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Research Article

Microdialysis Calibration Using Retrodialysis and Zero-Net Flux: Application to a Study of the Distribution of Zidovudine to Rabbit Cerebrospinal Fluid and Thalamus

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A retrodialysis (RD) method for the real-time calibration of on-line microdialysis (MD) procedures was investigated *in vitro* and *in vivo*. Calibration by retrodialysis was simultaneously validated through the use of a zero-net flux (ZNF) method, which assumes directional independence of diffusion of the solute. In RD, a calibrator with dialysance (PeA; effective permeability–surface area product) similar to that of the compound of interest is introduced into the perfusate. If the calibrator is suitable, its loss from the perfusate during RD is identical to the recovery of the solute of interest determined simultaneously by normal MD. Two antiviral nucleosides (AZT and AZdU) which differ structurally by only a methylene group were utilized as solute and calibrator, respectively. Both nucleosides exhibited similar recovery and loss at flow rates of 0.5 to 5 μL/min *in vitro*, indicating a similar PeA product in this flow domain. Furthermore, both compounds showed similar loss into the lateral ventricle or thalamus of rabbits ($n = 4$) during RD at a flow rate of 1 μL/min for 6 hr. The relative loss decreased rapidly within the first hour, reaching a relatively stable value after 2 hr. The significant reduction in the loss of AZdU and AZT *in vivo* compared with that *in vitro* likely results from a lower diffusion coefficient in tissue. The distribution of AZT between plasma and cerebrospinal fluid (CSF) in the ventricle and extracellular fluid (ECF) in thalamus was determined at steady state using calibration by RD and ZNF simultaneously. The relative loss of AZdU during continuous RD was not significantly different from the recovery of AZT determined by ZNF in the same animal. Since RD may allow for continuous monitoring of microdialysis recovery in real time, it may offer an advantage over the ZNF method of system calibration. The steady-state C_{csf}/C_p and C_{ecf}/C_p ratios for AZT in this study were 0.26 ± 0.08 and 0.18 ± 0.08. That these ratios are much less than unity suggests that carrier-mediated transport of AZT exists in the brain-to-plasma direction.

KEY WORDS: brain microdialysis; retrodialysis; zero-net flux; antiviral nucleosides.

INTRODUCTION

Frequent measurements of the concentration of an active substance in extracellular fluid in an animal are needed to reliably assess drug distribution to a target organ. Microdialysis has proven to be a useful technique because it permits continuous monitoring of the solute concentration in extracellular space in the same animal. This is accomplished without the removal or introduction of fluids, either of which could disturb homeostasis. This technique was initially employed in neurochemical studies (1,2). Recently it has been used in pharmacological and pharmacokinetic studies to determine the concentration of drug in brain (3–5), subcutaneous tissue (6), liver (7), and blood (8) and in assessing plasma protein binding (9).

The primary problem which limits the application of microdialysis in pharmacokinetic studies is the determination of relative recovery of the solute of interest *in vivo*. This results from the significant difference in diffusion behavior of a solute in tissue and in water. Relative recovery is defined as the ratio of the concentration of the compound of interest in dialysate to that in the medium surrounding the probe.

Through an examination of fundamental transport and physiological parameters, Lindefors *et al.* (10,11) developed a detailed mathematical model of microdialysis that recognizes the importance of tortuosity as well as diffusion and convection phenomena in the tissue, probe membrane, and probed medium. In addition, Bungay *et al.* (12) presented a steady-state diffusion model that considered the effects of microvasculature transport, metabolism, and intra/extracellular space exchange. Furthermore, Morrison *et al.* (4) extended the model of Bungay *et al.* and described transient probe response when metabolism or microvasculature transport play major roles. These models have greatly advanced our understanding of factors governing spatial and time-dependent solute distribution.

In an effort to characterize the concentration of solutes in extracellular fluids from a knowledge of dialysate concen-

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trations, Jacobson *et al.* (13) described an approach utilizing a steady-state mass transfer relationship which considers changes in dialysate concentration at different flow rates. This method is also called the flow-rate or stop-flow method. Lonnroth *et al.* (6) developed a method where recovery *in vivo* is estimated from dialysate concentrations when a wide range of concentrations of the solute of interest are perfused, while maintaining the extracellular concentration *in vivo* at steady state. This is referred to as the concentration difference method or zero-net flux method (ZNF). Stahle *et al.* (14) compared these two methods and concluded that both gave satisfactory results, with the zero-net flux method of Lonnroth *et al.* possibly yielding more accurate predictions.

Because the ZNF method requires that the study subject be examined under steady-state conditions prior to the actual experiment, the total study time is extended. In view of observed time-dependent changes in relative recovery (15,16), it is possible that recovery calculated using a zero-net flux approach may not be applicable during the subsequent experimental period. Retrodialysis (RD), which allows for continuous assessment of recovery *in vivo* during the study period (5,17), may avoid this potential problem. RD is technique for probe calibration involving the measurement of diffusive loss of molecules (e.g., a RD calibrator) from the dialysis perfusate into the environment surrounding the probe, under sink conditions.

In this study, two compounds (AZT as the solute of interest and AZdU as the RD calibrator) which differ structurally by only a methylene group were utilized. Both compounds are anti-HIV nucleosides with comparable polarity and molecular weight (267 and 253, respectively) and similar pharmacokinetic behavior (18,19). Because of AZT's limited lipid solubility, its distribution to brain tissue extracellular fluid was questionable. Thus, concentrations of this agent in brain tissue ECF were of interest, particularly in subcortical regions, such as the thalamus, where evidence of viral infection was obtained in early studies.

