deguchi et al., 1991):

$$CL_{vivo} = FC_d/C_{isf} = F\{1 - \exp(-PA_{vivo}/F)\} \quad (2)$$

where C_{isf} is the unbound concentration in the ISF. Additionally, the effective dialysis coefficient (R_d), which is the ratio of the in vivo and in vitro permeability rate constants, was defined as follows (Deguchi et al., 1991):

$$R_d = PA_{vivo}/PA_{vitro} \quad (3)$$

To evaluate the in vivo permeability rate constant of drug ($PA_{vivo,drug}$), antipyrine, which does not undergo binding to plasma proteins and is rapidly transported through the blood-brain barrier, was used as an in vivo reference compound. The PA_{vivo} value of antipyrine was determined from Eqn 2 on the basis of the assumption that the concentration of antipyrine in brain ISF is identical to that of plasma at steady state; i.e., $C_{isf} = C_p$, thus, $R_{d,ref}$ can be obtained from Eqn 3.

Assuming $R_{d,drug} = R_{d,ref}$ and rearranging Eqns 1–3, the equation for extrapolation of the unbound concentration in the ISF ($C_{isf,u,drug}$) is obtained as follows:

$$C_{isf,u,drug} = C_{d,vivo,drug} / \{1 - \exp(-R_{d,ref}PA_{vitro,drug}/F)\} \quad (4)$$

where $C_{d,vivo,drug}$ is the dialysate concentration determined by microdialysis. Eqn 4 has been demonstrated to be useful in extrapolating the data on muscle, liver and lung (Deguchi et al., 1992). Eqn 4 was therefore employed in the analysis of brain microdialysis data.

Recovery method This is the procedure which has conventionally been used. Assuming that the in vivo microdialysis recovery is the same as that in vitro, the unbound concentration can be obtained as follows:

$$C_{isf,u,drug} = C_{d,vivo,drug}(C_r/C_d) \quad (5)$$

Finally, we can assess the validity of the extrapolation method by comparing the unbound concentration in ISF as estimated using Eqns 4 and 5 with that predicted from the plasma unbound concentration at steady state by assuming that the unbound concentration in brain ISF is equal to that in the plasma.

Data analysis for evaluation of damage

In the evaluation of damage after implantation of the transcranial-type cannula, the concentration of [^{14}C]sucrose in ISF may increase gradually with time. Therefore, the concentration in each dialysate sample represents the average value in ISF during the sampling period. Since the present reference method is valid only under steady-state conditions, quantitative evaluation of the damage required the use of Eqn 6 as an approximation for the ratio of average ISF and average plasma concentration of [^{14}C]sucrose (ISF/plasma) as expressed by:

$$\text{ISF/plasma} = \int_{t_i}^{t_{i+1}} C_{isf}\,dt \Big/ \int_{t_i}^{t_{i+1}} C_p\,dt$$

$$= C_d(t_{i+1} - t_i)/\{1 - \exp(-R_{d,ref}PA_{vitro}/F)\} \Big/ \int_{t_i}^{t_{i+1}} C_p\,dt \quad (6)$$

$\int_{t_i}^{t_{i+1}} C_p dt$ was calculated using the pharmacokinetic parameters estimated by fitting the plasma concentration-time curve for [^{14}C]sucrose using a nonlinear least-squares regression method (MULTI program) (Yamaoka et al., 1981).

Results

In vitro microdialysis of [^{3}H]water, caffeine and aminopyrine

The in vitro permeability rate constants of [^{3}H]water, caffeine and aminopyrine were determined in RHB at 37 °C. The PA_{vitro} values (means ± S.E.; expressed in μl/min) of [^{3}H]water, caffeine and aminopyrine estimated directly from Eqn 1 were 4.44 ± 0.11 ($n = 15$), 0.924 ± 0.026 ($n = 9$) and 0.654 ± 0.018 ($n = 9$), respectively.

TABLE 1

Comparison between the reference and recovery methods for the extrapolation of concentrations of [^{3}H]water in the brain ISF [a]

Flow rate (μl/min)	Dialysate (×10^6)(dpm/ml)	Concentration in ISF (×10^6)(dpm/ml)		
		Predicted [b]	Observed	
			Reference method [c]	Recovery method [d]
2.5	1.72 ± 0.10 (9)	3.85 ± 0.10 (3)	3.91 ± 0.35 (9)	2.22 ± 0.17 (9)
5.0	1.04 ± 0.07 (9)	3.76 ± 0.19 (3)	3.59 ± 0.28 (9)	1.78 ± 0.13 (9)
10.0	0.627 ± 0.057 (9)	3.46 ± 0.42 (3)	3.77 ± 0.38 (9)	1.68 ± 0.17 (9)
25.0	0.278 ± 0.028 (9)	3.95 ± 0.24 (3)	4.00 ± 0.43 (9)	1.65 ± 0.18 (9)
50.0	0.134 ± 0.009 (9)	3.90 ± 0.18 (3)	3.85 ± 0.23 (9)	1.54 ± 0.09 (9)

[a] Values are means ± S.E. with the number of points being shown in parentheses.
[b] Values obtained based on the assumption that the plasma concentration is equal to the concentration of ISF at steady state.
[c] ISF concentration estimated using the reference method.
[d] ISF concentration estimated via the recovery method.
The fixed R_d value of antipyrine employed in the extrapolation was 0.389, which was the mean for all studies with antipyrine ($n = 108$).

