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# Volume Shifts and Protein Binding Estimates using Equilibrium Dialysis: Application to Prednisolone Binding in Humans

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**Abstract** □ Sizable volume shifts can occur during equilibrium dialysis. This net movement of water, presumably caused by the osmotic effect of plasma proteins, reduces the concentration of binding proteins. In this paper the theory of protein binding estimation is extended, equations are developed for calculating the unbound and bound drug concentrations at dialysis equilibrium by correcting for the dilution of the proteins, and the equations are applied to a study of prednisolone. To demonstrate the importance of correcting for the volume shift, the parameters of a model in which prednisolone binds to corticosteroid-binding globulin, a protein with a limited capacity, and albumin were estimated. Unbound and bound concentrations were determined by correcting for both volume shifts (average 31%) and loss of drug to the buffer side, by correcting only for loss of drug to buffer side, and by making no correction at all (the usual method of treating equilibrium dialysis data). The error introduced by neglecting volume shifts was analyzed by comparing the parameter values obtained using the three methods. The results confirm the need to adjust for volume shifts and imply that reported binding constants obtained by equilibrium dialysis may be in error for many substances.

**Keyphrases** □ Equilibrium dialysis—measurement of protein binding, effect of volume shifts, theoretical model, application to prednisolone in humans □ Protein binding—determined by equilibrium dialysis, effect of volume shifts, theoretical model, application to prednisolone in humans □ Prednisolone—protein binding as determined by equilibrium dialysis, effect of volume shifts, application of theoretical model, humans

Binding of drugs to plasma proteins is important in pharmacokinetics and pharmacodynamics. Equilibrium dialysis is commonly employed for estimation of binding, but it has limitations. With the introduction of translucent cells, it has become evident that sizable volume shifts occur across the dialysis membrane. We have investigated the importance of these volume shifts in the estimation of plasma protein binding parameters and have developed a procedure to correct for them. The procedure is applied to a study of prednisolone in humans.

The binding of prednisolone in plasma is thought to involve two proteins, corticosteroid-binding globulin (transcortin) and albumin. In the range of concentrations associated with therapy (1), the plasma protein binding of prednisolone is concentration dependent largely because of saturable binding to sites on corticosteroid-binding globulin. It has been shown *in vitro* that glucocorticoid activity is a function of unbound concentration and that the activity can be altered by the addition or removal of the globulin (2). Definition of concentration–effect relationships for prednisolone, therefore, requires the ability to estimate unbound prednisolone concentrations. Estimates of unbound concentration *in vivo* can be obtained by measurement of total prednisolone concentration (bound plus unbound) and application of a suitable model for predicting the unbound concentration from the total concentration.

There are several complications in the use of equilibrium dialysis to estimate plasma protein binding. These include binding of drug to the dialysis cell or membrane, transfer of substantial amounts of drug from the plasma to the buffer side of the membrane, and osmotic volume shifts of fluid to the plasma side. Some of these problems have been discussed elsewhere (3). In this paper, a method is described for calculating the magnitude of osmotic volume shifts and for estimating the parameters that reflect binding *in vivo*.

## THEORETICAL

Figure 1 is a schematic representation of equilibrium in a dialysis device containing plasma on one side and buffer solution on the other, with and without a volume shift. The volume of the plasma side is increased and the buffer side is decreased, because of a net osmotic transfer of water across the membrane. Osmotic equilibrium may or may not be reached at the time equilibrium is virtually achieved with respect to the drug. The derivations which follow assume conservation of the mass of prednisolone in the system and of the total volume of the two half-cells. Symbols and abbreviations are defined in Appendix I.