The objective of this work was to investigate the use of retrodialysis of AZdU for the real-time calibration of microdialysis recovery of AZT from ventricular cerebrospinal fluid (CSF) and from the extracellular space in the thalamus of rabbits receiving constant-rate intravenous infusions of AZT. In addition, the agreement between retrodialysis and the zero-net flux method of Lonnroth *et al.* for the calibration of microdialysis recovery *in vivo* and *in vitro* was assessed.

THEORETICAL

Considering the case of laminar flow in a hollow cylindrical dialysis fiber introduced into the tissue (Fig. 1), and ignoring diffusion in the direction of flow, it can be shown (16,20) that

$$\frac{C_o - C_e}{C_o - C_i} = \exp^{(-PeA/Q)} \quad (1)$$

Here C_e denotes the effluent concentration; C_i, the influent concentration; C_o, the solute concentration outside the probe membrane; Pe, the effective solute permeability; A, the membrane surface area; and Q, perfusate (dialysate) flow

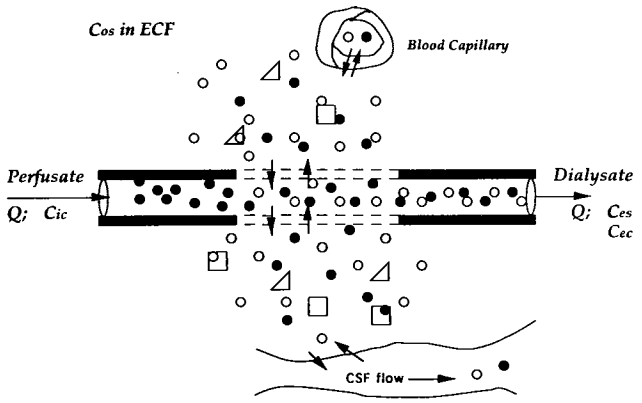

Fig. 1. Microenvironment within and surrounding the microdialysis probe *in vivo*. The solid and dashed line segments schematically represent the nonpermeable probe wall and semipermeable membrane, respectively. Open and filled circles represent molecules of solute of interest and retrodialysis calibrator, respectively. Squares and triangles represent macromolecules which may bind solute and/or calibrator but which are not recovered by dialysis. Arrows indicate direction of transport. Abbreviations are as defined in the text.

rate. The apparent permeability–area product (PeA), or dialysance, has dimensions of volume per unit time. PeA is a function of mass transport (diffusive) resistance. In general, it varies with radial distance, and depends on the physiochemical properties of the solute, characteristics of the medium, and interactions between solute and medium (16). A solute and its retrodialysis calibrator should exhibit the same PeA product during microdialysis and retrodialysis.

During microdialysis and retrodialysis *in vitro*, the overall resistance to diffusion includes that associated with dialysate, probe membrane, aqueous diffusion layer, and medium surrounding the probe. However, during microdialysis and retrodialysis *in vivo*, additional resistance is derived from interstitial space, intra-/extracellular space exchange, microvasculature transport, and metabolism in tissue (16). Furthermore, dialysis membrane composition, effective surface area, exact placement in tissue, and microenvironment of the probe are potential sources of variation in recovery from one study preparation to another (7). In addition, time-dependent changes in the probe membrane and/or its environment may occur within a study. To evaluate these diffusive resistances separately would be extremely difficult. However, retrodialysis may provide a practical means of continuously monitoring recovery without characterizing these individual barriers to diffusion. In the following, three simplifying assumptions are made: (a) the transport of a solute and its calibrator is a simple diffusion process and the overall diffusion flux is rate-limited by the slowest step, (b) sink conditions exist for the loss of calibrator from the probe and for recovery of the solute, and (c) there is no thermodynamic interaction between the calibrator and the solute.

During microdialysis, the concentration of the solute of interest in the perfusion medium is normally equal to zero. The recovery of solute (R_s) is defined by its effluent concentration (C_{es}) in relation to the donor solution concentration (C_{os}). This ratio depends on Pe, A, and Q, as follows (13):

$$R_s = \frac{C_{es}}{C_{os}} = 1 - \exp^{(-[PeA]_s/Q)} \quad (2)$$

With retrodialysis, the calibrator is introduced into the perfusate, and its relative loss during perfusion is measured. The fraction (R_c) of the calibrator recovered (i.e., not lost) is defined as the ratio of its concentration in the effluent (C_{ec}) to that in the influent perfusate (C_{ic}). As in microdialysis, the fraction recovered during retrodialysis is related to Pe, A, and Q, as follows:

$$R_c = \frac{C_{ec}}{C_{ic}} = \exp^{(-[PeA]_c/Q)} \quad (3)$$

From Eqs. (2) and (3), the relative loss of the calibrator (L_c) during retrodialysis is equal to the microdialytic recovery of the solute if both exhibit a similar PeA product. Thus,

$$L_c = 1 - R_c = 1 - \exp^{(-[PeA]_c/Q)} \quad (4)$$

The purpose of including a calibrator in the perfusate is to estimate the recovery of the solute of interest from the donor solution by equating R_s to L_c. An obvious application of this occurs in microdialysis *in vivo* when the solute concentration in the donor solution (often tissue extracellular fluid) is unknown but can be calculated if its relative recovery can be estimated *in vivo*. The concentration of the solute in tissue extracellular fluid is then calculated as

$$C_{os} = \frac{C_{es}}{L_c} \quad (5)$$

In general, the dialysance of a calibrator, $[PeA]_c$, may be different from that of the solute, $[PeA]_s$. Here, retrodialysis loss of the calibrator (L_c) is not equal to the microdialysis recovery of the solute (R_s). Assume $[PeA]_c$ is a fraction, f, of $[PeA]_s$:

$$[PeA]_c = f \cdot [PeA]_s \quad (6)$$

When Pe$A \ll Q$, it can be seen through a Taylor expansion that

$$\frac{L_c}{R_s} = \frac{1 - \exp^{(-f\cdot[PeA]_s/Q)}}{1 - \exp^{(-[PeA]_s/Q)}} \approx f \quad (7)$$

This relationship shows that f, the ratio of the dialysance of the calibrator to that of its solute, is approximated by the ratio of the calibrator loss to solute recovery, in a range of flow rates where Pe$A \ll Q$.