Unbound concentrations of [^{3}H]water, caffeine and aminopyrine in brain interstitial fluid

Plasma and dialysate concentrations of [^{3}H]water reached a steady state within 20 min after infusion. The steady-state total plasma concentrations of [^{3}H]water, regarded as the ISF unbound concentration, are listed in Table 1. As an observed value, the brain ISF concentration was extrapolated via the reference method (Eqn 4) from the dialysate concentration, PA_{vitro} value and a fixed $R_{d,ref}$ value for antipyrine (0.389 ± 0.011, mean $\pm$ S.E., $n = 108$), which is the mean value obtained from the studies for caffeine and aminopyrine, and the independent study for antipyrine. The results are summarized in Table 1. The ISF concentration was also calculated with the aid of Eqn 5 according to the recovery method, the values being summarized on the right-hand side of Table 1. Good agreement was found between the predicted and observed values at each flow rate estimated via the reference method. However, significant differences were observed between the predicted and observed values calculated using the recovery method.

In the cases of aminopyrine and caffeine, their respective steady-state total plasma concentrations were 92.3 ± 1.8 and 43.5 ± 1.2 (means $\pm$ S.E., expressed in μg/ml; $n = 12$ in both cases). No appreciable binding to plasma proteins of aminopyrine was observed. The plasma unbound fraction of caffeine was determined to be 0.893 ± 0.019 (mean $\pm$ S.E., $n = 3$). Using the total plasma concentration and the plasma unbound fraction, the unbound concentration of caffeine in brain ISF was estimated as the predicted value with the assumption that the unbound concentration in brain ISF is equal to that in the plasma, the results being shown in Table 2. As the observed value, the unbound concentrations of aminopyrine and caffeine in brain ISF were also estimated via the reference method from the dialysate concentration, the $R_{d,ref}$ value for antipyrine determined at each flow rate, and the PA_{vitro} value. These results are also summarized in Table 2. Additionally, the unbound concentration was also extrapolated using the recovery method, the values being listed in Table 2. Fairly good agreement was obtained between the predicted and observed concentrations as estimated via the reference method at each flow rate. However, the unbound concentrations calculated using the recovery method were obviously underestimated in comparison with those predicted (Table 2).

TABLE 2

Comparison between the reference and recovery methods for the extrapolation of unbound concentrations of caffeine in brain ISF [a]

Flow rate (μl/min)	Dialysate (μg/ml)	R_d	Unbound concentration in ISF (μg/ml)		
			Predicted [b]	Observed	
				Reference method [c]	Recovery method [d]
Aminopyrine					
2.5	11.9 $\pm$ 0.4 (9)	0.491 $\pm$ 0.023 (9)	90.8 $\pm$ 3.3 (3)	112 $\pm$ 3 (9)	58.3 $\pm$ 1.7 (9)
10.0	3.68 $\pm$ 0.07 (9)	0.537 $\pm$ 0.023 (9)	97.8 $\pm$ 4.6 (3)	108 $\pm$ 3 (9)	60.9 $\pm$ 1.4 (9)
50.0	0.698 $\pm$ 0.015 (9)	0.496 $\pm$ 0.038 (9)	90.1 $\pm$ 4.9 (3)	102 $\pm$ 4 (9)	51.6 $\pm$ 1.2 (9)
Caffeine					
2.5	5.90 $\pm$ 0.22 (9)	0.399 $\pm$ 0.014 (9)	36.4 $\pm$ 1.0 (3)	41.4 $\pm$ 1.4 (9)	18.1 $\pm$ 0.9 (9)
10.0	2.02 $\pm$ 0.04 (9)	0.435 $\pm$ 0.010 (9)	41.0 $\pm$ 1.3 (3)	47.1 $\pm$ 1.0 (9)	22.0 $\pm$ 0.8 (9)
50.0	0.374 $\pm$ 0.006 (9)	0.382 $\pm$ 0.008 (9)	41.3 $\pm$ 2.3 (3)	46.7 $\pm$ 0.9 (9)	17.3 $\pm$ 0.6 (9)

[a] Values are means $\pm$ S.E., with the number of points being indicated in parentheses.
[b] Values determined based on the assumption that the unbound concentration in the plasma is equal to that in ISF.
[c] ISF unbound concentration estimated using the reference method.
[d] ISF concentration evaluated via the recovery method.

