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Fluid shift and protein leakage corrections in protein binding equilibrium dialysis experiments

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Summary

Conventional equilibrium dialysis methods used in the estimation of ligand–macromolecule binding parameters (affinities, capacities, unbound ligand fractions, etc.) tacitly assume and require that no ligand–macromolecule binding occurs on the buffer side of the dialysis chamber. Unfortunately, this is almost never a valid assumption, as small amounts of plasma proteins are invariably detected in the buffer chamber. Provided the extent of protein leakage is in the usual region (about 0.1%) and the extent of ligand binding does not exceed approximately 99%, errors associated with conventional free fraction estimations obtained from calculations ignoring the effect of protein leakage are usually small (about 1–10% error). As ligand binding exceeds 99%, significant errors may ensue. A generalized theoretical equilibrium dialysis method is developed which permits the estimation of association constants, the number of binding sites and free fraction determinations for a model employing any number of classes of binding sites. Application of the method requires a minimum of two experimental runs for each class of binding sites; volume shifts are automatically adjusted for by the method.

Introduction

Although recent papers have addressed the problem of plasma volume changes (shifts) during equilibrium dialysis experiments (Behm and Wagner, 1979; Huang, 1983; Lima et al., 1983; Lockwood and Wagner, 1983; Tozer et al., 1983; Boudinot and Jusko, 1984; Curry and Hu, 1984), little attention has focused on the influence of protein leakage from plasma to buffer. Bowers et al. (1984) calculated that a 1% leakage of binding protein would cause a 9% overestimate in free fraction for a drug 90% bound to protein. Khor et al. (1985), assuming a single class of binding sites, developed a simple method for determination of the number of sites binding the ligand, the association constant and the free fraction of ligand in plasma prior to dialysis. Volume shifts are automatically adjusted for by the method. From a single experimental run, the following information is required: (1) plasma and buffer volumes before and after dialysis; and (2) total protein and drug concentrations in buffer and plasma before and after dialysis. For drugs extensively bound to plasma protein (> 99%), the usual amount of plasma to buffer protein leakage (about 0.1% for a

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12,000–14,000 molecular weight cutoff membrane) was demonstrated to cause a significant error in free fraction estimation when the leakage factor was ignored. A hypothetical but realistic example utilizing S-warfarin human plasma protein binding data indicated free fraction estimates (therapeutic levels) may be overestimated by approximately 50% (0.0043 estimate vs 0.0028 actual). The reason for the error is that with 0.1% protein leakage into buffer, approximately 35% of drug in buffer is actually bound. A major drawback to the method is the requirement that there be only one class of binding sites. This paper considers the more generalized case in which any number of classes of sites may exist. Complete characterization of system parameters requires a minimum of two experimental dialysis runs per class of sites.

Theoretical

Although the method may (and will) be generalized for any number of classes of sites, temporarily consider a two class example. We make the following assumptions: (1) the activities of components equal their concentrations; (2) protein affinities and binding capacities remain constant and are equal in plasma and dialysate environments; (3) non-specific adsorption, Donnan equilibria and hindered passage of free drug are absent; (4) pH fluctuations, anticoagulant effects and other experimental variables are absent; (5) only one protein binds drug; (6) neither drug nor protein decomposes; and (7) despite protein transfer between compartments, an equilibrium may nonetheless be established. Note that no assumptions are made regarding initial volumes, volume shifts and binding linearity. The method has added flexibility in that plasma and buffer may be mixed prior to experimentation. For example, 1 ml of a 9 : 1 plasma–buffer mixture may be dialyzed against 2 ml of a 95 : 5 buffer–plasma mixture. The method will nonetheless calculate free drug fraction in the 1 ml of undiluted plasma prior to dialysis. Required for the analysis are total drug and protein concentrations in plasma and dialysate at equilibrium. Let:

P_1 = free protein site concentration (class I) in plasma after dialysis;
P_2 = free protein site concentration (class II) in plasma after dialysis;
P_1' = free protein site concentration (class I) in buffer after dialysis;
P_2' = free protein site concentration (class II) in buffer after dialysis;
P_1^0 = free protein site concentration (class I) in plasma before dialysis;
P_2^0 = free protein site concentration (class II) in plasma before dialysis;
DP_1 = drug–protein complex concentration (class I) in plasma after dialysis;
DP_2 = drug–protein complex concentration (class II) in plasma after dialysis;
DP_1' = drug–protein complex concentration (class I) in buffer after dialysis;
DP_2' = drug–protein complex concentration (class II) in buffer after dialysis;
DP_1^0 = drug–protein complex concentration (class I) in plasma before dialysis;
DP_2^0 = drug–protein complex concentration (class II) in plasma before dialysis;
n_1 = number of equivalent and independent protein groups (class I) capable of binding drug;
n_2 = number of equivalent and independent protein groups (class II) capable of binding drug;
K_1 = class I association constant;
K_2 = class II association constant;
D = free drug concentration in plasma and buffer after dialysis;
D^0 = free drug concentration in plasma before dialysis;
M = total drug concentration in plasma after dialysis;
M^0 = total drug concentration in plasma before dialysis;
Q = total drug concentration in buffer after dialysis;
X = total protein concentration in plasma after dialysis;
X^0 = total protein concentration in plasma before dialysis;
Y = total protein concentration in buffer after dialysis;

a = Y/X (by definition);
β = $(DP_1 + DP_2)/D$ = ratio of bound to free drug concentration in plasma after dialysis;
fu^0 = free fraction of unbound drug in plasma before dialysis.

Conservation of mass requires the following:

$$P_1 + DP_1 = n_1 \cdot X \quad (1)$$

$$P_2 + DP_2 = n_2 \cdot X \quad (2)$$

$$D + DP_1 + DP_2 = M \quad (3)$$

$$P_1' + DP_1' = n_1 \cdot Y \quad (4)$$

$$P_2' + DP_2' = n_2 \cdot Y \quad (5)$$

$$D + DP_1' + DP_2' = Q \quad (6)$$

The law of mass action requires:

$$K_1 = \frac{DP_1}{(P_1)(D)} = \frac{DP_1'}{(P_1')(D)} \quad (7)$$

$$K_2 = \frac{DP_2}{(P_2)(D)} = \frac{DP_2'}{(P_2')(D)} \quad (8)$$

From Eqn. 7:

$$\frac{DP_1}{P_1} = \frac{DP_1'}{P_1'} \quad (9)$$

From Eqns. 1 and 4, respectively:

$$P_1 = (n_1 \cdot X) - DP_1 \quad (10)$$

$$P_1' = (n_1 \cdot Y) - DP_1' \quad (11)$$

Substitution of Eqns. 10 and 11 into 9 gives (with rearrangement):

$$DP_1' = \frac{Y \cdot DP_1}{X} \quad (12)$$

Let a = Y/X. Therefore:

$$DP_1' = a \cdot DP_1 \quad (13)$$

Similarly:

$$DP_2' = a \cdot DP_2 \quad (14)$$

Substitution of Eqns. 13 and 14 into Eqn. 6 gives:

$$D + (a \cdot DP_1) + (a \cdot DP_2) = Q \quad (15)$$

From Eqn. 3:

$$(a \cdot D) + (a \cdot DP_1) + (a \cdot DP_2) = a \cdot M \quad (16)$$

Subtracting Eqn. 16 from Eqn. 15 gives:

$$D = \frac{Q - (a \cdot M)}{1 - a} \quad (17)$$

Although expressions are being derived for two classes of binding sites, this equation is valid regardless of class number. Mass balance must be preserved and total drug and protein concentration in buffer and plasma after dialysis must be experimentally determined (these qualifications are also true for all subsequent calculations).

From Eqns. 1 and 7, respectively:

$$DP_1 = (n_1 \cdot X) - P_1 \quad (18)$$

$$DP_1 = K_1(P_1)(D) \quad (19)$$

Equating Eqns. 18 and 19 gives:

$$P_1 = \frac{n_1 \cdot X}{(K_1 \cdot D) + 1} \quad (20)$$

Substituting Eqn. 20 into Eqn. 18 gives:

$$DP_1 = \frac{n_1 \cdot X \cdot K_1 \cdot D}{1 + (K_1 \cdot D)} \quad (21)$$

Similarly:

$$DP_2 = \frac{n_2 \cdot X \cdot K_2 \cdot D}{1 + (K_2 \cdot D)} \quad (22)$$

After dialysis, the ratio of bound-to-free drug concentration on the plasma side, β, is:

$$\frac{DP_1 + DP_2}{D} = \frac{M - D}{D} = \beta \quad (23)$$

Combining Eqns. 21, 22 and 23 gives:

$$\beta + [\beta \cdot D \cdot I(1)] + [\beta \cdot D^2 \cdot I(2)] - [X \cdot J(1)] - [X \cdot D \cdot J(2)] = 0 \quad (24)$$

where (for the two class model):

$$I(1) = K_1 + K_2 \quad (25)$$

$$I(2) = K_1 \cdot K_2 \quad (26)$$

$$J(1) = (n_1 \cdot K_1) + (n_2 \cdot K_2) \quad (27)$$

$$J(2) = (n_1 \cdot K_1 \cdot K_2) + (n_2 \cdot K_1 \cdot K_2) \quad (28)$$

Complete characterization of the system requires solution to Eqn. 24. This is readily accomplished. Begin by calculating D from Eqn. 17; this requires experimental measurement of total drug and protein concentration in both plasma and buffer after dialysis (equilibrium). The value of β is then calculated from Eqn. 23. At this point, the free fraction of drug in the plasma compartment at equilibrium is calculable (independent of the number of classes of sites). This may be all that is desired by the investigator. However, due to volume shifts (protein dilution, etc.) and possible dependence of free drug fraction on drug concentration, it may not reflect free fraction in plasma before dialysis. Additionally, the investigator may wish values for the association constants and number of sites for each class. Since Eqn. 24 has 4 unknowns, a minimum of 4 experimental runs are required for its solution; precision is enhanced by studying binding over a sufficient drug concentration range such that each class of sites significantly contributes to free drug fraction over some region (Klotz, 1982, 1983, 1985). Since no assumptions are made about volume shifts and binding linearity, these experimental factors are inconsequential.

Consider next the case for a single class of sites, viz. $n_2 = K_2 = I(2) = J(2) = 0$. Eqn. 24 then reduces to:

$$\beta + [\beta \cdot D \cdot I(1)] - [X \cdot J(1)] = 0 \quad (29)$$

where:

$$I(1) = K_1 \quad (30)$$

$$J(1) = n_1 \cdot K_1 \quad (31)$$

Since Eqn. 29 has two unknowns [I(1) and J(1)], at least two experimental runs are required for its solution (remember that D is calculated from Eqn. 17, β is calculated from Eqn. 23 and X is experimentally measured).

In the case of 3 classes of sites, Eqn. 24 takes on the form:

$$\beta + [\beta \cdot D \cdot I(1)] + [\beta \cdot D^2 \cdot I(2)] + [\beta \cdot D^3 \cdot I(3)] - [X \cdot J(1)] - [X \cdot D \cdot J(2)] - [X \cdot D^2 \cdot J(3)] = 0 \quad (32)$$

where (for the three class site model):

$$I(1) = K_1 + K_2 + K_3 \quad (33)$$

$$I(2) = (K_1 \cdot K_2) + (K_1 \cdot K_3) + (K_2 \cdot K_3) \quad (34)$$

$$I(3) = K_1 \cdot K_2 \cdot K_3 \quad (35)$$

$$J(1) = (n_1 \cdot K_1) + (n_2 \cdot K_2) + (n_3 \cdot K_3) \quad (36)$$

$$J(2) = [n_1 \cdot K_1(K_2 + K_3)] + [n_2 \cdot K_2(K_1 + K_3)] + [n_3 \cdot K_3(K_1 + K_2)] \quad (37)$$

$$J(3) = [n_1 \cdot K_1(K_2 \cdot K_3)] + [n_2 \cdot K_2(K_1 \cdot K_3)] + [n_3 \cdot K_3(K_1 \cdot K_2)] \quad (38)$$

Solution of the six unknowns of Eqn. 32 requires at least 6 experimental runs.

