The Binding of Cyclosporin A to Human Plasma: An *in Vitro* Microdialysis Study*

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Purpose. The human plasma binding of cyclosporin A was studied *in vitro* using the technique of microdialysis. The effect of temperature on the overall binding interaction between cyclosporin A and human plasma was also investigated.
Methods. Flow-through loop-type microdialysis probes were constructed from fused silica tubing and regenerated cellulose tubing with a MWCO of 13000 daltons. Probes were perfused with phosphate buffer (0.5 μl/min) and the concentration of ^{3}H-cyclosporin A in the well-mixed medium (plasma or buffer) was 1200 ng/ml. Relative recoveries of cyclosporin A from plasma or buffer were determined for each probe by separate experiments to measure the solute gain or loss with reference to the perfusate.
Results. Recoveries determined by loss were significantly greater than those determined by gain and in each case temperature dependent, with higher recoveries at higher temperatures. The plasma free fraction of cyclosporin A calculated from the recovery data and the perfusate to plasma concentration ratios was dependent on temperature in a log-linear fashion. Mean ± s.d. plasma free fractions expressed in percent were 33.5 ± 4.6, 17.9 ± 3.6, 6.2 ± 0.8, 3.0 ± 0.6, and 1.5 ± 0.2 at temperatures of 4, 10, 20, 30, and 37°C, respectively. Assuming that the enthalpy of binding is constant over the temperature range studied and pseudo-first order conditions exist, the binding reaction at these temperatures was spontaneous, endothermic ($\Delta H = 74.0$ kJ/mole), and entropically driven ($\Delta S = 0.274$ kJ/mole/deg).
Conclusions. These results show that the free fraction of cyclosporin A in human plasma is dependent on temperature with the fraction unbound decreasing with temperature in the range of 4 to 37°C. The thermodynamic parameters for the binding of cyclosporin A to plasma components indicate that the reaction is a spontaneous endothermic reaction that is mainly entropy driven, similar to the partitioning of lipophilic molecules from an aqueous to a hydrophobic phase. Moreover, these results show that microdialysis is a feasible method to determine the binding interactions between plasma and cyclosporin A, which indicates the method may be suitable for other difficult binding studies where the solutes have nonspecific binding to separation devices.

KEY WORDS: cyclosporin A; plasma free fraction; microdialysis; thermodynamics of binding.

INTRODUCTION

Cyclosporin A is a useful immunosuppressive drug in solid organ and bone marrow transplantations (1). However, the effective use of cyclosporin A in this setting is limited by significant toxicity (2–5) and the considerable interpatient variability in the pharmacokinetic (6, 7) and subsequent pharmacodynamic behavior of the drug (8–10). It has been suggested that this interpatient variability may be due, in part, to variable distribution characteristics that result from interindividual variability in the fraction of drug that is unbound in blood or plasma (11–15). Therefore, it is important to know the determinants of plasma binding of cyclosporin A to rationally design dosage regimens that will maximize efficacy and minimize toxicity.

Cyclosporin A is a cyclic peptide consisting of eleven amino acids with a molecular weight of 1202 daltons. The physicochemical properties of cyclosporin A are generally characterized by a high lipid solubility, imparted to some extent by intramolecular hydrogen bonding, and the molecule avidly binds nonspecifically to a variety of materials, including the commonly used membranes for ultrafiltration and equilibrium dialysis (11, 16). Therefore, these methods have failed to yield reproducible results in the determination of the plasma free fraction of cyclosporin A. The accepted standard technique for the determination of the free fraction of cyclosporin A is ultracentrifugation (11, 17), and there has been an attempt to use stainless-steel equilibrium dialysis chambers (18). The ultracentrifugation technique is laborious, technically difficult, and does not easily lend itself to manipulation of experimental conditions that are necessary to critically examine the determinates of binding. We therefore propose the use of technique of microdialysis (19) for protein binding studies of cyclosporin A. Microdialysis has been used to study the protein binding of drugs that do not suffer from the inherent nonspecific binding problems of cyclosporin A and has compared favorably with ultrafiltration (20) and equilibrium dialysis (21).

The primary objective of this study was to examine the feasibility of determining the free fraction of cyclosporin A in human plasma using the technique of microdialysis. Additionally, the effect of temperature on the overall interaction of cyclosporin A and plasma components was investigated to gain further insights into the general nature of these interactions, and to provide a qualitative means of validation for the method.