**Conservation of Volume**—The total volume of the cell is unchanged by dialysis; therefore:

$$\underset{\text{(before)}}{V_P + V_B} = \underset{\text{(after)}}{V'_P + V'_B} \qquad \text{(Eq. 1)}$$

Letting $\delta$ be the fractional increase in $V_P$ due to osmotic water shift, then:

$$V'_P = V_P(1 + \delta) \qquad \text{(Eq. 2)}$$

and



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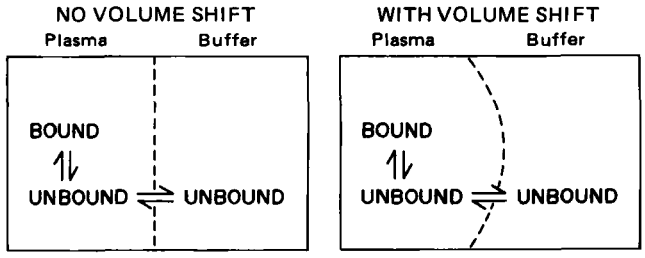


**Figure 1**—*Schematic diagram of a dialysis cell at equilibrium with respect to drug distribution. Whether there is a volume shift (on right) or not (on left), the unbound concentrations on both sides of the membrane (---) are identical. The amount bound on the plasma side is unchanged, but the bound concentration on the plasma side is decreased as the fluid shifts from the buffer side to the plasma side.*

$$V'_B = V_B - V_P \cdot \delta \qquad \text{(Eq. 3)}$$

**Conservation of Mass**—The total amount of drug is unchanged by dialysis; therefore $(C_P \cdot V_P + C^*_B \cdot V_B)$ (before) = $(C'_P \cdot V'_P + C'_B \cdot V'_B)$ (after), where $C^*_B$ is the concentration of labeled drug added to the buffer before dialysis. Substituting for $V'_P$ and $V'_B$ using Eqs. 2 and 3 gives $C_P \cdot V_P + C^*_B \cdot V_B = C'_P \cdot V_P(1 + \delta) + C'_B(V_B - V_P \cdot \delta)$. Therefore:

$$C'_P = \frac{C_P \cdot V_P + C^*_B \cdot V_B}{V_P(1 + \delta) + \frac{C'_B}{C'_P}(V_B - V_P \cdot \delta)} \qquad \text{(Eq. 4)}$$

**Conservation of Radiolabel**—When radiolabeled drug is added to the buffer side before dialysis to produce a concentration of $D_B$ disintegrations/min/ml, then the concentration of radioactivity on the plasma and buffer sides after dialysis are $D'_P$ and $D'_B$, *i.e.*, $D_B \cdot V_B$ (before) = $(D'_P \cdot V'_P + D'_B \cdot V'_B)$ (after). Substituting for $V'_P$ and $V'_B$ using Eqs. 2 and 3 and solving for $\delta$:

$$\delta = \frac{R \cdot (D_B - D'_B) - D'_P}{D'_P - D'_B} \qquad \text{(Eq. 5)}$$

where $R$ is the ratio of $V_B$ to $V_P$.

After dialysis, the ratio of unbound drug concentration to total drug concentration $(C'_B/C'_P)$ is the same as the ratio of the radiolabel concentrations $(D'_B/D'_P)$; therefore, Eq. 4 can be simplified by substituting Eq. 5 for $\delta$ and by replacing $C'_B/C'_P$ with $D'_B/D'_P$, giving:

$$C'_P = \frac{(C_P + C^*_B \cdot R)}{R} \cdot \frac{D'_P}{D'_B} \qquad \text{(Eq. 6)}$$

and

$$C'_B = \frac{(C_P + C^*_B \cdot R)}{R} \cdot \frac{D'_B}{D_B} \qquad \text{(Eq. 7)}$$

The bound concentration on the plasma side, $C'_{bnd}$, is then obtained from the difference between $C'_P$ and $C'_B$:

$$C'_{bnd} = \frac{(C_P + C^*_B \cdot R)}{R} \cdot \frac{(D'_P - D'_B)}{D_B} \qquad \text{(Eq. 8)}$$

**Volume Shift Correction**—The amount of drug bound to plasma proteins depends, among other factors, on the amount of binding proteins present and the unbound concentration. At dialysis equilibrium the shift in volume from the buffer side to the plasma side is simply the transfer of buffer solution, containing drug at the unbound concentration, from the buffer to the plasma side as shown in Fig. 1. This transfer does not change the unbound concentration, but the *bound* concentration on the plasma side is decreased by the transfer of fluid. The amount of binding protein is not influenced by the transfer nor is the unbound equilibrium concentration; therefore, the total *amount bound* is the same *with or without* a volume shift.

