A Modified Equilibrium Dialysis Technique for Measuring Plasma Protein Binding: Experimental Evaluation with Diazepam and Nortriptyline

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INTRODUCTION

The binding of endogenous or exogenous molecules (e.g., drugs or toxicants) to constituents of the blood (e.g., proteins, red blood cells, etc.) has been of interest to biological scientists for many years. The generally accepted standard procedure for the measurement of binding to plasma proteins continues to be equilibrium dialysis. Conventional equilibrium dialysis physically separates, by a semipermeable dialysis membrane, plasma or protein solution which contains drug from the buffer, which is initially devoid of drug. The fundamental assumption behind the use of this method is that only the unbound drug is capable of penetrating the semipermeable membrane and it is that form which reaches equilibrium in the entire system. Once equilibrium is achieved, measurement of drug concentrations on both sides of the membrane allows calculation of the unbound fraction of drug in the plasma sample or the protein solution. For drugs highly bound to plasma proteins, the drug concentration on the buffer side at equilibrium (i.e., unbound drug concentration) may be too low to be accurately determined by an available analytical method. Accurate quantification of unbound fraction for this type of compound at relevant *in vivo* plasma concentrations is often only possible with the use of a radioactive tracer form of the drug. If no radioactive form is available, another sensitive analytical method must be found.

In order to overcome the difficulty of estimating low unbound drug concentrations on the buffer side, we propose here a method in which a certain percentage of buffer is replaced with plasma or protein solution (containing the drug) prior to dialysis. Figure 1 illustrates the schematics of the conventional and modified equilibrium dialysis methods. It is hypothesized that this buffer replacement method will result in increased total drug concentration on the buffer side at equilibrium, in turn affording a more accurate analytical determination of drug concentration. A theoretical treatment has permitted the estima-

tion of the unbound fraction of a drug when protein is present on the buffer side as a consequence of membrane leakage during the equilibrium dialysis experiment (1). Conceptually, that condition is no different from intentionally adding protein (or plasma sample) to the buffer prior to the dialysis experiment.

In the present study, we examined the feasibility of applying a modified dialysis method, the "buffer replacement method," for measuring protein binding of two highly protein bound drugs, diazepam and nortriptyline.

METHODS

Materials

Diazepam and nortriptyline hydrochloride were purchased from Sigma Chemical Company (St. Louis, MO). [2-^{14}C]-diazepam was purchased from Amersham Corp. (Arlington Heights, IL) and [^{3}H]-nortriptyline was received as a gift from Burroughs Wellcome Company (Research Triangle Park, NC). To minimize decomposition, stock solutions of [2-^{14}C]-diazepam and [^{3}H]-nortriptyline were stored at $-20°C$ in the absence of light. Bovine serum albumin, Fraction V fatty acid free (Sigma Chemical Company, St. Louis, MO) and bovine α_1-acid glycoprotein (Sigma Chemical Company, St. Louis, MO), purified from Cohn Fraction VI, were used as the binding proteins for diazepam and nortriptyline, respectively.

General Equilibrium Dialysis Procedure

The equilibrium dialysis apparatus used in our experiments consisted of a 5-cell Spectrum Equilibrium Dialyzer (Spectrum Medical Industries, Inc., Los Angeles, CA). Dialysis membranes with a molecular weight cutoff of 12–14,000 Daltons (Spectrum Medical Industries, Inc.) were used. In the conventional dialysis experiments, Sorensen's phosphate buffer (0.067 M, pH 7.4) was added to one side of the membrane (referred to as the buffer side) prior to dialysis. The other side (referred to as the plasma side) contained albumin or α_1-acid glycoprotein solution to which labeled and unlabeled drug had been added. To preclude the phenomenon of a volume shift, the buffer solution was supplemented with dextran (average M.W. 72,600, Sigma Chemical Company, St. Louis, MO) at a concentration of one half of the protein concentration used on the other side of the dialysis membrane (2). Prior to dialysis, in the modified dialysis experiments, protein solution identical to that used in the conventional dialysis experiments was prepared and added to one side of the dialysis membrane. Sorensen's buffer with a certain fraction replaced with the protein solution (containing the drug) was added to the other side. All dialysis experiments were performed at 37°C with the apparatus rotating during the experiment.
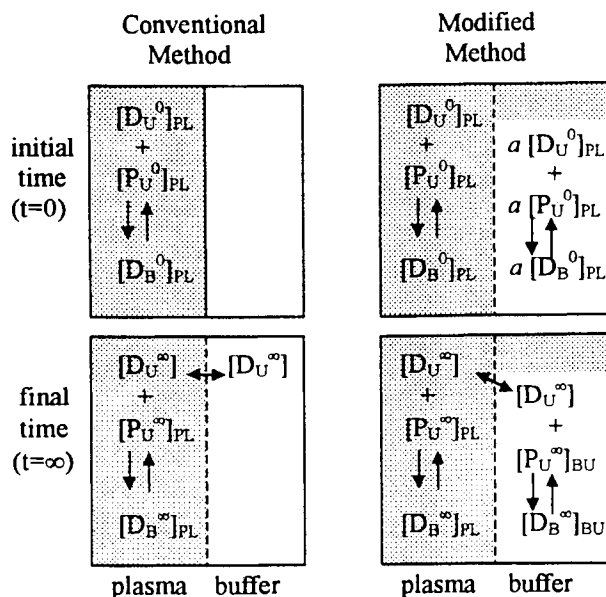
Determination of Time to Reach Equilibrium