MATERIALS AND METHODS

Microdialysis Probes

Flow-through loop-type probes were constructed using fused-silica tubing (Polymicro Technologies, Inc. Phoenix, AZ, 85017) with a 75 μm I.D. and 150 μm O.D. The dialysis membrane (20mm length) was regenerated cellulose Spectro/Pro in vivo microdialysis hollow fibers (Spectrum Medical Industries, Inc., Los Angeles, CA 90060) with a MWCO of 13000 daltons. A stainless steel wire was inserted into the membrane lumen to avoid kinking. The dialysis membrane was connected to the fused silica tubing with superglue. The integrity of the probe was assessed for leaks first visually using a stereomicroscope (Nikon SMZ-2T) and then gravimetrically with a degassed phosphate buffer collection over a specific interval and buffer flow rate, and probes with a collected perfusate weight that was within 5% of nominal were deemed intact and acceptable for use.

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Perfusion

The perfusate was a phosphate buffer (0.1 M, pH 7.4 at 37°C), filtered and degassed. The flow rate for these dialysis experiments was 0.5 μl/min. A dual syringe pump (Harvard 22, Harvard Apparatus, South Natick, MA 01760) with a 1 ml gastight syringe (1000 series, Hamilton Co., Reno, NV 89520) was employed to yield a precise and even flow.

Specimen for Dialysis

Phosphate buffer or human plasma (Biological Specialty Corp., Landsdale, PA 19446) served as the medium for microdialysis. Plasma was received frozen on dry ice, thawed at room temperature, then aliquotted into single use volumes and stored at −20°C until use. Medium for dialysis (2 ml) was placed into a cylindrical glass vial (12 × 35 mm) that was then fitted into a custom plexiglass chamber thermostatted with water at the temperature of interest using a recirculator (D1-G, Haake, Paramus, NJ 07652). Temperature in the chamber immediately adjacent to the specimen vial was monitored using a digital probe thermometer (Fisher Scientific, Plano, TX 75074) and was maintained within ± 0.5°C. The concentration of cyclosporin A (Sandoz Research Institute, East Hanover, NJ 07936) in the medium was 1000 ng/ml of unlabelled compound and radiolabelled tracer was added (^{3}H-cyclosporin A, specific activity 411 GBq/mmol, Amersham, Arlington Heights, IL 60005) to give a final concentration of 1200 ng/ml. The radiochemical purity was checked by HPLC and the only radioactivity above background was observed for the peak corresponding with the retention of cyclosporin A. The medium was well-mixed throughout the experiment using a glass micromagnetic stir bar.

Cyclosporin Analysis

The dpm of ^{3}H-cyclosporin A was determined using liquid scintillation counting (Packard Tricarb 2500) using 5 ml of BCS scintillant (Amersham) in a 7 ml plastic vial. Routine counting efficiencies under these conditions were approximately 50% (external standard method).

Microdialysis Collections

Normal recovery buffer. A microdialysis probe was pretreated in buffer containing labelled and unlabelled cyclosporin A overnight, and then placed in a vial containing buffer with labelled cyclosporin A at the desired concentration, 1200 ng/ml. At a flow rate of 0.5 μl/min, using a gastight syringe that has never been exposed to cyclosporin A, (cyclosporin binds to teflon), the probe was perfused overnight with drug-free buffer, then dialysate (Cb_{out}) was collected at 60 min intervals for four intervals. At the end of the interval collections, the medium (Cb_m), in this case buffer, in the vial was sampled (10 μl triplicate) using a 10 μl Hamilton micoliter syringe (no teflon). The relative recovery due to gain of cyclosporin A by the probe in buffer (normal recovery, buffer; Rnb) was calculated as:

$$Rnb = Cb_{out}/Cb_m \quad (1)$$

Reverse recovery buffer. The same probe is then placed in a vial containing buffer with no cyclosporin A. Labelled cyclosporin A in buffer is then perfused through the probe at a flow rate of 0.5 ml/min (using a specific gastight syringe for this purpose) for 240 minutes. Dialysate (Cb_{out}) is then collected in the same manner as before for six 10 min intervals. The fused silica tubing on the inflow side of the membrane is then cut just proximal to the membrane and perfusate (Cb_{in}) is collected for six 10 min intervals. This procedure is necessary to guarantee the accurate determination of the concentration entering the probe because of binding of cyclosporin A to syringe components. The relative recovery due to loss of cyclosporin A through the probe in buffer (reverse recovery, buffer; Rrb) was calculated as:

$$Rrb = ((Cb_{in} - Cb_{out})/Cb_{in})_{buffer} \quad (2)$$

The recovery calculated by equation 2 is the probe extraction fraction measured by retrodialysis from buffer to buffer (delivery) and assumes sink conditions for the buffer in the vial.