The theoretical basis of this conclusion has been previously reported (4) for the method of ultrafiltration. In ultrafiltration, plasma filtrate contains drug at the same concentration as that unbound in the plasma. Continued filtration concentrates the protein and the bound drug, but the amount of drug bound remains unchanged. In contrast to ultrafiltration, a volume shift in equilibrium dialysis produces a decrease in the protein and bound drug concentrations as a result of the net transfer of water and unbound drug to the plasma side. In both cases, the concentration of unbound drug remains the same.

**Conservation of Amount Bound**—The amount bound postdialysis is $C'_{bnd} \cdot V'_P$. From the argument in the previous paragraph, the amount bound if no volume shift had occurred would have been the same. Therefore:

$$C^0_{bnd} \cdot V_P = C'_{bnd} \cdot V'_P \qquad \text{(Eq. 9)}$$

where $C^0_{bnd}$ is the bound drug concentration expected had no volume shift occurred. Substituting for $V'_P$ from Eq. 2 and simplifying:

$$C^0_{bnd} = C'_{bnd}(1 + \delta) \qquad \text{(Eq. 10)}$$

Now substituting for $C'_{bnd}$ from Eq. 8 and for $\delta$ from Eq. 5:

$$C^0_{bnd} = \frac{(C_P + C^*_B \cdot R) \cdot [R(D_B - D'_B) - D'_B]}{D_B \cdot R} \qquad \text{(Eq. 11)}$$

**Nonlinear Binding**—The sum of the estimates of the unbound (Eq. 7) and bound (Eq. 11) concentrations gives the expected total drug concentration in the plasma postdialysis had no volume shift occurred. This value is not the same as the drug concentration in plasma before dialysis, because some drug had been transferred to the buffer during dialysis. To predict the bound and unbound concentrations in the original plasma sample, an appropriate protein binding model, such as the one below (5), must be used:

$$C^0_{bnd} = \frac{CAP_1 \cdot C'_B}{K_{d1} + C'_B} + \frac{CAP_2 \cdot C'_B}{K_{d2} + C'_B} \qquad \text{(Eq. 12)}$$

where $CAP_1$ and $CAP_2$ are the binding capacities of two classes of binding protein sites with equilibrium dissociation constants $K_{d1}$ and $K_{d2}$, respectively. If the concentrations of the binding proteins are measured, $CAP_1$ and $CAP_2$ can be expressed as $n_1 \cdot P_1$ and $n_2 \cdot P_2$, where $P_1$ and $P_2$ are the concentrations of the binding proteins ($P_1 = P_2$ if both classes of binding sites are on the same protein) and $n_1$ and $n_2$ are the numbers of the respective binding sites in each class. If the protein concentrations are not measured, the binding capacities are best expressed as $CAP_1$ and $CAP_2$.

If $K_d \gg C'_B$ (as is the case for prednisolone), the model expressed by Eq. 12 reduces to:

$$C^0_{bnd} = \frac{CAP_1 \cdot C'_B}{K_{d1} + C'_B} + S \cdot C'_B \qquad \text{(Eq. 13)}$$

where $S$ is a constant $(CAP_2/K_{d2})$.

Once the parameters of a model such as Eq. 12 are estimated, the unbound $(C_u)$ and bound $(C_{bnd})$ concentrations in the original plasma sample can be determined by simultaneous solution of Eq. 13 and the relationship, $C_P = C_{bnd} + C_u$. The unbound and bound concentrations are then:

$$C_u = \frac{C_P - L + \sqrt{(C_P - L)^2 + M \cdot C_P}}{N} \qquad \text{(Eq. 14)}$$

and

$$C_{bnd} = C_P - C_u \qquad \text{(Eq. 15)}$$

where $L = S \cdot K_{d1} + CAP_1 + K_{d1}$, $M = 4 \cdot K_{d1} \cdot (S + 1)$, and $N = 2 \cdot (S + 1)$.