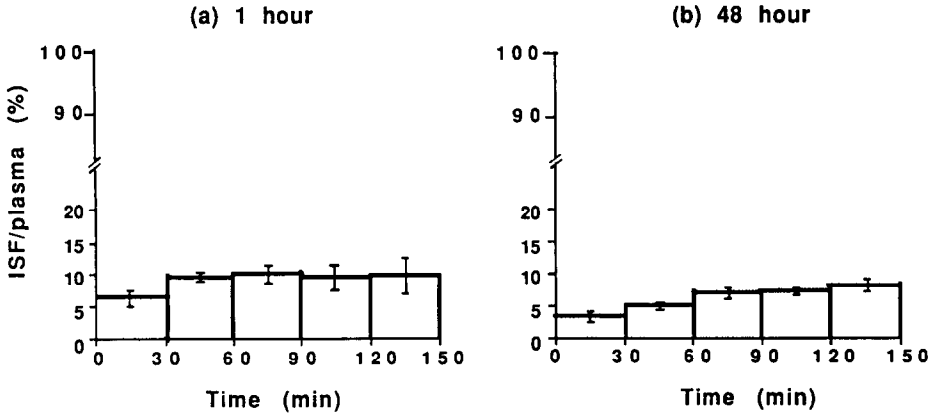

Fig. 1. Percentage ratio of the average ISF and average plasma concentrations (ISF/plasma, %) of [^{14}C]sucrose as a function of time. Brain microdialysis was performed at 1 h (a) and 48 h (b) after implantation of the transcranial-type microdialysis cannula in order to evaluate damage to the blood-brain barrier. The ISF/plasma ratio was calculated according to Eqn 6. Each point and the corresponding vertical bar represent the mean ± S.E. of three independent experiments.

Evaluation of damage to the blood-brain barrier

In order to evaluate the damage to blood-brain barrier due to the implantation of a transcranial-type microdialysis cannula, the ratio of the average ISF concentration and average plasma concentration of [^{14}C]sucrose (ISF/plasma, %) was determined using Eqn 6. As shown in Fig. 1, the ISF/plasma ratios ranged from 6.3 to 10.0% at 1 h following implantation of the cannula, whilst the corresponding range at 48 h after implantation was found to be 3.32–8.03%.

Discussion

The true unbound drug concentration in the ISF is not readily extrapolated from the dialysate concentration. This is due to the fact that in vivo microdialysis involves the existence of many complex processes in the tissue water, compared with in vitro microdialysis (Benveniste, 1989; Benveniste and Huttemeier, 1990; Deguchi et al., 1991). Although several methods of extrapolation have been proposed for evaluation of the true in vivo unbound concentration in ISF (Lonnroth et al., 1987; Amberg et al., 1989; Benveniste et al., 1989; Lindefors et al., 1989; Bungay et al., 1990; Stahle et al., 1991), the advantage of the technique proposed in the present study (the reference method) is that the effective dialysis coefficient (R_d) for the reference compound, antipyrine, is used to correct the difference in permeability rate constant between in vivo and in vitro microdialysis. Moreover, this procedure does not involve large uncertainties such as the behavior of diffusion in vivo except for the assumption that the effective dialysis coefficient of a test drug is equal to that of the reference compound. This assumption is based on previous findings that the R_d value is independent of both the molecular weight and the plasma membrane permeability of the substrates employed in the dialysis for the erythrocyte suspension (Deguchi et al., 1991). Moreover, verification of the validity of the above assumption has recently been demonstrated in muscle, liver and lung in which the permeability of low molecular weight substances across the capillary wall is not restricted (Deguchi et al., 1992).

On the other hand, Jacobson et al. (1985) estimated the ISF concentration using basically the same equation as Eqn 2 via fitting of the curves for the experimental data determined at various dialysis flow rates. In contrast, the reference method does not require a curve fitting procedure, and allows estimation of the ISF concentration solely from the data at constant dialysis flow rate.

For the verification of the present reference method, we assumed that the brain ISF concen-

tration of the substrate is equal to the plasma unbound concentration at steady state. This assumption can hold true for transport of the substrate across the blood-brain barrier by a process of simple and/or facilitated diffusion, and when it is not metabolized in the brain. Therefore, the following substrates were selected: [^{3}H]water, antipyrine, aminopyrine and caffeine, which are not metabolized in the brain and for which no specific mechanism of transport exists, such as that of active transport or protein-mediated transport systems. As shown in Tables 1 and 2, the observed values for [^{3}H]water, caffeine and aminopyrine, obtained by extrapolation according to the reference method were very close to those predicted, suggesting that the reference method is useful for reliable estimation of the in vivo unbound drug concentration in brain ISF; although the PA_{vitro} values of the above compounds differ several-fold. On consideration of the results listed in Table 1, it appears that [^{3}H]water should be suitable as an alternative reference compound in the method above, as concluded on the basis of the following reasons: (1) [^{3}H]water becomes distributed homogeneously throughout the whole body after intravenous bolus administration; (2) intravenous infusion is not required in order to maintain a steady state.