Reverse recovery plasma. The inlet fused silica tubing of the probe is reconnected using fused silica connections and the probe is then placed in blank plasma that has equilibrated to the selected temperature. The probe is then perfused with the labelled cyclosporin A buffer solution at 0.5 μl/min for 240 minutes. Then the dialysate (Cb_{out}) is collected in the same manner as before for six 10 min intervals. The fused silica tubing on the inflow side of the membrane is then cut as above and perfusate is then collected for six 10 min intervals. The reverse recovery in plasma (Rrp) was calculated as:

$$Rrp = ((Cb_{in} - Cb_{out})/Cb_{in})_{plasma} \quad (3)$$

The recovery calculated by equation 3 is the probe extraction fraction measured by retrodialysis from buffer to plasma (delivery) and assumes sink conditions for diffusable drug in the plasma in the vial.

Concentration ratio in plasma. The same probe is then placed in plasma in a vial that contains labelled drug. At this point, unlabelled buffer is perfused through the probe at 10 μl/min and the buffer is collected for one 10 min interval. This collection is analyzed gravimetrically and if the weight is within 5% of nominal the integrity of the probe is considered adequate and the experiment continues. The probe is then perfused at 0.5 μl/min overnight, then the dialysate (Cb_{out}) was collected at 60 min intervals for four intervals. Collection was done directly into scintillation vials as before. At the end of the interval collections, the medium (Cm) in the vial (plasma) was sampled (10 μl triplicate) using the 10 μl Hamilton microliter syringe (no teflon). The concentration ratio in plasma was calculated as:

$$P_{ratio} = (Cb_{out}/Cm)_{plasma} \quad (4)$$

Normal recovery in plasma. The normal recovery in plasma was calculated knowing the reverse recovery from plasma and the relationship between the measured normal and reverse recoveries from buffer. This calculation assumes that the difference between the normal (gain to dialysate) and reverse (loss from dialysate) recoveries from plasma is due to the same mechanism that would affect the relationship between the normal and reverse recoveries from buffer.

One such possible mechanism may be that some of the drug may be captured by the membrane upon traversing through the membrane, which may be likely for a compound such as

cyclosporin A. Therefore the normal recovery from plasma (Rnp) was calculated as follows:

$$Rnp = Rrp * (Rnb/Rrb) \quad (5)$$

Free fraction in plasma. The free fraction (fu) of cyclosporin A in plasma was calculated as follows:

$$fu = P/Rnp = (Cb_{out}/Cm)_{plasma}/Rnp \quad (6)$$

Effects of Temperature on Binding

The procedure to determine the plasma binding of cyclosporin A as outlined above was followed with replication ($n = 5$ to 8) at the temperatures of 4, 10, 20, 30, and 37°C.

Thermodynamic Parameters

From the following scheme:

$$[D] + [P] \Leftrightarrow [DP] \quad (7)$$

where:

[D] = the concentration of unbound drug,
[P] = the concentration of unbound plasma binding sites,
[DP] = the concentration of bound drug.

The equilibrium association constant (Keq) of the above binding reaction is

$$Keq = [DP]/([D][P]) \quad (8)$$

Assuming pseudo-first order conditions, an apparent equilibrium constant (Kapp) can be defined as

$$Kapp = Keq * [P] \quad (9)$$

Therefore, Kapp is the ratio of the bound to free concentration, or conversely, the ratio of the fraction bound to the fraction free

$$Kapp = (1 - fu)/fu, \quad (10)$$

where

$$fu = [D]/([D] + [DP]) \quad (11)$$

From the study of the effect of temperature on the plasma binding of cyclosporin A, thermodynamic functions of the binding process can be calculated. The standard enthalpy change for the binding process, ΔH°_{bind}, may be obtained from the slope of the plot of ln Kapp versus 1/T (van't Hoff plot) using ordinary least squares regression,