**Other Dialysis Systems**—The expressions for the unbound (Eq. 7) and bound (Eq. 11) postdialysis concentrations corrected for volume shift are simplified if the initial plasma and buffer volumes are equal, as shown in Appendix II. The relationships for measurement of protein binding when no radiolabel is added are given in Appendix III. Appendix IV provides appropriate relationships for calculating the fraction unbound, a binding parameter commonly used in pharmacokinetics.

**Error Introduced by not Recognizing Volume Shifts**—The method described above (method I) accounts for volume shifts and for movement of drug from the plasma to the buffer side during dialysis. Behm and Wagner (3) proposed a method for calculating the value of $C'_P$ with the assumption that no volume shift occurs, but accounting for movement of drug from plasma to buffer. On rearrangement and accounting for added radiolabel, their method (method II) becomes:

$$C'_P = \frac{(C_P + C^*_B \cdot R)}{1 + f_u' \cdot R} \qquad \text{(Eq. 16)}$$

where $f_u'$ is the ratio of concentrations of drug (or radiolabel) in the buffer cell to the plasma cell after dialysis ($C'_B/C'_P$ or $D'_B/D'_P$). Unbound $(C'_B)$ and bound $(C'_{bnd})$ concentrations are:

$$C'_B = C'_P \cdot f_u' \qquad \text{(Eq. 17)}$$

$$C'_{bnd} = C'_P \cdot (1 - f_u') \qquad \text{(Eq. 18)}$$

A third method (method III) commonly used for protein binding measurements (6), ignores both the volume shift and the shift of drug from the plasma cell to the buffer cell. The unbound and bound concentrations are then given by:

$$C'_B = (C_P + C^*_B \cdot R) \cdot f_u' \quad \text{(Eq. 19)}$$

$$C'_{bnd} = (C_P + C^*_B \cdot R) \cdot (1 - f_u') \quad \text{(Eq. 20)}$$

## EXPERIMENTAL

Nine patients who had received kidney transplants volunteered for a comparative bioavailability study of prednisone and prednisolone oral tablets *versus* intravenous prednisolone (1). Plasma samples obtained from this study were stored at −70° before analysis by high-performance liquid chromatography (HPLC) for total prednisolone concentration (7).

The protein binding of prednisolone was determined by equilibrium dialysis, using acrylic plastic equilibrium dialysis cells[1] with a 1-ml maximum capacity/cavity. The membrane employed[2] had a molecular weight cut-off of 12,000–14,000. One-half milliliter of plasma was equilibrated against 0.5 ml of Krebs–Ringer buffer, (pH 7.4, 0.153 *M*), containing 4.5 ng of [6,7-(*n*)-$^3$H]prednisolone (specific activity 43 Ci/mmole)[3]. Dialysis was continued for 16 hr in a shaking incubator[4] at a water temperature of 37°. One-tenth milliliter of dialyzed plasma and 0.1 ml of dialyzed buffer were then transferred to individual glass scintillation vials, to which was added 10 ml of scintillation fluid[5]. After shaking, the vials were counted in a scintillation counter[6]. The counting efficiency was determined by the channels-ratio method of quench correction. Additional correction was made for background counts. The purity (98%) of the radioactive prednisolone was confirmed by TLC and prednisolone was found to be stable in the dialysis cell during the equilibration period.

The bound and unbound concentrations after dialysis were computed after correction for volume shift (method I, Eqs. 7 and 11), by the method proposed by Behm and Wagner (3) (method II, Eqs. 17 and 18), and by the standard method (method III, Eqs. 19 and 20). The fractional increase in the plasma cell volume was calculated using Eq. 5. These calculations were performed using the PROPHET computer system (8). The binding parameters of Eq. 13 were estimated using MKMODEL (9) and unweighted nonlinear least-squares regression (10, 11).