Before determining protein binding parameters by using the equilibrium dialysis method it was necessary to establish the time required to reach equilibrium. In experiments involving diazepam, unlabeled and labeled diazepam solutions were prepared and mixed with the albumin solution giving final concentrations of 2.29 μg/mL (8.04×10^{-6} M), 5.0×10^{-5} mCi/mL,

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Conventional Method

Modified Method

initial time (t=0)

final time (t=∞)

plasma   buffer   plasma   buffer

Fig. 1. Schematic representations of equilibrium dialysis in the conventional method (left) and in the modified method (right) at time zero prior to dialysis (top) and once equilibrium has been achieved (bottom). The shaded and unshaded areas represent plasma and buffer, respectively. The vertical dashed line represents the semi-permeable dialysis membrane. In the conventional method, prior to dialysis, the drug is present only in the plasma and there is no drug on the buffer side. In the modified method, prior to dialysis, a fraction ("a") of buffer volume has been replaced with plasma containing the drug (e.g., 5% or 10%; taken from the same plasma sample as on the plasma side). The buffer containing plasma and drug are mixed to form a homogenous solution (although the shaded area on the buffer side of the modified method should really be uniformly dispersed, it is intentionally illustrated in this way to show the volume that the plasma occupies on the buffer side). The resulting initial conditions on the buffer side indicate that each form of the drug and plasma protein is present at a concentration equal to the volume fraction of buffer replaced with plasma (e.g., 0.05 or 0.1) times the original concentration on the plasma side. $[P_U^0]_{PL}$: initial unbound protein concentration on the plasma side prior to dialysis; $[P_U^x]_{PL}$: final unbound protein concentration on the plasma side after equilibrium is achieved; $[P_U^x]_{BU}$: final unbound protein concentration on the buffer side after equilibrium is achieved; $[D_U^0]_{PL}$: unbound drug concentration in plasma prior to dialysis; $[D_B^0]_{PL}$: bound drug concentration in plasma prior to dialysis; $[D_U^x]$: unbound drug concentration after equilibrium is achieved; $[D_B^x]_{PL}$: bound drug concentration on the plasma side after equilibrium is achieved; $[D_B^x]_{BU}$: bound drug concentration on the buffer side after equilibrium is achieved; a: volume fraction of plasma (containing drug) on the buffer side (v/v) prior to dialysis.

and 4 g/dL (6.02 × 10⁻⁴ M), respectively. In experiments involving nortriptyline, protein solutions contained unlabeled and labeled nortriptyline, and α₁-acid glycoprotein at final concentrations of 5 μg/mL (1.67 × 10⁻⁵ M), 1.26 × 10⁻⁴ mCi/mL and 1 g/dL (2.56 × 10⁻⁴ M), respectively. In the conventional dialysis experiments, Sorensen's phosphate buffer was used to dialyze against the protein solution containing the drug. In the modified dialysis experiments, 10% or 20% volume of the buffer solution was replaced with protein solution containing the unlabeled and labeled drugs (volume fraction of buffer replaced by the protein solution, "a", = 0.1 or 0.2, respectively). Samples from both sides of the dialysis membrane were col-

lected at different times after initiation of dialysis. The radioactivity associated with the samples was determined by liquid scintillation counting.