$$\ln Kapp = -\Delta H^{o}_{bind}/RT + \Delta S^{o}_{app}/R \quad (12)$$

$$slope = -\Delta H^{o}_{bind}/R \quad (13)$$

where R is the gas constant, T is the absolute temperature, and ΔS^{o}_{app} is the apparent change in entropy for the binding process. This analysis assumes that the enthalpy of binding does not change over the temperature range studied; 4 to 37°C. An apparent free energy change, ΔG^{o}_{app}, of the binding process is related to the apparent equilibrium constant, Kapp, through the relationship,

$$\Delta G^{o}_{app} = -RT*\ln(Kapp) \quad (14)$$

An apparent standard entropy change, ΔS^{o}_{app}, for the binding process can be calculated from the following relationship:

$$\Delta G^{o}_{app} = \Delta H^{0}_{bind} - T\Delta S^{o}_{app} \quad (15)$$

The van't Hoff form of the equation (eq. 12) is a linearization of a nonlinear equation. In order to investigate the degree of bias that may be introduced in the determination of the enthalpy of binding parameter using the linearization technique, the following equation was fit using nonlinear least squares analysis (PCNONLIN) to estimate ΔH^{o}_{app} without prior data transformation:

$$Kapp = (1 - fu)/fu = e^{-\Delta H/RT} * e^{\Delta S/R} \quad (16)$$

Statistical Analysis

The normal and reverse recoveries of the microdialysis probes at each temperature were compared using a paired t-test with a p value less than 0.05 considered significant.

RESULTS

Comparison of Relative Recovery from Buffer

The mean ± S.D. relative recoveries of cyclosporin A at each temperature, calculated from the extraction fraction (reverse recovery) and calculated from the gain to the dialysate (normal recovery), are listed in Table I. A significant difference was observed between the normal and reverse recoveries in each case, with the reverse recovery being greater than the normal recovery. Moreover, there was a positive correlation between temperature and recovery for both the reverse and normal recoveries.

Free Fraction of Cyclosporin A in Plasma

The mean ± S.D. free fractions of cyclosporin A in plasma calculated using equation 6 were 0.335 ± 0.046, 0.179 ± 0.036, 0.062 ± 0.008, 0.030 ± 0.006, 0.015 ± 0.002, at the temperatures of 4, 10, 20, 30, and 37°C, respectively. The free fraction in plasma was related to temperature in a log-linear fashion, as can be seen in Figure 1.

Thermodynamic Parameters of the Binding Interaction

The thermodynamic parameters characterizing the interaction between cyclosporin A and plasma are listed in Table II. The ΔH°_{bind} determined from the van't Hoff analysis (linear

Table I. Relative Microdialysis Probe Recoveries of Cyclosporin A in Vitro Determined by Loss from the Perfusate (Rr, Retrodialysis) and by Gain into the Perfusate from the Medium (Rn, Normal Recovery), Expressed in Percentage as the Mean ± Standard Deviation.

Temperature	Recovery by loss (Rr)	Recovery by gain (Rn)	Ratio (Rr/Rn)
4°C	17% ± 3%[a]	11% ± 1%[a]	0.68 ± 0.12
10°C	26% ± 4%[b]	20% ± 3%[b]	0.75 ± 0.15
20°C	36% ± 5%[b]	30% ± 7%[b]	0.82 ± 0.13
30°C	55% ± 8%[a]	44% ± 4%[a]	0.80 ± 0.05
37°C	58% ± 6%[a]	49% ± 7%[a]	0.85 ± 0.07

[a] Rn significantly different than Rr by paired t-test with $p < 0.01$.
[b] Rn significantly different than Rr by paired t-test with $p < 0.05$.

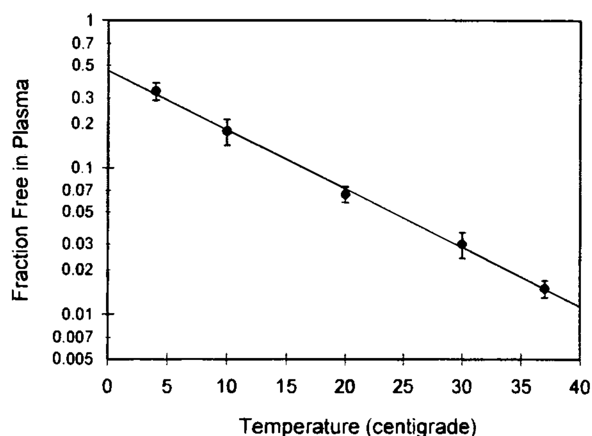

Fig. 1. The effect of temperature on the free fraction of cyclosporin A in human plasma. The solid circles are the mean ± S.D. (n = 5–6) free fraction. The solid line is the linear regression fit of the log transformed data (R^2 = 0.998).