## RESULTS AND DISCUSSION

The distribution of volume shifts was estimated from 388 samples used in this study (Fig. 2). The mean volume shift was 0.31 ± 0.15 (mean ± *SD*). There was no correlation between volume shift and predialysis prednisolone concentration. On dialysis of the radiolabel in buffer against an equal volume of buffer, there was no volume shift and negligible binding (<2%) to cell walls or membrane. The binding parameters estimated in the samples from the intravenous and two oral studies by each of the three methods are shown and compared in Table I. The relatively large standard deviations mostly reflect interindividual differences.

Figure 3 shows the fit of the model (Eq. 13) to bound and unbound concentrations obtained by method I from an individual subject. Using average parameter values, graphs of bound against unbound concentrations were simulated (Fig. 4) using MKMODEL (7). In a similar fashion, the unbound concentrations predicted by the binding parameters from each method were plotted as a function of total concentration (Fig. 5). Finally, the unbound fraction was calculated for each method as a function of the total concentration (Fig. 6).

Measurement of drug (or radiolabel) on the plasma side after dialysis is not required when using the volume correction method. However, this measurement can be used to estimate the magnitude of the volume shift. A mean volume shift of 31% was estimated with the aforementioned dialysis conditions. The most likely cause of the shift is the osmotic effect of the impermeable plasma proteins; its magnitude is presumably controlled by the duration of dialysis, the concentration of protein, the area and thickness of the membrane, and other factors that determine the rate of osmotic equilibration.

[1] Technilab Instruments.
[2] Spectrapor No. 2, Spectrum Medical.
[3] Amersham Corp.
[4] Dubnoff Metabolic Shaking Incubator; Precision Scientific Co., Chicago, Ill.
[5] Aquasol, New England Nuclear.
[6] Model 3320, Packard.

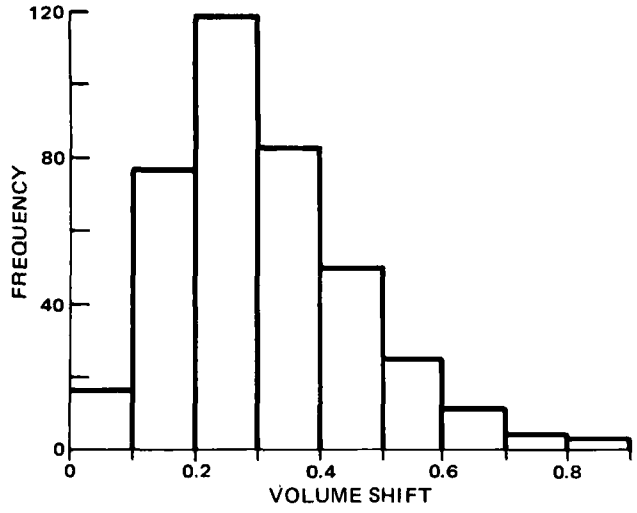


**Figure 2**—*Distribution of volume shifts (expressed by the fractional increase in $V_p$) calculated using Eq. 5 for 388 protein binding measurements. The statistics of the distribution are:* N = *388; mean* ± SD = *0.31* ± *0.15; median = 0.29; geometric mean = 0.27; range = 0.005–0.86; skewness = 0.91; kurtosis = 1.03.*

Failure to recognize and compensate for volume shifts in equilibrium dialysis can be a serious source of error. For example, if the volume shift determined in this study had been ignored, the pharmacokinetic parameters, clearance, and volume of distribution based on the unbound concentration, would have been underestimated by ~30%.

We contend that the proposed method (method I) for estimating the concentration of bound and unbound drug after equilibrium dialysis is superior to the other methods. This contention is based on the extension of the theoretical basis for equilibrium dialysis outlined in *Theoretical.*

It is clear from Table I that estimates of protein binding parameters are dependent on the method chosen for calculation of bound and unbound concentrations. The disparity between the predictions based on these parameter estimates, for bound as a function of unbound concentration and for unbound concentration or fraction unbound as a function of total drug concentration, are shown in Figs. 4–6. These differences are largely explained by the failure of methods II and III to account for the shift of fluid between the dialysis chambers.