For a 4 class model, the appropriate equation: is:

$$\beta + [\beta \cdot D \cdot I(1)] + [\beta \cdot D^2 \cdot I(2)] + [\beta \cdot D^3 \cdot I(3)] + [\beta \cdot D^4 \cdot I(4)] - [X \cdot J(1)] - [X \cdot D \cdot J(2)] - [X \cdot D^2 \cdot J(3)] - [X \cdot D^3 \cdot J(4)] = 0 \quad (39)$$

where:

$$I(1) = K_1 + K_2 + K_3 + K_4 \tag{40}$$

$$I(2) = (K_1 \cdot K_2) + (K_1 \cdot K_3) + (K_1 \cdot K_4) + (K_2 \cdot K_3) + (K_2 \cdot K_4) + (K_3 \cdot K_4) \tag{41}$$

$$I(3) = (K_1 \cdot K_2 \cdot K_3) + (K_1 \cdot K_2 \cdot K_4) + (K_1 \cdot K_3 \cdot K_4) + (K_2 \cdot K_3 \cdot K_4) \tag{42}$$

$$I(4) = K_1 \cdot K_2 \cdot K_3 \cdot K_4 \tag{43}$$

$$J(1) = (n_1 \cdot K_1) + (n_2 \cdot K_2) + (n_3 \cdot K_3) + (n_4 \cdot K_4) \tag{44}$$

$$J(2) = n_1 \cdot K_1(K_2 + K_3 + K_4) + n_2 \cdot K_2(K_1 + K_3 + K_4) + n_3 \cdot K_3(K_1 + K_2 + K_4) + n_4 \cdot K_4(K_1 + K_2 + K_3) \tag{45}$$

$$J(3) = n_1 \cdot K_1(K_2 \cdot K_3 + K_2 \cdot K_4 + K_3 \cdot K_4) + n_2 \cdot K_2(K_1 \cdot K_3 + K_1 \cdot K_4 + K_3 \cdot K_4) + n_3 \cdot K_3(K_1 \cdot K_2 + K_1 \cdot K_4 + K_2 \cdot K_4) + n_4 \cdot K_4(K_1 \cdot K_2 + K_1 \cdot K_3 + K_2 \cdot K_3) \tag{46}$$

$$J(4) = n_1 \cdot K_1(K_2 \cdot K_3 \cdot K_4) + n_2 \cdot K_2(K_1 \cdot K_3 \cdot K_4) + n_3 \cdot K_3(K_1 \cdot K_2 \cdot K_4) + n_4 \cdot K_4(K_1 \cdot K_2 \cdot K_3) \tag{47}$$

A generalized expression may now be posited for N classes of sites:

$$\beta + [\beta \cdot D \cdot I(1)] + [\beta \cdot D^2 \cdot I(2)] + [\beta \cdot D^3 \cdot I(3)] + [\beta \cdot D^4 \cdot I(4)] + \ldots + [\beta \cdot D^N \cdot I(N)] - [X \cdot J(1)] - [X \cdot D \cdot J(2)] - [X \cdot D^2 \cdot J(3)] - [X \cdot D^3 \cdot J(4)] \ldots - [X \cdot D^{(N-1)} \cdot J(N)] = 0 \tag{48}$$

where:

$$I(1) = \sum_{i=1}^{N} K_i \tag{49}$$

$$I(2) = \sum_{i=1}^{N-1} K_i \left(\sum_{j=i+1}^{N} K_j \right) \tag{50}$$

$$I(3) = \sum_{i=1}^{N-2} K_i \left[\sum_{j=i+1}^{N-1} K_j \left(\sum_{k=j+1}^{N} K_k \right) \right] \tag{51}$$

$$I(4) = \sum_{i=1}^{N-3} K_i \left[\sum_{j=i+1}^{N-2} K_j \left\{ \sum_{k=j+1}^{N-1} K_k \left(\sum_{m=k+1}^{N} K_m \right) \right\} \right] \tag{52}$$

$$I(N) = \prod_{i=1}^{N} K_i \tag{53}$$

$$J(1) = \sum_{i=1}^{N} n_i \cdot K_i \tag{54}$$

$$J(2) = \sum_{i=1}^{N} n_i \cdot K_i \left(\sum_{\substack{j=1 \\ j \neq i}}^{N} K_j \right) \tag{55}$$

$$J(3) = \sum_{i=1}^{N} \left\{ n_i K_i \left[\sum_{\substack{j=1 \\ j \neq i}}^{N} K_j \left(\sum_{\substack{k>j \\ k \neq i}}^{N} K_k \right) \right] \right\} \tag{56}$$