An additional technique which has been successfully used to estimate microdialysis recovery *in vivo* was introduced by Lonnroth *et al.* (6). Often termed the difference method, it is referred to here as the zero-net flux (ZNF) technique. This approach has been used to determine concentrations of ions, glucose, and theophylline *in vivo* or *in vitro* (3,6,14). The method requires that the concentration of solute outside the probe is fixed, e.g., at steady state. Then, the direction of the gradient across the dialysis membrane depends upon whether the concentration in the perfusion medium is greater or less than that in the fluid surrounding the membrane. By varying the concentration in the perfusion medium over a sufficiently large range, the magnitude and direction of the gradient is altered. Using symbols described above, the following relationships can be derived:

$$C_{es} = R_s \cdot C_{os} + (1 - L_s) \cdot C_{is} \quad (8)$$

and

$$C_{es} - C_{is} = R_s \cdot C_{os} - L_s \cdot C_{is} \quad (9)$$

The concentration in the medium surrounding the probe, C_{os}, at which the net flux across the membrane is zero, can be estimated from Eq. (9), by linear regression of the concentration difference ($C_{es} - C_{is}$) on C_{is}, as the intercept on the X axis. The slope is $-L_s$, where L_s is equal to R_s, the relative recovery of the solute. When the net flux is zero ($C_{es} - C_{is} = 0$), we get

$$R_s \cdot C_{os} - L_s \cdot C_{is} = 0 \quad (10)$$

Thus, C_{os} equals C_{is} when R_s equals L_s. Since this analysis depends upon the linear relationship expressed by Eq. (9), it assumes that recovery, the slope of the line, is independent of solute concentration.

MATERIALS AND METHODS

Drugs and Reagents

We used zidovudine (3′-azido-3′-deoxythymidine: AZT; Burroughs Wellcome Co., Research Triangle Park, NC), 3′-azido-2′,3′-dideoxyuridine (AZdU; Triton Biosciences Inc., Alameda, CA), β-hydroxypropyl theophylline (BHPT; Sigma Chemical Co., St. Louis, MO), ammonium phosphate monobasic (AR grade), isopropyl alcohol, and methyl alcohol (Mallinckrodt, Inc., St. Louis, MO). Solvents were of HPLC grade, and all other chemicals were AR grade.

Surgery

Guide cannula placement was accomplished using micromanipulators mounted on a stereotaxic frame fitted with a rabbit adapter (David Kopf Instruments, Tujunga, CA). Cannulas were positioned in the thalamus and ventricle of adult male New Zealand White rabbits ($n = 8$), weighing 3–4 kg, under anesthesia as before (5). Briefly, 0.4 mg of atropine sulfate (Vedco, Inc., St. Joseph, MO) was injected intramuscularly (i.m.) into the thigh. A half-hour later, a half-dose of atropine sulfate was given again, and 1 mg/kg of acepromazine maleate (Aveco Co., Inc., Fort Dodge, IA) and 50 mg/kg of ketamine (Fort Dodge Lab., Fort Dodge, IA) were given i.m. When the rabbit was anesthetized, a dose of penicillin G potassium (Marsam Pharmaceuticals Inc., Cherry Hill, NJ) equivalent to 0.3 million units (USP) was given, i.m. Dialysis guide cannulas were positioned (21) in the right lateral ventricle (coordinates, relative to bregma: anterior, 1.0 mm; lateral, 2.5 mm; and ventral, 7.0 mm) and thalamus (coordinates: posterior, 4.0 mm; lateral, 4.0 mm; and ventral, 10.0 mm).

To obtain the CSF directly, a cannula (PE-50) was inserted into the cisterna magna through a 4-mm-diameter hole drilled 5 mm laterally from the sagittal suture and 5 mm anterior to the lambda. Dental cement (L. D. Caulk Company, Milford, DE) was used to anchor and fix the guide cannulas and the PE-50 cannula in position. The surgical incision was sutured to cover the cement, leaving the cannulas exposed. Microdialysis was performed one week after recovery from surgery.

Probes and Cerebrospinal Fluid (CSF) Perfusate

Microdialysis probes (CMA/10; BAS, West Lafayette, IN) were used as the sampling device for *in vitro* and *in vivo* experiments. The probe has a 3-mm membrane length, 400-μm ID, 500-μm OD, and 20,000 molecular weight cutoff. The dead volume of the probe is 4 μL.

Simulated CSF solution was freshly prepared (22) and filtered (PC membrane, 47 mm, 0.4 μm; Nucleopore, Pleasanton, CA) before use. Concentrations (m *M*) of ions in the final solution were Mg (1.1), Na (144.2), Ca (1.35), Cl (129.5), K (3.0), and PO_4 (0.242), and the pH was 7.6. Solutions of AZT and AZdU were prepared directly by dissolving them in CSF.

Experimental Setup for On-Line Microdialysis

An on-line microdialysis-HPLC analysis system was used in this study. Various flow rates of dialysis perfusate were achieved using a Harvard microdialysis pump (Model 22; Harvard Apparatus, Inc., South Natick, MA) adapted with 1-mL plastic syringes (Monoject, St. Louis, MO). Dialysis effluent was collected directly into an injection loop (5 μL) before injection. In this setup, two loops were fitted into a multifunctional 10-port valve (Valco Instruments, Co. Inc., Houston, TX) controlled by a digital valve sequence programmer (Valco). The use of two loops allows for parallel experiments at two sampling sites.