The proposed method corrects for volume changes whether nonlinear binding is absent or present. The method is potentially required for any substance, drug or hormone, whose binding is estimated by equilibrium dialysis. Furthermore, the results of this study imply that all equilibrium dialysis binding data in the literature may be in error to the extent that volume shifts occurred and were ignored.

## APPENDIX I: GLOSSARY OF TERMS

Volume Terms

| | |
|---|---|
| $V_P$ | Volume before dialysis on plasma side of dialysis cell |
| $V'_P$ | Volume after dialysis on plasma side of dialysis cell |
| $V_B$ | Volume before dialysis on buffer side of dialysis cell |
| $V'_B$ | Volume after dialysis on buffer side of dialysis cell |
| $R$ | Ratio of $V_B$ to $V_P$ |
| $\delta$ | Fractional increase in $V_P$ due to volume change during dialysis |

Concentration Terms

| | |
|---|---|
| $C_P$ | Total plasma drug concentration before dialysis, including radiolabeled drug if added to plasma |
| $C'_P$ | Total drug concentration on plasma side after dialysis |
| $C'_B$ | Drug concentration after dialysis on buffer side (unbound drug concentration on both sides at equilibrium) |
| $C'_{bnd}$ | Bound drug concentration on plasma side after dialysis |
| $C^0_{bnd}$ | Bound drug concentration that would have been observed after dialysis if no volume shift had occurred |
| $C_{bnd}$ | Bound drug concentration in plasma before dialysis |
| $C_u$ | Unbound drug concentration in plasma before dialysis |
| $C^*_B$ | Concentration of radiolabelled drug in buffer before dialysis |

**Table I—Prednisolone Protein Binding Parameters**

| Method | Binding Capacity ($CAP_1$), ng/ml | | Dissociation Constant ($K_{d1}$), ng/ml | | Nonspecific Binding Constant ($S$) | |
|---|---|---|---|---|---|---|
| | iv[a] | po[b] | iv[a] | po[b] | iv[a] | po[b] |
| I | 168 ± 52[c] | 199 ± 154 | 18 ± 6 | 42 ± 73 | 2.1 ± 0.9 | 2.3 ± 1.9 |
| II | 202 ± 100 | 205 ± 146 | 31 ± 21 | 33 ± 26 | 1.2 ± 0.5 | 1.4 ± 1.2 |
| III | 241 ± 114 | 247 ± 183 | 40 ± 27 | 41 ± 32 | 1.3 ± 0.4 | 1.5 ± 1.2 |

[a] IV = intravenous prednisolone ($N$ = 9). [b] PO = values for prednisolone following oral prednisone or oral prednisolone ($N$ = 9 for both drugs). [c] Mean ± $SD$.

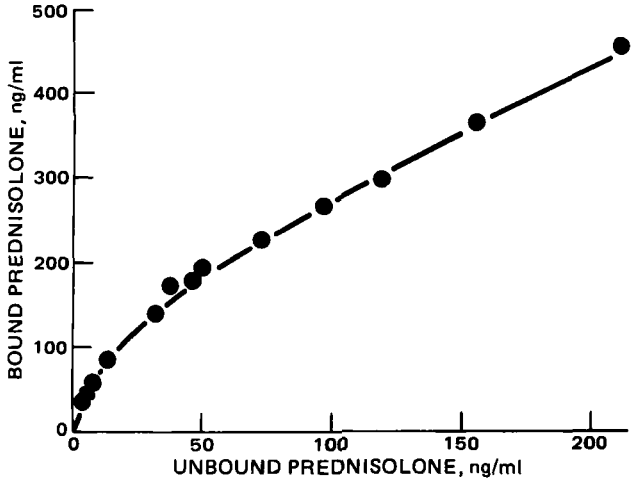


**Figure 3**—*Typical data of one individual for the determination of binding parameters. The bound concentration was determined by method I (see text) which corrects for volume shifts. The line was obtained by fitting the parameters of Eq. 13 to the data using unweighted nonlinear least-squares regression.*