Determination of Unbound Fraction (f_u)

The values of f_u were determined at the same unlabeled and labeled drug concentrations used in determination of time to reach equilibrium. For diazepam, two different initial protein concentrations, 4 g/dL and 2 g/dL, each with and without a 10% volume replacement on the buffer side ("a" = 0.1) were used. Additional f_u values were determined at a protein concentration of 4 g/dL and a 20% volume fraction of replacement, "a" = 0.2. For nortriptyline, f_u values were determined at a protein concentration of 1 g/dL with no replacement on the buffer side and with two different volume fractions of replacement ("a" = 0.1 and 0.2). Samples from both sides of the dialysis membrane were collected after the system had reached equilibrium. The radioactivity associated with the samples was determined by liquid scintillation counting.

Data Analysis

When the traditional equilibrium dialysis method was used, the values of f_u were obtained by dividing the drug concentrations on the buffer side (i.e., unbound drug concentrations) by the drug concentrations on the plasma side (i.e., total drug concentrations). For the proposed buffer replacement method, the unbound drug concentrations were estimated indirectly by applying the following equation taken from Khor et al. (1),

$$[D_U^x] = \frac{[D_T^x]_{BU} - a[D_T^x]_{PL}}{1 - a} \qquad (1)$$

where $[D_U^x]$ denotes the unbound drug concentration after equilibrium is achieved; $[D_T^x]_{BU}$ represents total drug concentration (bound and unbound drug) on the buffer side after equilibrium is achieved and $[D_T^x]_{PL}$ represents total drug concentration on the plasma side after equilibrium is achieved. The f_u values were then determined from the ratio of the calculated unbound drug concentrations to the measured drug concentrations on the plasma side (i.e., total drug concentrations). This f_u value was compared with the value obtained from the traditional method to determine whether comparable f_u values could be estimated from these methods.

The magnitude of the improvement of drug concentration on the buffer side using the proposed method was evaluated with the ratio term, R. R was calculated by dividing the experimentally determined drug concentration on the buffer side obtained from the modified method by that obtained from the traditional method.

RESULTS

Time to Reach Equilibrium

Figure 2 illustrates the f_u values of diazepam as a function of time when different volume fractions of replacement were used. It can be seen that in all cases the f_u values became essentially constant after 4 to 6 hours. No significant differences were observed in the times needed to reach equilibrium for diazepam with a replacement volume fraction up to 20%. The
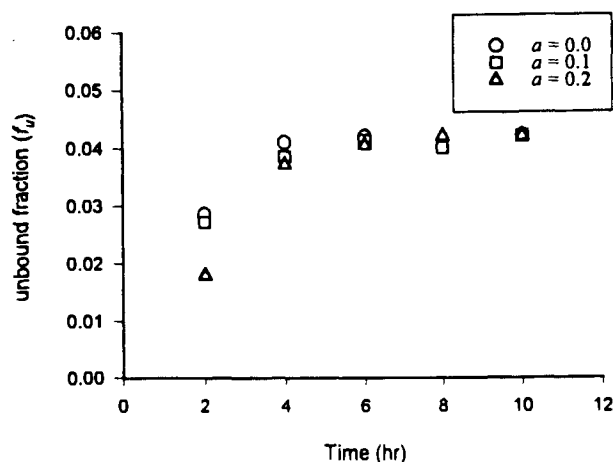
Fig. 2. The f_u values of diazepam as a function of time when different volume fractions of replacement were used. The following conditions were used in the experiment: initial unlabeled drug concentration on the plasma side, 8.04×10^{-6} M; initial protein concentration on the plasma side, 4 g/dL (6.02×10^{-4} M); and three different volume fractions of buffer replacement, "a" = 0.0 (no buffer replacement, traditional dialysis method), "a" = 0.1 (10% of the buffer was replaced with the protein solution containing the drug), "a" = 0.2 (20% of the buffer was replaced with the protein solution containing the drug). Each data point represents the mean of two determinations.