Table II. Thermodynamic Parameters for Cyclosporin A Interaction with Human Plasma

Temperature (kelvin)	Kapp	ΔH°bind[a] (kJ/mole)	ΔG°app (kJ/mole)	TΔS°app (kJ/mole)
277	2.03	74.00	−1.63	75.63
283	4.78	74.00	−3.68	77.68
293	15.45	74.00	−6.67	80.67
303	33.98	74.00	−8.88	82.88
310	68.30	74.00	−10.89	84.89

[a] determined from the linear fit of equation 12. (Van't Hoff analysis)

transformation) was 74 kJ/mole. The relationship between cyclosporin A binding and temperature that is expressed by equation 12 is shown in Figure 2. A nonlinear regression analysis of the data using equation 16 yields essentially the same result for the enthalpy of binding, i.e. the ΔH°_{bind} determined in this manner was 74.9 kJ/mole.

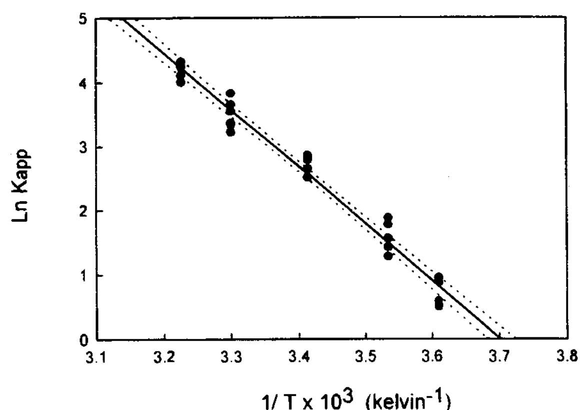

Fig. 2. Van't Hoff plot of the apparent equilibrium constant versus reciprocal temperature. The solid circles represent all data points, the solid line is the regression fit, and the dotted lines are the 95% confidence limits. Ln Kapp = −8900 * 1/T + 33, R^2 = 0.975.

DISCUSSION

This report describes a new technique, in vitro microdialysis, to measure the free fraction of cyclosporin A in plasma. The method is reproducible and allows precise control of important experimental variables, such as temperature. Like ultracentrifugation, the microdialysis method avoids the problem of variable nonspecific binding to ultrafiltration and equilibrium dialysis membranes, which in previous studies has led to non-reproducible results (11, 16). The free fraction of cyclosporin A in plasma at 37°C determined by microdialysis (1.5% ± 0.2) compares favorably with: 1) ultracentrifugation methods (11) where the free fraction in normal plasma ranged from 3.2 to 4.3%, and 2) equilibrium dialysis using stainless steel chambers (12) where the free fraction was 1.37% ± 0.42. Because of the technical difficulty in determining the free fraction of cyclosporin A, indirect modeling techniques have been employed to estimate the free fraction as a model dependent parameter. These models have included blood/plasma distribution models (22, 23), models based on physicochemical principles (24), and a physiological model of renal clearance (25). The cyclosporin A plasma free fractions estimated from these models range from 4% to 14%. It is unclear why these model dependent parameter estimates are generally greater than free fractions determined using the microdialysis technique.

The increase in free fraction of cyclosporin A when the temperature is lowered from 37° to 4°C is similar to that reported by Neiderberger et al. (26), Legg and Rowland (11), and Lemaire and Tillement (17). Lemaire and Tillement, using ultracentrifugation, found the plasma percent free at 4°C and 20°C to be 30% and 5–10% (17), respectively, which compares well with results from the current study, where the mean ± S.D percent free at 4°C was 33.5% ± 4.6% and at 20°C, it was 6.2% ± 0.8%. The positive enthalpy of binding (74 kJ/mole), precisely determined from both a linear transformation method (Van't Hoff plot) or direct fitting of the nonlinear relationship between free fraction and temperature, indicates the binding interaction is a spontaneous endothermic reaction that is entropy driven, similar to the partitioning of lipophilic molecules from an aqueous solution into a hydrophobic phase. This observation is consistent with the hypothesis that cyclosporin A binding to plasma components (such as lipoproteins) is a simple partitioning phenomenon. The fact that cyclosporin A binding has been shown to be concentration independent (nonsaturable) also supports this mechanism (11, 17).