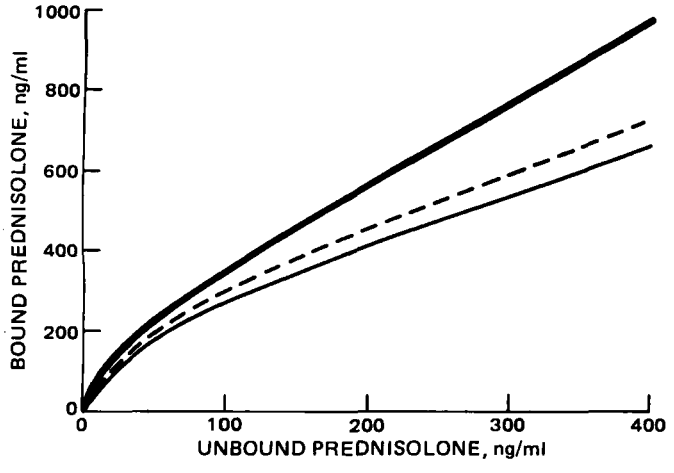


**Figure 4**—*Simulated relationships between bound and unbound prednisolone concentrations. Parameter values, from fits of individual data to Eq. 13 using the three methods, were averaged for the simulation. The average parameter values for $CAP_1$, $K_{d1}$, and $S$ were: (—) method I, 168, 18, 2.1: (—) method II, 202, 31, 1.2; (------) method III, 241, 40, 1.3.*

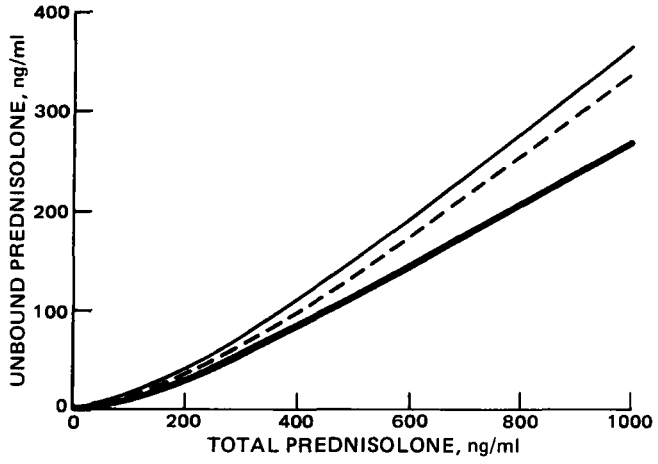


**Figure 5**—*Simulated relationship between unbound and total prednisolone concentrations using the three methods for determining prednisolone binding. The average parameter values are given in Fig. 4. Key: (—) method I; (—) method II; (---) method III.*

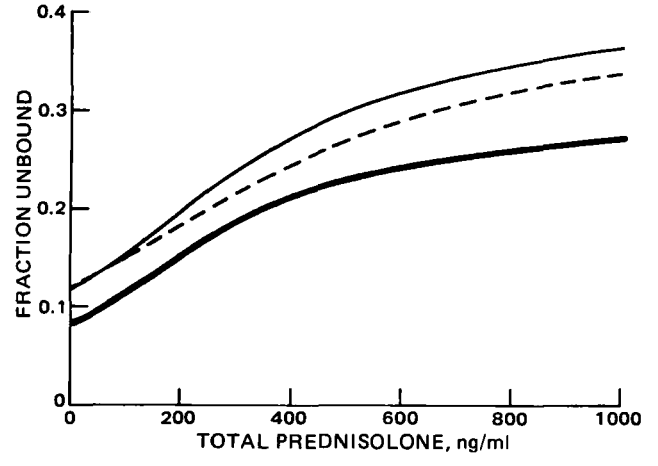


**Figure 6**—*Simulated relationship between the unbound fraction and the total prednisolone concentration using the three methods for determining prednisolone binding. The average parameter values are given in Fig. 4. Key: (—) method I; (—) method II; (---) method III.*

$D_P$ Radiolabel concentration (dpm/ml) in plasma before dialysis (label added to plasma)