In Vitro Dialysis Experiments

Effect of Flow Rate on Microdialysis Recovery or Retrodialysis Loss of AZT and AZdU in Vitro. To determine microdialysis recovery *in vitro*, probes were immersed in an unstirred solution (0.5 μg/mL) of AZT and AZdU in CSF (C_{os}) at room temperature (25°C). These were perfused with blank CSF (C_{is} = 0) at each of five flow rates (0.5, 1, 2, 3, and 5 μL/min). Time was allowed for system equilibration when the flow rate was changed. At each flow rate, four perfusate samples at steady state were collected on-line and injected for analysis. Samples of the probed solution were also injected to determine C_{os} for each compound.

The loss of AZT and AZdU during retrodialysis was examined using the same probes (n = 4) and flow rates. Here, a solution of AZT and AZdU (0.5 μg/mL) was perfused through probes immersed in blank CSF.

Accuracy in Determination of Concentration of AZT Using the Zero-Net Flux Method with Simultaneous Retrodialysis in Vitro. Here, a solution of known concentration of AZT (0.49 μg/mL by direct assay) in CSF was used as the surrounding medium. The dialysis probe was perfused at a flow rate of 1 μL/min with a solution containing a fixed concentration of AZdU (0.15 μg/mL) and varying concentrations of AZT (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/mL). For each AZT concentration, three to four dialysates at steady state were injected for analysis of AZT and AZdU. The concentrations in each sample were calculated using a standard curve, which was prepared immediately before or after the experiment on the same day. The recovery of AZT and the concentration of AZT in the probed medium were obtained from regression analysis of the data, using a zero-net flux plot [Eq. (9)]. The relative loss of AZdU by retrodialysis during each perfusion interval was also calculated from Eq. (4).

In Vivo Dialysis Experiments

Retrodialysis Loss of AZT and AZdU in Vivo. An experiment to examine relative loss-time profile of AZT and AZdU into rabbit thalamus and lateral ventricle (n = 4) *in vivo* was performed at flow rate of 1 μL/min. Retrodialysis was conducted for 6 hr, during which the rabbit was conscious but restrained and allowed free access to water. To confirm integrity of the probes and assess the difference in relative loss *in vitro* and *in vivo, in vitro* dialysis was conducted before and after the *in vivo* experiment. During the *in vitro* study, a concentration of 0.15 μg/mL of AZT and AZdU in CSF solution was retrodialyzed using 50 mL of blank CSF as surrounding medium. Four dialysate samples at steady state were collected to calculate the relative loss of AZT and AZdU. In the *in vivo* experiment, probes removed from the *in vitro* system were inserted immediately into the guide cannulas in the ventricle and thalamus, and retrodialysis was continued using the same solutions of AZT and AZdU. Dialysis samples were collected beginning immediately after the probes were inserted. Sufficient time was allowed to clear the system prior to collecting dialysates when probes were repositioned. The relative loss of AZT and AZdU was estimated as above.

Accuracy in Determination of Concentration of AZT Using the Zero-Net Flux Method with Simultaneous Retrodialysis in Vivo. This study was similar in design to that performed *in vitro*. Here, the concentrations of AZT in ventricle and thalamus were maintained at steady state by constant iv infusion of AZT (n = 4). Infusion was maintained through a catheter inserted in the marginal ear vein (I-Cath Delmed, New Brunswick, NJ). To ensure steady state, iv infusion was started at least 6 hr before dialysis commenced. Three of the animals were infused at a rate of 1 mg/hr-kg, and one animal was infused at twice this rate.

Dialysis probes were inserted into the ventricle and thalamus guide cannulas and perfused immediately. A series of solutions in which the AZdU concentration was fixed (0.15 μg/mL), and AZT concentrations varied (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/mL), was selected for these studies. Three or four dialysis samples at steady state collected on-line were injected for each AZT concentration in perfusate. Compared to dialysis *in vitro*, longer times (about 1.5 hr) were needed to achieve a steady-state loss during each period. Blood samples were collected at the midpoint of each period from another ear vein catheter. Plasma was separated by centrifugation, transferred to vacutainers, and stored at −20°C until analysis. CSF (30 μL) was sampled from the cisterna magna of one rabbit at the same time that blood was collected and transferred to a tube containing 20 ng of BHPT as internal standard. The CSF samples were immediately diluted with 30 μL of mobile phase and mixed well prior to analysis.

Sample Analysis

Plasma samples were analyzed off-line for AZT using an HPLC method (23). This method allows quantification of AZT in concentrations as low as 0.010 μg/mL in plasma. CSF samples were analyzed for AZT after supplementing

with the internal standard, by direct injection using the same HPLC system.

Microdialysis samples collected on-line were injected directly onto a microbore hypersil ODS column [20 cm × 2.1 mm (i.d.), 5-μm average particle size; Hewlett-Packard, Avondale, PA 19311]. The mobile phase consisted of 15% by volume of acetonitrile in 10 m*M* monobasic ammonium phosphate, and the flow rate was 0.2 mL/min using a Waters 510 HPLC pump (Millipore Co., Milford, MA 01757). Column effluent was monitored at 254 nm using a Waters 440 absorbance detector (Waters Associates, Inc., Milford, MA 01757) and peak heights were calculated using an electronic integrator (Model 3390, Hewlett-Packard). Under these experimental conditions, AZdU and AZT are well separated, with retention times of 5.1 and 6.9 min, respectively. The limit of quantitation is 0.010 μg/mL for both AZT and AZdU, corresponding to 50 pg on column.

Data Analysis

Differences were considered significant when $P < 0.05$ using ANOVA. The clearance of AZT at steady state was calculated as the infusion rate (k_0) divided by the steady-state plasma concentration (C_{pss}). Simple linear regression was performed to determine the recovery of AZT during ZNF, according to Eq. (9), and to establish a relationship between retrodialysis loss of AZdU and ZNF recovery of AZT.