Since the reference method has been derived from the tube model and is applicable under steady-state conditions (Deguchi et al., 1991), comparison is readily performed of the in vivo dialysis clearance with the blood-brain barrier permeability surface product of a drug in the same dimension. In the case where the in vivo dialysis clearance is greater than the blood-brain barrier permeability surface product, microdialysis of the brain would result in the substrate concentration in brain ISF decreasing significantly. Thus, in the present study, substances which are highly permeable through the blood-brain barrier (Greig, 1989) were used in order to maintain steady-state conditions in brain ISF. On the other hand, the in vivo permeability rate constant at steady state is a function of the in vivo diffusional distance of the outer side of the membrane (Deguchi et al., 1991). When the system is not under steady-state conditions, several factors, namely, blood-brain barrier permeability, rates of uptake and exchange in ISF and parenchymal cells, and metabolic rate in brain tissue may reflect the magnitude of the in vivo diffusional distance, and/or the in vivo permeability rate constant. This could, perhaps, become a critical factor during brain microdialysis under non-steady-state conditions, since numerous drugs or other substances have a low degree of permeability across the blood-brain barrier.

The determination of substrates in brain ISF by microdialysis obviously requires that the procedure for cannula implantation does not damage the blood-brain barrier. Although the integrity of the blood-brain barrier only a few hours after implantation has been evaluated with α-aminoisobutyrate (Benveniste et al., 1984) and sodium technitate (Tossman and Ungerstedt, 1986), there are as yet no reports on the integrity of blood-brain barrier at 48 h or more after the implantation of cannula. Consequently, we evaluated the damage to blood-brain barrier at 1 and 48 h after implantation with [^{14}C]sucrose, as an extracellular marker, since the neuropil in brain tissue has been reported to be normal at those time points (Benveniste and Diemer, 1987). In the case where the blood-brain barrier is largely destroyed by cannula implantation, a high ISF/plasma ratio would be expected, since sucrose may permeate freely from the microvasculature into the ISF at the site of injury of the blood-brain barrier. As is clearly seen in Fig. 1, the ISF/plasma ratios of [^{14}C]sucrose estimated from the initial 30 min dialysate samples were 7% at 1 h and 3.7% at 48 h after transcranial-type cannula implantation. On the other hand, the ISF concentration of sucrose (C_{isf}) in the intact animal can be estimated via the numerical solution of Eqn 7 using the reported values for the permeability rate constant of [^{14}C]sucrose (P, 1.2×10^{-7} cm/s) (Levin et al., 1976), capillary surface areas (S, 33–171 cm^2/cm^3) (Levin et al., 1976) and the sucrose space (V_{isf}, 0.176 ml/g) (Patlak and Fenstermacher, 1975).

$$dC_{isf}/dt = (PS)(C_p - C_{isf})/V_{isf} \quad (7)$$

where C_p represents the plasma concentration which is fixed in the multiexponential equation using the pharmacokinetic parameters estimated via the fitting of the plasma concentration-time curve.

Then, the ISF/plasma ratio of sucrose at the initial time point of 30 min following the administration of [^{14}C]sucrose was estimated from the following expression:

$$\text{ISF/plasma} = \int_0^{30} C_{isf}\, dt \Big/ \int_0^{30} C_p\, dt \qquad (8)$$

The calculated values amounted to 0.022–0.110, in good agreement with the ISF/plasma ratios obtained from microdialysis. The data suggest that the blood-brain barrier did not suffer any significant damage. Moreover, the finding that the ISF/plasma ratio at 48 h after cannula implantation decreased 50% in comparison with that at 1 h implies that repair of the blood-brain barrier progresses further with time. Based on the above evidence, we concluded that the blood-brain barrier was not significantly damaged on implantation of the transcranial-type microdialysis cannula, and remained intact. Moreover, the above results suggest that microdialysis experiments can be performed at 1 h after probe implantation. The advantage of the reference method is that even in the chronic experiment, the in vivo unbound concentration in brain ISF could be determined, since reduction in the extent of dialysis clearance of the membrane by collagen deposits around the fiber wall (Benveniste and Diemer, 1987) can be compensated by that of the reference compound.

In conclusion, in order to evaluate the in vivo unbound concentration in brain ISF, extrapolation using the reference compound was demonstrated to provide a satisfactory estimate of the in vivo unbound concentration in brain ISF. Moreover, it has been shown that damage in the blood-brain barrier does not occur to any significant extent on implantation of the transcranial-type microdialysis cannula.

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