$D'_P$ Radiolabel concentration (dpm/ml) on plasma side after dialysis

$D_B$ Radiolabel concentration (dpm/ml) in buffer before dialysis

$D'_B$ Radiolabel concentration (dpm/ml) on buffer side after dialysis

Binding Parameters

$CAP_1$, $CAP_2$ Binding capacities for drug to plasma proteins at two different sites

$K_{d1}$, $K_{d2}$ Equilibrium dissociation constants for two different sites

$S$ Ratio of $CAP_2$ to $K_{d2}$

$f_u$ Ratio of $C_u$ to $C_P$

$f_u'$ Ratio of $C'_B$ to $C'_P$

## APPENDIX II: EQUAL PREDIALYSIS VOLUMES

In the derivations herein, the volume of the plasma and buffer cell contents are assumed to be unequal at the start of dialysis. It is common practice, as in these studies, to use equal volumes. This simplifies the expressions for unbound (Eq. 7) and bound (Eq. 11) concentrations incorporating the volume correction, that is:

$$C'_B = (C_P + C'_B)\frac{D'_B}{D_B} \qquad \text{(Eq. A1)}$$

$$C^0_{bnd} = C_P + C^*_B - 2 \cdot C'_B \quad \text{(Eq. A2)}$$

## APPENDIX III: DIRECT DRUG MEASUREMENT

If concentration of drug (no radiolabel added) is measured, the volume-corrected bound concentration postdialysis is given by:

$$C^0_{bnd} = C_P - C'_B\,(R + 1) \quad \text{(Eq. A3)}$$

From mass balance, $V_P C_P = C'_P \cdot V'_P + C'_B \cdot V'_P$ and using Eqs. 2 and 3, the volume shift $\delta$ is:

$$\delta = \frac{C_P - C'_P - R \cdot C'_B}{(C_P - C'_B)} \quad \text{(Eq. A4)}$$

## APPENDIX IV: THE FRACTION UNBOUND

**Label Added to Buffer**—The fraction unbound, $f_u'$, after dialysis and corrected for volume shift, can be calculated from the concentrations of radioactivity in the buffer before and after dialysis. The relationship is:

$$f_u' = \frac{D'_B}{R(D_B - D'_B)} \quad \text{(Eq. A5)}$$

when radiolabel is initially added to the buffer. The relationship is obtained from Eqs. 7 and 11 and the definition of fraction unbound, $f_u' = C'_B/(C^0_{bnd} + C'_B)$. The fraction unbound after dialysis, when no radiolabel is added and drug is initially present in plasma, is:

$$f_u' = \frac{C'_B}{C_P - R \cdot C'_B} \quad \text{(Eq. A6)}$$

This relationship is obtained from Eqs. A3 and A4.

**Label Added to Plasma Before Dialysis**—Relationships similar to Eqs. 7, 11, and A5, can be derived for the situation in which radiolabel is added to the plasma before dialysis. These relationships, corrected for volume shift, are:

$$C'_B = C_P \cdot \frac{D'_B}{D_P} \quad \text{(Eq. A7)}$$

$$C^0_{bnd} = \frac{C_P \cdot [D_P - D'_B(R + 1)]}{D_P} \quad \text{(Eq. A8)}$$

$$f_u' = \frac{D'_B}{D_P - R \cdot D'_B} \quad \text{(Eq. A9)}$$

where $C_P$ is the drug concentration, including radiolabel, and $D_P$ is the disintegrations per min per ml in the plasma before dialysis.

**Fraction Unbound When Nonlinear Binding Occurs**—The fractions unbound calculated by Eqs. A5, A6, and A9 are the values expected at the postdialysis total plasma concentration; the fraction unbound in the plasma sample drawn from a subject or patient will be different, because of loss of drug to the buffer side. The fraction unbound in the original sample can then be determined from the unbound and bound concentrations as calculated in Eqs. 14 and 15 and from the definition of $f_u$, *i.e.*, $f_u = C_u/(C_u + C_{bnd})$.

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