RESULTS

Effect of Flow Rate On Microdialysis Recovery or Retrodialysis Loss of AZT and AZdU *in Vitro*

As flow rates were increased from 0.5 to 5 μL/min, the relative recovery and loss of AZT and AZdU decreased (n = 4 at each flow rate) from approximately 40 to 5% (Fig. 2). Both compounds exhibited a similar recovery when they were dialyzed together and a similar loss when retrodialyzed simultaneously ($P > 0.05$) at each flow rate employed. There was also no significant difference between the recovery and the loss of both compounds at any flow rate ($P > 0.05$). These results suggest that AZT and AZdU exhibit similar diffusivities across the microdialysis membrane and the efficiency of diffusion (dialysance) is the same in both directions, i.e., the Pe*A* products for both compounds are similar and independent of direction across the membrane.

Linear relationships were obtained from Eqs. (2) and (4):

$$\ln(1 - R_s) = -\frac{[PeA]_s}{Q} \tag{11}$$

and

$$\ln(1 - L_c) = -\frac{[PeA]_c}{Q} \tag{12}$$

Application of these equations in modeling dialysis and retrodialysis data revealed an excellent fit (r = 0.999) shown in the inset in Fig. 2. This suggests that the assumption of constant Pe*A* is valid in this flow domain. The Pe*A* values (μL/min) for AZT and AZdU (mean ± SD; n = 4) obtained were 0.26 ± 0.02 and 0.27 ± 0.02, respectively, using microdialysis data and 0.23 ± 0.04 and 0.24 ± 0.04, respectively, using retrodialysis data. None of these values were significantly different ($P > 0.05$).

Accuracy in Determination of Concentration of AZT Using the Zero-Net Flux Method with Simultaneous Retrodialysis *in Vitro*

Figure 3 depicts the results of zero-net flux study, expressed as the mean ± SD of the pooled data (n = 12 to 16) from four probes at each of seven concentrations. Retrodialysis was performed simultaneously in the four probes, and these pooled results are also plotted (mean ± SD; n = 84). Linear regression of the AZT concentration difference ($C_{es} - C_{is}$) on the concentration in perfusate (C_{is}) showed a good correlation for all four probes. The regression slope, which equals the recovery or loss of AZT at this flow rate, was 0.24 ± 0.05 (four probes). The concentration of AZT in the outer

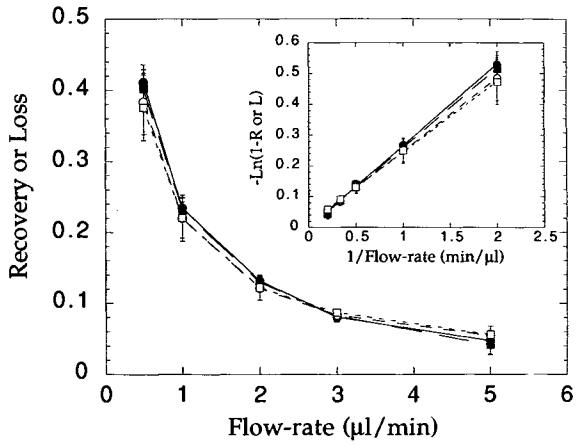

Fig. 2. Effect of flow rate on recovery of AZT and loss of AZdU during microdialysis and retrodialysis *in vitro*. Inset: Linear relationship between the transformation of recovery (R) or loss (L) and dialysate flow rate during microdialysis and retrodialysis *in vitro*. AZT: recovery (□); loss (■). AZdU: recovery (○); loss (●). Mean ± SD for four probes is shown.

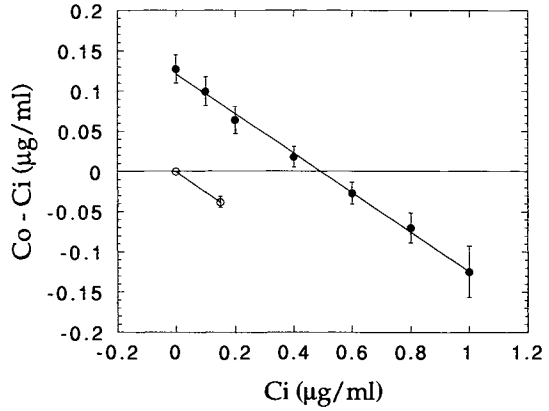

Fig. 3. Results of microdialysis (mean ± SD of pooled data from four probes) using the zero-net flux method (●) and simultaneous retrodialysis with AZdU (○) *in vitro*. Axes refer to solute and calibrator concentrations for ZNF and RD methods, respectively. The slopes are equal to recovery of AZT and loss of AZdU (○).

medium was well predicted using the zero-net flux method, with the predicted value being 100 ± 6% of the assayed value (Table I).

From retrodialysis, the relative loss of AZdU (RD loss) was obtained over the entire time course during which solutions of varying concentrations of AZT were perfused. The loss of AZdU was not statistically different from the recovery of AZT (ZNF recovery) obtained using zero-net flux method (Table I). The agreement between the ZNF recovery and the RD loss suggests that there is no interaction across the probe membrane in opposite directions. The AZT concentration in the probed medium can also be calculated using AZdU as RD calibrator by rearranging Eq. (9), assuming $L_c = R_s$, as follows:

$$C_{os} = \frac{C_{es} - (1 - L_c) \cdot C_{is}}{L_c} \quad (13)$$

The calculated AZT concentrations in the probed medium [Eq. (13)] are also given in Table I. The predicted value is 95 ± 4% of the assayed value.