profiles of f_u values as a function of time for nortriptyline under three different "a" values are shown in Fig. 3. The time required to reach equilibrium was about 6 to 8 hours when there was no replacement on the buffer side (i.e., conventional dialysis method), but increased to approximately 20 and 32 hours with replacement volume fractions of 0.1 and 0.2, respectively.
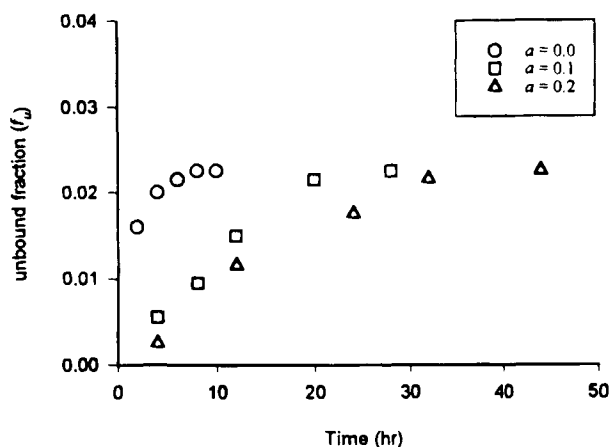
Fig. 3. The f_u values of nortriptyline as a function of time when different volume fractions of replacement were used. The following conditions were used in the experiment: initial unlabeled drug concentration on the plasma side, 1.67×10^{-5} M; initial protein concentration on the plasma side, 1 g/dL (2.56×10^{-4} M); and three different volume fractions of buffer replacement, "a" = 0.0 (no buffer replacement, traditional dialysis method), "a" = 0.1 (10% of the buffer was replaced with the protein solution containing the drug), "a" = 0.2 (20% of the buffer was replaced with the protein solution containing the drug). Each data point represents the mean of two determinations.

Determination of the f_u and R Values

The experimentally determined values of the unbound fraction of diazepam obtained from both the conventional dialysis method and the buffer replacement method are summarized in Table 1. Also listed is the enhancement in the drug concentration on the buffer side by employing the buffer replacement method (i.e., R). When the initial protein concentration on the plasma side was 4 g/dL, the f_u values were essentially identical with or without buffer replacement (0.041 ± 0.002 vs. 0.042 ± 0.002 vs. 0.042 ± 0.004 for dialysis without replacement and, with 0.1 or 0.2 volume fraction of buffer replaced with protein solution, respectively). Similarly, at a lower initial protein concentration (2 g/dL), the f_u values with or without buffer replacement were virtually identical (0.079 ± 0.004 vs. 0.077 ± 0.001 without and with 10% volume fraction of replacement, respectively). The major advantage of the proposed method became obvious when the R value was estimated for each experimental condition. At the same protein concentration (4 g/dL), the more buffer replaced with protein solution containing drug, the higher the R value (5.79 vs. 3.42 for 20% and 10% replacement, respectively). Furthermore, with the same fraction of replacement, the higher the protein concentration, the higher the resulting R value (3.42 vs. 2.16 for protein concentrations of 4 g/dL and 2 g/dL, respectively).

The experimental values for f_u and R for nortriptyline obtained from the conventional dialysis method and the buffer replacement method are also summarized in Table 1. At the fixed protein concentration, f_u values estimated with or without buffer replacement were consistent (0.022 ± 0.002, 0.021 ± 0.002, and 0.021 ± 0.001 for replacement volume fractions of 0.0, 0.1, and 0.2, respectively). Furthermore, a significant increase in the R values was observed when the modified method was applied to nortriptyline. At a fixed protein concentration (1 g/dL), the larger the volume fraction of the buffer replaced with the protein solution, the higher the resulting R value (5.74 vs. 10.2 with 10% and 20% replacement, respectively).

DISCUSSION

The purpose of this study was to experimentally evaluate a proposed method, which we have called the "buffer replacement

Table 1. Experimental Determinations of the Unbound Fraction (f_u) and the Enhancement in the Drug Concentration on the Buffer Side (R) Obtained from Both the Conventional Dialysis Method and the Buffer Replacement Method

Drug	$[P^0_T]_{PL}$, g/dL	a	f_u	R
Diazepam	4	0	$0.041^a \pm 0.002$	N/A
~	4	0.1	0.042 ± 0.002	3.42
	4	0.2	0.042 ± 0.004	5.79
	2	0	0.079 ± 0.004	N/A
	2	0.1	0.077 ± 0.001	2.16
Nortriptyline	1	0	0.022 ± 0.002	N/A
	1	0.1	0.021 ± 0.002	5.74
	1	0.2	0.021 ± 0.001	10.2