$$J(4) = \sum_{i=1}^{N} \left\{ n_i K_i \left(\sum_{\substack{j=1 \\ j \neq i}}^{N} K_j \left[\sum_{\substack{k>j \\ k \neq i}}^{N} K_k \left(\sum_{\substack{m>k \\ m \neq i \neq j}}^{N} K_m \right) \right] \right) \right\} \tag{57}$$

$$J(N) = \left(\prod_{i=1}^{N} K_i\right)\left(\sum_{i=1}^{N} n_i\right) \quad (58)$$

Note that the number of I and J terms always equals the number of classes of sites.

In practice, one could use matrix algebra to solve for I and J values in Eqns. 24, 32 or 39 (Eves, 1980):

$$U^T \cdot U \cdot V = U^T \cdot W \quad (59)$$

where U is the matrix of the coefficients, V is the matrix of the variables, W is a vector and U^T is the transpose of U.

Once association constants have been determined, the free fraction of drug in plasma prior to dialysis may be calculated:

$$fu^0 = \frac{D^0}{M^0} = \frac{D^0}{D^0 + DP_1^0 + DP_2^0 + \ldots DP_N^0}$$

$$= \left[1 + \left(K_1 \cdot P_1^0\right) + \left(K_2 \cdot P_2^0\right) + \ldots \left(K_N \cdot P_N^0\right)\right]^{-1} \quad (60)$$

where M^0 is measured before dialysis and D^0 is calculated from the expression:

$$\left[D^0 \cdot X^0 \sum_{i=1}^{N}\left(\frac{n_i \cdot K_i}{1 + \left(K_i \cdot D^0\right)}\right)\right] + D^0 = M^0 \quad (61)$$

This relationship, based on mass balance, is a generalized form of Eqn. 3, where the bracketed term on the left side is equal to bound drug concentration (see, for example, Eqns. 21 and 22). The only unknown in Eqn. 61 is D^0, and it can be solved by expanding to a (N + 1) degree polynomial.

For the two-class model, Eqn. 61 expands to:

$$(D^0)^3 + A(D^0)^2 + B(D^0) + C = 0 \quad (62)$$

where:

$$A = \left[(n_1 + n_2)X^0\right] + (1/K_1) + (1/K_2) - M^0 \quad (63)$$

$$B = \left[(n_1 \cdot X^0)/K_2\right] + \left[(n_2 \cdot X^0)/K_1\right] + \left[1/(K_1 \cdot K_2)\right] - (M^0/K_1) - (M^0/K_2) \quad (64)$$

$$C = -M^0/(K_1 \cdot K_2) \quad (65)$$

All terms on the right hand sides of Eqns. 63–65 are known, so that A, B and C may be solved and substituted into Eqn. 62 to calculate D^0.

Discussion

As a verification of the mathematical model, binding parameters reported to characterize the interaction between racemic warfarin and bovine serum albumin (BSA) will be used to generate theoretical data (2 classes of sites). Reported parameters are for illustrative purposes only; human and bovine data will therefore be used jointly. For a drug as extensively bound to plasma proteins as warfarin (> 99% in human plasma, Banfield et al., 1983), any method that fails to incorporate the influence of trace protein concentrations within the putative protein free buffer compartment will inevitably give false results (Khor et al., 1985).

Using a dynamic dialysis procedure, the following parameters were reported (Meyer and Guttman, 1970): $n_1 = 1$; $n_2 = 6$; $K_1 = 6.24 \times 10^6$ liters/mole; and $K_2 = 2.61 \times 10^3$ liters/mole. Assume the BSA concentration in the "plasma" compartment before dialysis is 7.428×10^{-4} moles/liter. Further assume volume shifts do not occur (to simplify the simulation), plasma and buffer volumes before dialysis are equal and protein leakage is 0.1%. Therefore: X = (7.428×10^{-4} M) (0.999) and Y = (7.428×10^{-4} M) (0.001). Assigned values for D are substituted into Eqns. 1 and 7; the two equations are solved for the two unknowns, DP_1 and P_1. Similarly, DP_2 and P_2 are solved from Eqns. 2 and 8. Eqns. 4, 5, 7 and 8 are used analogously to solve for DP_2' and P_2'. Values for M and Q are then solved from Eqns. 3 and 6, permitting the determination (from Eqn. 24) of I(1), I(2), J(1) and J(2) from four theoretical data