The relative recovery of a microdialysis probe is a critical parameter in microdialysis sampling, and it is necessary to determine the actual concentration of a solute in the sampled medium (27, 28). The recovery of the microdialysis probe for cyclosporin A was determined by both measuring the loss of cyclosporin A from the perfusate to the medium (retrodialysis) and the gain of cyclosporin A from the medium to the perfusate (normal dialysis) in the lumen of the probe. Wang et al. (29) have reported that retrodialysis with calibrator molecules that have a similar dialysance is a useful method to determine probe recoveries both in vitro and in vivo. In the present study, cyclosporin A itself was used as a retrodialysis calibrator for recovery determination, and a directional dependence for recovery was observed. It is unclear why the recovery by retrodialysis should be larger than by normal dialysis (see Table I), however one possibility is that some cyclosporin A is captured by the

membrane upon diffusion from the medium to the perfusate and vice versa. This observation has been reported by Lindefors et al. (27) for ^{3}H-sucrose, where the recovery from the outer medium was 0.7 times the loss when ^{3}H-sucrose was in the perfusate. These authors explained the directional dependence of recovery by the solute interaction with the membrane. The tendency for a solute to interact with the membrane may vary with the physicochemical characteristics of the molecule, and given that cyclosporin is known to bind to membranes, this may be a logical explanation for the differences observed in the retrodialysis and normal dialysis recoveries. However, it is important to note that a steady-state level of membrane "capture" of the solute has been achieved, and experiments were performed to validate the steady-state condition with regard to both retrodialysis and normal dialysis recoveries. Figure 3 shows the recovery values during each time interval in a binding experiment, and no time-dependent change in recovery was seen for the four to six intervals collected for either retrodialysis or normal dialysis recoveries. Even though the directional dependence in recovery may be due to membrane nonspecific binding, because the recovery differences are time independent, a precise correction factor can be used to determine the free fraction.

This study demonstrates that the examination of the binding of cyclosporin A to human plasma is feasible using the microdialysis technique with careful attention given to the issue of relative recovery. This technique offers advantages, particularly in control of experimental conditions and precision of measurement, over other methods of binding determination for compounds that exhibit nonspecific binding. These findings have important implications for future binding studies of cyclosporins and other lipophilic molecules that have a high degree of nonspecific binding, especially where binding studies are necessarily important for understanding the mechanism of action or the biodistribution for many of these compounds.

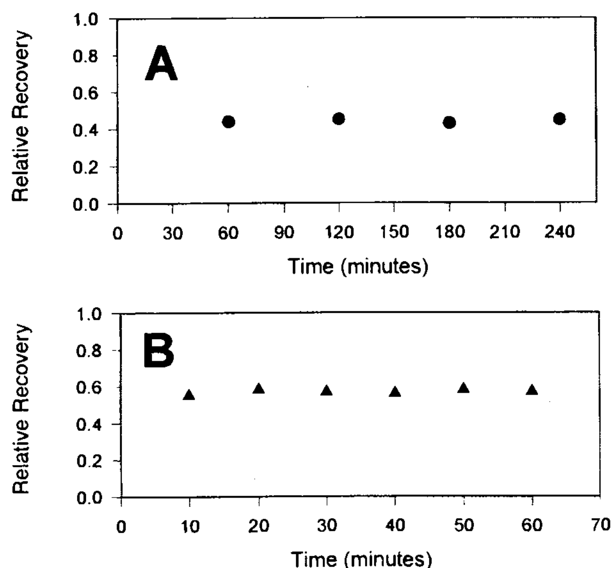

Fig. 3. Relative recovery by gain (panel A) and by loss (panel B) of a microdialysis probe during the time course of a representative binding experiment. The mean ± S.D. of the recovery by gain (panel A) was 0.44 ± 0.01, and the recovery by loss (panel B) was 0.57 ± 0.01. The medium was phosphate buffer at 37°C.

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