Note: $[P^0_T]_{PL}$ denotes total initial protein concentration on the plasma side and "a" denotes the volume fraction of replacement.
[a] Data are presented as mean $\pm$ standard deviation of three determinations.

method," for characterizing plasma protein binding. The method was proposed in order to overcome the analytical sensitivity issues encountered experimentally with ligands that are highly bound to plasma proteins. The two drugs selected for study, diazepam and nortriptyline, are extensively bound (to different proteins) and have equilibrium association binding constants which differ by a factor of about 5 (4×10^4 vs. 2×10^5 M^{-1}). In order to establish the utility of this method, unbound fractions were determined with the proposed approach and compared with values obtained from conventional equilibrium dialysis.

The experimental binding results obtained in this study (i.e., f_u) for both diazepam and nortriptyline using the proposed buffer replacement method agreed well with those obtained from the traditional dialysis method (Table 1). There was a two- to ten-fold enhancement in the concentration of drug on the buffer side when the proposed buffer replacement method was used. This analytical advantage seems to be dependent on the volume fraction of replacement, protein concentration, and the association binding constant of the compound. These results provide experimental validation for the feasibility of using the proposed buffer replacement method to determine protein binding parameters for highly bound drugs.

In these experiments, the time for diazepam to reach equilibrium changed minimally when a fraction of the buffer solution was replaced with protein. However, delay in the time for nortriptyline to reach equilibrium was apparent when 10% of the buffer solution was replaced with protein. This indicates that the time needed to reach equilibrium with the "buffer replacement" method will increase more significantly when the drug has a higher affinity to protein. Furthermore, for nortriptyline, the larger the volume fraction of the buffer replaced with protein solution, the more substantial was the delay in time to reach equilibrium. Previous work (3,4) has examined factors affecting time to reach equilibrium in the conventional dialysis system. It was found that the dialysis system requires more time to reach equilibrium when a greater amount of drug needs to be transported across the membrane. In our buffer replacement method, a fraction of the buffer was replaced with protein solution containing drug, resulting in a smaller amount of drug needed to be transported across the membrane. One would then expect that the buffer replacement method requires less time to reach equilibrium which is not consistent with our observations. The buffer replacement method also results in a smaller unbound drug concentration gradient between plasma and buffer compartments than that in the conventional dialysis system. Under our testing conditions, the unbound drug concentration gradient may represent the true driving force of changes in the total drug concentration in either compartment. The delay in time to reach equilibrium using the modified method may, therefore, be attributed to the smaller concentration gradient

across the membrane. Further theoretical and experimental analyses of factors governing the delay in time needed to reach equilibrium are required to confirm our hypothesis.

The R values for both diazepam and nortriptyline increased as more buffer was replaced with protein solution containing the drug. Furthermore, a larger initial protein concentration also led to a larger R value. In comparing results obtained from diazepam and nortriptyline for a 10% buffer replacement, the R value for diazepam (Ka $\cong 4 \times 10^4$ M^{-1}) was about 3.42, and the R value for nortriptyline (Ka $\cong 2 \times 10^5$ M^{-1}) was about 5.74. This suggests that buffer replacement for compounds having a large Ka will result in a larger R value. Such a system, however, may result in a prolonged time to reach equilibrium. In the latter instance, a smaller replacement volume could be used to achieve the desired increase in drug concentration yet not unduly prolong time to reach equilibrium.

In summary, the experimental studies reported here demonstrate that the modified equilibrium dialysis method, the "buffer replacement" method, can be applied to the measurement of plasma protein binding. This has been demonstrated for two highly plasma protein bound drugs (96%–98% bound), diazepam and nortriptyline. The primary advantage to the use of the proposed method is an increase in the measurable ligand concentration which, in turn, provides more accurate estimation of binding (i.e., f_u). In contrast, a disadvantage of the proposed method is that the time to reach equilibrium inevitably increases for compounds with a Ka value about equal to or greater than 10^5 M^{-1}. This disadvantage can be minimized by judicious selection of the "a" value (i.e., fraction of buffer volume replaced). Further experience with the proposed method will allow additional testing of its applicability and feasibility.

ACKNOWLEDGMENTS

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