TABLE 1

HYPOTHETICAL PROTEIN BINDING VARIABLES AND PARAMETERS FOR RACEMIC WARFARIN BINDING TO BOVINE SERUM ALBUMIN (SEE TEXT FOR DISCUSSION)

M^0 (moles/liter)	D (moles/liter)	Calculated apparent unbound fraction (uncorrected for protein leakage)		Calculated unbound fraction (corrected for protein leakage)		True unbound fraction of drug in BSA solution before dialysis
		After dialysis Q/M	Before [a] dialysis D_0^{app}/M^0	After dialysis D/M	Before [b] dialysis D^0/M^0	fu^0
3.11450×10^{-8}	6.70×10^{-12}	0.00121617	0.00027163	0.00021538	0.00021532	0.00021538
3.90761×10^{-6} [c]	8.45×10^{-10}	0.00121730	0.00027306	0.00021651	0.00021644	0.00021648
1.36906×10^{-5} [c]	3.00×10^{-9}	0.00122017	0.00027672	0.000219396	0.000219325	0.00021939
8.61601×10^{-4}	9.80×10^{-6}	0.01250588	0.0116831	0.01151641	0.0123140	0.0123142
1.206086×10^{-3}	3.70×10^{-5}	0.0326486	0.0282388	0.03167931	0.0336453	0.0336454
1.309948×10^{-3}	4.60×10^{-5}	0.03739352	0.0316842	0.03642899	0.0386443	0.0386443

[a] D_0^{app} is the apparent free drug concentration in protein solution before dialysis calculated from incorrect n_i and K_i values obtained by assuming the absence of protein leakage.

[b] The slight differences between D^0/M^0 and fu^0 values are due to rounding off errors.

[c] Therapeutic plasma concentration range (window) in humans.

sets. Eqns. 25–28 are then used to solve n_1, n_2, K_1 and K_2. The unbound fraction of drug in the original "plasma" sample before dialysis (fu^0) is solved from Eqns. 60–65. Table 1 illustrates the magnitude of the errors obtained by neglecting the influence of protein leakage. For M^0 values approximating therapeutic plasma levels in man, neglect of the influence of protein leakage results in equilibrium free fraction (Q/M) errors of approximately 460%.

Taking into account protein leakage, and using 4 values of D ranging from 0.54×10^{-5} to 0.41×10^{-4} moles/liter, n_1, n_2, K_1 and K_2 were calculated with errors of 0.1% or less. However, when protein leakage was ignored (taken not to exist), the following parameter values were obtained: n_1

TABLE 2

THEORETICAL PROTEIN BINDING VARIABLES FOR RACEMIC WARFARIN BINDING TO BOVINE SERUM ALBUMIN

It was assumed equal volumes of protein solution (7.428×10^{-4} moles/liter) and buffer were dialyzed, that there were no volume shifts and that protein leakage into buffer was 0.1%. Binding parameters were taken from the example in the discussion section (see text for discussion).

Total drug conc. in protein solution before dialysis (M^0, moles/liter)	Free drug conc. in protein and buffer solution after dialysis (D, moles/liter)	Total drug conc. in protein solution after dialysis (M, moles/liter)	Protein solution, post-dialysis	
			Percent class I sites occupied	Percent class II sites occupied
0.311450 [a] $\times 10^{-7}$	6.70×10^{-12}	$0.31107188 \times 10^{-7}$	0.00418	0.00000
0.259424×10^{-5}	5.60×10^{-10}	0.259108×10^{-5}	0.34822	0.00015
0.225423×10^{-4}	5.00×10^{-9}	0.225148×10^{-4}	3.0256	0.0013
0.148915×10^{-3}	4.00×10^{-8}	0.148726×10^{-3}	19.97439	0.0104
0.125272×10^{-2}	4.10×10^{-5}	0.121055×10^{-2}	99.61065	9.66658

[a] Below this concentration, unbound drug fraction in protein solution is independent of drug concentration, i.e. binding is linear.