Retrodialysis Loss of AZT and AZdU *in Vivo*

Figures 4a and b show the relative loss of AZT and AZdU into the thalamus and ventricle, respectively, in four rabbits over a period of 6 hr. These figures also summarize the relative loss of both compounds *in vitro* immediately prior to and following the *in vivo* studies. There was no significant difference in AZT and AZdU loss *in vitro* or *in vivo*. Additionally, the loss of AZT and AZdU *in vivo* were significantly less than those *in vitro* for each probe. During retrodialysis *in vivo*, the loss of AZT and AZdU into both thalamus and ventricle showed a rapid decrease within the first hour, reaching a relatively stable value after 2 hr. The average loss of AZdU and AZT (mean ± SD) into the thalamus after 2 hr was 0.10 ± 0.02 and 0.098 ± 0.024, respectively; loss into the ventricle was 0.13 ± 0.03 and 0.13 ± 0.03, respectively. The loss into the ventricle, however, was greater than that into the thalamus ($P < 0.05$). The magnitude of loss of AZdU and AZT into the thalamus relative to the initial loss *in vitro* was 39 ± 5 and 38 ± 4%, respectively; that into the ventricle was 50 ± 22 and 48 ± 17%, respectively. Probes inserted in the thalamus exhibited a greater rebound in recovery than those inserted in the ventricle when they were later calibrated *in vitro*, for reasons that are unclear.

Table I. Measurement of Known Concentrations of AZT (C_{os} = 0.49 μg/mL) Using the ZNF Method and Simultaneous RD *in Vitro* (n = 4)

Probe no.	RD L_{azdu} (mean ± SD)	ZNF R_{azt}	Predicted concentration (μg/mL) By RD (mean ± SD)	Predicted concentration (μg/mL) By ZNF
1	0.30 ± 0.03	0.31	0.47 ± 0.04	0.47
2	0.24 ± 0.04	0.24	0.44 ± 0.04	0.47
3	0.26 ± 0.04	0.24	0.49 ± 0.03	0.53
4	0.21 ± 0.03	0.18	0.47 ± 0.06	0.50
Mean	0.25	0.24	0.47	0.49
SD	0.04	0.05	0.02	0.03

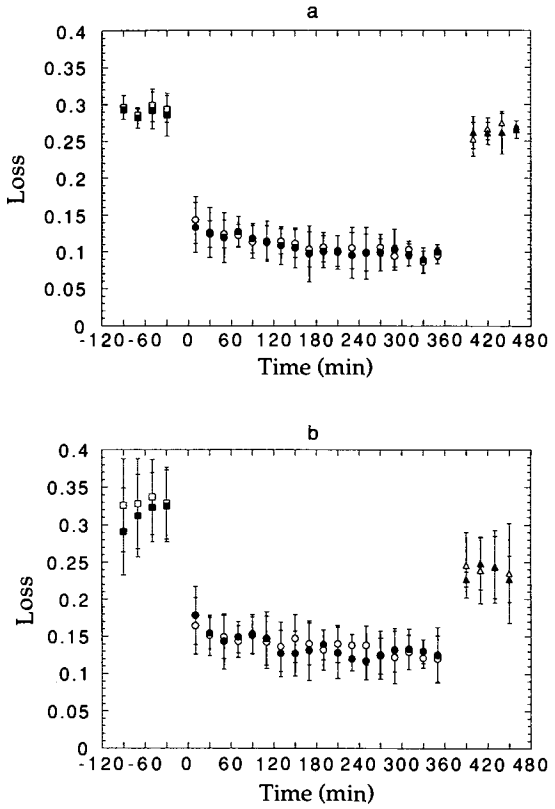

Fig. 4. Relative loss of AZT (●) and AZdU (○) into thalamus (a) and ventricle (b) of rabbits (n = 4) compared with corresponding *in vitro* loss before (■, □) and after (▲, △) the experiment *in vivo* (mean ± SD) during retrodialysis at a flow rate of 1 μL/min.

Accuracy in Determination of Concentration of AZT Using the Zero-Net Flux Method with Simultaneous Retrodialysis *in Vivo*

Figure 5 illustrates representative results obtained using ZNF and RD in a rabbit with dialysis probes inserted in both the thalamus and the ventricle. Good correlations between the concentration differences ($C_{es} - C_{is}$) and concentrations in perfusate (C_{is}) were obtained for thalamus (r^2 = 0.97) and ventricle CSF (r^2 = 0.99). Relative recoveries of AZT were estimated as the negative slope of the linear regression line [Eq. (9)]. Tissue concentrations (C_{csf} and C_{ecf}) were calculated directly from the intercepts of the regression lines on the X axis as before. The relative loss of AZdU in thalamus and ventricle probes during the entire experiment was also obtained simultaneously by retrodialysis. These results (mean ± SD; n = 25) for the same animal are included in Fig. 5, and the data for all animals are summarized in Table II. The loss of AZdU, calculated from Eqs. (3) and (4), is equivalent to the slope of the line segment shown for the RD data in Fig. 5.

Simple linear regression of the average loss of AZdU during RD upon the relative recovery of AZT estimated using ZNF (Fig. 6) yielded a linear relationship with a slope of 0.97 and an intercept of 0.008 (line of identity rather than

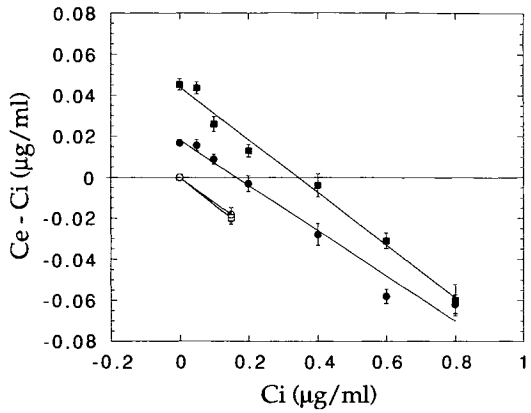

Fig. 5. Microdialysis of the ventricle (squares) and thalamus (circles) in one rabbit using zero-net flux method (ZNF; filled symbols) and simultaneous retrodialysis (RD; open symbols) at a flow rate of 1 μL/min. Rabbit received a constant-rate iv infusion of AZT for at least 6 hr prior to ZNF and RD calibration. Axes refer to solute and calibrator concentrations for ZNF and RD methods, respectively. Ordinate values are mean ± SD for ZNF data (filled symbols; n = 3 or 4 at each concentration) and for RD data (open symbols; n = 25). The ZNF data for ventricle and thalamus were fitted by simple linear regression. Slopes are estimates of recovery of AZT (ZNF) and loss of AZdU (RD).

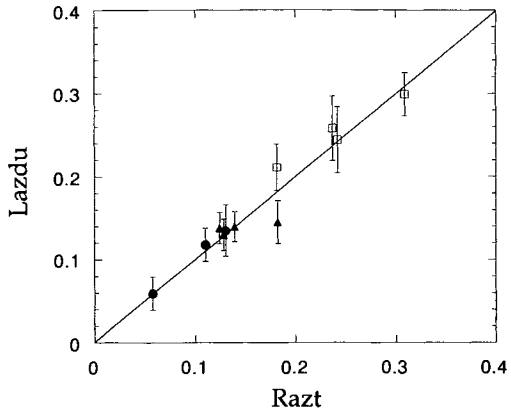

Fig. 6. Agreement between retrodialysis loss of AZdU and the zero-net flux recovery of AZT, *in vivo* (thalamus, ●; ventricle, ▲) and *in vitro* (□). The line of identity is shown.

regression line shown) and good correlation (r^2 = 0.94). Thus, calibration of probe recovery was comparable by both methods in the *in vitro* and *in vivo* studies.

Agreement in the concentrations of AZT measured directly in CSF and those determined by ZNF regression or RD calibration [using Eq. (13)] demonstrates that both ZNF and RD provide a similar accuracy in predicting the concentration of AZT in ventricular CSF (Fig. 7). The concentrations of AZT in the thalamus and ventricle calculated using the ZNF method and RD, as well as the steady-state concentrations in plasma, are listed in Table III. There was no significant difference between the concentrations determined by ZNF and RD. The ratios of free concentrations of AZT in the ventricle and thalamus over that in the plasma at steady state are also provided in Table III.

DISCUSSION

Theoretical analysis of relative recovery and loss during microdialysis and retrodialysis shows that dialysance (PeA product) plays a major role in determining recovery and loss *in vitro* or *in vivo*. This term has dimensions of flow, and may be thought of as an effective clearance, expressing the flux of solute across the probe membrane per unit concentration of solute.

Dialysance and the resultant recovery are not easily estimated in routine microdialysis experiments. This in part prompted the work of Jacobson, who described a flow-rate method (13) and the studies by Lonnroth *et al.*, who introduced the zero-net flux or difference method (6), as well as diffusion models (4,10–12,15). Retrodialysis calibration may be a useful technique for quantitative microdialysis, if a retrodialysis calibrator, which satisfies the three assumptions described earlier, is available. These assumptions may be met more readily *in vitro* than *in vivo* since the rate-limiting barrier for diffusion *in vitro* is usually the dialysis membrane. Exceptions to this occur when membrane hydraulic conductivity and transmembrane pressure differences, both hydrostatically and osmotically derived, are different during microdialysis and retrodialysis (16).

In the present studies *in vitro*, a similar PeA product for two model compounds AZT and AZdU during microdialysis and retrodialysis at different flow rates, as well as the agree-

Table II. Recovery of AZT Obtained Using the ZNF Method and Loss of AZdU During RD in Thalamus and Ventricle of Rabbits

Rabbit no.	ZNF R_{azt}		RD L_{azdu} (mean ± SD)	
	Thalamus	Ventricle	Thalamus	Ventricle
1	0.058	0.12	0.059 ± 0.020	0.14 ± 0.02
2	0.13	0.18	0.14 ± 0.03	0.15 ± 0.03
3	0.11	0.13	0.12 ± 0.02	0.13 ± 0.02
4	—	0.14	—	0.14 ± 0.02
Mean	0.099	0.14	0.10	0.14
SD	0.037	0.03	0.04	0.01

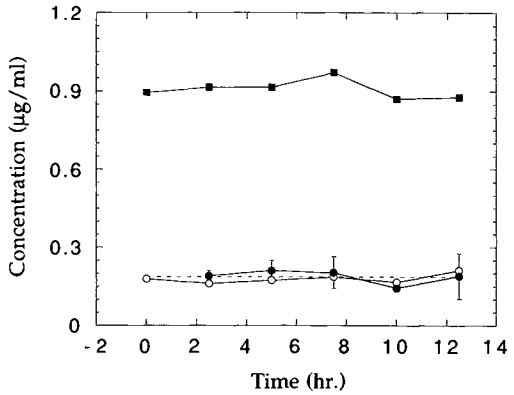

Fig. 7. Steady-state concentrations of AZT in CSF measured directly (○), estimated by the ZNF method (-------), and by RD (●; mean ± SD) in a microdialyzed rabbit receiving a constant-rate iv infusion of AZT. AZT plasma concentrations in the same animal (■) are also plotted. The concentration estimated by the ZNF method is a single value calculated as the X-axis intercept of the regression line expressed in Eq. (9).

Table III. Distribution of AZT Among Plasma, Ventricular CSF, and Thalamus ECF Determined at Steady State Using ZNF and RD Simultaneously

Parameter	Rabbit No. 1	2	3	4	Mean ± SD
k_o (mg/hr · kg)	2.0	1.0	1.0	1.0	
C_{pss} (μg/mL)	1.8 ± 0.11	0.50 ± 0.06	0.86 ± 0.07	0.91 ± 0.04	
CL (mL/min · kg)	18.4	33.2	19.4	18.3	22.3 ± 7.3
C_{csf} (μg/mL)	—	—	—	0.18 ± 0.02	
ZNF					
C_{csf} (μg/mL)	0.48	0.10	0.34	0.18	
C_{ecf} (μg/mL)	0.44	0.059	0.16	—	
C_{csf}/C_p	0.27	0.20	0.40	0.20	0.27 ± 0.09
C_{ecf}/C_p	0.24	0.12	0.19	—	0.18 ± 0.06
RD					
C_{csf} (μg/mL)	0.47 ± 0.08	0.10 ± 0.03	0.32 ± 0.05	0.19 ± 0.06	
C_{ecf} (μg/mL)	0.45 ± 0.14	0.049 ± 0.015	0.15 ± 0.03	—	
C_{csf}/C_p	0.23 ± 0.05	0.21 ± 0.06	0.37 ± 0.06	0.21 ± 0.02	0.25 ± 0.08
C_{ecf}/C_p	0.26 ± 0.05	0.078 ± 0.048	0.18 ± 0.03	—	0.17 ± 0.09

ment between ZNF recovery and RD loss, suggested that dialysance is equal in both directions in a dialysis device. AZT recovery or loss may be predicted by AZdU recovery or loss, or vice versa.

Microdialysis or retrodialysis *in vivo* is a much more complicated process than that *in vitro*. Benveniste *et al.* (15) and Lindefors *et al.* (10,11) have concluded that the probe membrane need not be considered to be a major diffusion barrier for recovery under conditions *in vivo*. This is consistent with what we have observed in the present study. Both recoveries and losses of AZT and AZdU *in vivo* were significantly less than those *in vitro*. It should be noted that although the *in vitro* studies were carried out at 25°C, the *in vitro* recoveries were significantly greater than those determined at 37°C *in vivo*. Greater differences would be expected if the *in vitro* studies were performed at the higher temperature, since diffusivity is temperature dependent. It has been shown that solute recovery may increase by 30% with temperature increases from 23 to 37°C (6,10,25). In addition, the relative recovery *in vivo* is time dependent. Others have shown (15,16) that recovery decreases rapidly with the time, achieving a steady-state value which depends on the diffusivity of the solute *in vivo*. The relative loss-time profiles of AZT obtained *in vivo* in the present study are qualitatively similar to those measured for sucrose (26). However, whether the loss-time profiles reflect the recovery-time profile as a mirror image needs further study. That sink conditions apparently exist for the loss of AZT and AZdU during retrodialysis *in vivo* may be related to their rapid removal from extracellular fluid into capillary blood by an active transport system (5,19,27).

Since AZT exhibits negligible (5–10%) protein binding in rabbit plasma (24), unbound concentration ratios (brain/plasma) significantly less than unity at steady state suggests the existence of active transport of AZT from brain to plasma. In addition, the total clearance of this drug in the rabbit is independent of the dose. These results are consistent with those of previous studies which have shown that the total clearance of AZT and its distribution into thalamus and ventricular CSF are independent of plasma concentration in this range (5,24). That the clearance of AZT is not altered from control values (24) by the microdialysis procedure is supported by the measured C_{pss} in this study.

A stable steady-state plasma concentration of AZT was achieved in these infusion studies. It should be noted that the attainment of a stable plasma level does not insure that a specific tissue level will also be at steady state. The ZNF method requires the establishment of a steady state in the probed tissue, where RD may be applied under non-steady-state conditions. Demonstration of a stable plasma level in this study was necessary to justify a comparison of both methods of microdialysis calibration.

To be useful as a quantitative tool, microdialysis probes must be calibrated *in vivo*. It has been reported that tritiated water (28) and antipyrine (7), both of which distribute throughout body water, may be used as dialyzable references to correct for differences in efficiency among and within dialysis probes *in vivo*. Retrodialysis may also be a useful technique for this purpose. Caffeine has been utilized as a retrodialysis calibrator in the microdialysis of theophylline in rat brain (29). An ideal retrodialysis calibrator is one which demonstrates the same dialysance *in vivo* as the solute of interest. A radiolabeled form of the compound which exhibits the same diffusion coefficient may be a suitable choice. However, an unlabeled compound which is similar to the solute of interest in terms of its molecular size, degree of ionization, lipophilicity, and interaction with the probe membrane and dialysis components may serve as a calibrator. Indeed, the use of an unlabeled compound may be advantageous if it can be assayed simultaneously with the solute, thereby avoiding an additional analytical procedure. This would be a particular advantage where on-line analysis of the dialysate is performed. However, it should be noted that similar diffusivity of two compounds in an aqueous medium *in vitro* does not ensure that they will exhibit a similar dialysance *in vivo* and that one can be used as a retrodialysis calibrator for the other. In addition to molecular diffusivity in solution, one needs to consider potential differences in microvasculature transport, metabolism, and intra-/extracellular space exchange. Nevertheless, a compound

might serve as a retrodialysis calibrator of a solute if the relative loss of the calibrator during retrodialysis is proportional to the recovery of the solute during microdialysis.

In summary, microdialysis recovery can be monitored by retrodialysis loss. A comparison of retrodialysis and the zero-net flux method suggests that both methods yield the same degree of accuracy in prediction of recovery and therefore of the extracellular solute concentration in the probed region. However, retrodialysis may offer an advantage over the zero-net flux method of probe calibration since the latter method, which is utilized when concentrations in the probed tissue are maintained at steady state, may lengthen the study period. In addition, retrodialysis may be used to monitor recovery continuously and to correct for changes in probe efficiency among and within sampling devices during *in vivo* microdialysis.

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