$= 0.989$; $n_2 = 70.87$; $K_1 = 4.99 \times 10^6$; and $K_2 = 0.249 \times 10^3$. Whereas n_1 and K_1 are not poorly estimated, this cannot be said of the estimates of n_2 and K_2.

For a two-class system, the fractions of occupied protein sites are:

$$\text{Fraction of class I sites occupied} = DP_1/(n_1 \cdot X) \tag{66}$$

$$\text{Fraction of class II sites occupied} = DP_2/(n_2 \cdot X) \tag{67}$$

Table 2 illustrates binding to the two classes of sites used in the warfarin example from Table 1. Note that at lower warfarin concentrations, drug binding to class II sites is negligible. Under these conditions, the left side of Eqn. 24 is well approximated by the left side of Eqn. 29 (although this is not apparent upon casual inspection). As per the warfarin example, using Eqn. 29 rather than Eqn. 24 to calculate K_1 and n_1 at unbound drug concentrations of about $1.2\text{–}1.8 \times 10^{-7}$ moles/liter, gives errors of less than 0.16% for n_1 and K_1. It is this apparent absence of class II binding at low therapeutic drug concentrations that is probably responsible (analogously) for reports in which both one and two classes of binding sites have been reported for human plasma and albumin binding of warfarin (see Jusko and Gretch, 1976, for collated warfarin binding data). This "collapsing" of a two class model into an apparent one class model has been cogently discussed by Klotz (1982, 1983, 1985).

Under conditions in which binding to only one class of sites predominates, our previously published method (Khor et al., 1985) may be applied. In fact, the collapsing of the two class to a one class model was tacitly assumed in the warfarin example used in our initial report. In that method, one need only run one dialysis experiment to be able to solve for n_1, K_1 and free fraction of drug in plasma before dialysis (see warfarin example in that work).

As with the previous method (Khor et al., 1985), no assumptions are posited regarding the extent of protein contamination of buffer and vice versa. Consequently, plasma and buffer may be mixed in any reasonable proportion prior to dialysis. For example, 1 ml of 9 : 1 plasma–buffer may be dialyzed against 1 ml of 9 : 1 buffer–plasma. Volume shifts are inconsequential, viz. they do not affect the results. Additionally, binding may be non-linear.

Another potential application of this method is for obtaining initial estimates of protein binding parameters. For example, Thompson et al. (1984) fitted the following equation to protein binding data:

$$\frac{DP_1 + DP_2}{D} = \frac{n_1 \cdot P_1 \cdot K_1}{1 + (K_1 \cdot D)} + \frac{n_2 \cdot P_2 \cdot K_2}{1 + (K_2 \cdot D)} \tag{68}$$

where $n_1 \cdot P_1$, $n_2 \cdot P_2$, K_1 and K_2 were iterated. Using our procedure, initial estimates of these parameters could have readily been obtained.

The mathematical model does have restrictions. The assumption that only one protein binds drug would invalidate the procedure if smaller molecular weight fragments (impurities, bacterial degradation products, etc.) also bound drug. It is also assumed that although protein leakage and volume shifts occur, a state mimicing equilibrium exists at the time the experiment is terminated. To obtain good results, experiments should be run over markedly different drug concentrations so that the data reveal the influence of the requisite number of classes of binding sites. One would not for example, only run BSA–warfarin binding studies at low drug concentrations. Under these conditions, BSA class II sites would not be appreciably occupied, and consequently the experimental data would permit considerably less precise and accurate estimates of parameters characterizing binding to this class of sites. As noted by Klotz (1983), "there is no substitute for the collection of precise data over a wide range of concentrations of ligand".

If K_i and/or n_i are dependent on protein concentration, e.g. as with some corticosteroids (Brunkhorst and Hess, 1965; Kerkay and Westphal, 1969; Boudinot and Jusko, 1984), the equations are not valid. As noted by Mueller and Potter (1981), this could occur if: (1) binding of one ligand to a macromolecule creates an energeti-

cally favorable site for another ligand molecule (allosteric positive co-operativity); (2) the protein or plasma contains agents which compete with drug for protein binding sites in a concentration dependent fashion; and (3) self-association of the macromolecule, as in the formation of dimers, is concentration dependent.

Finally, the method requires the application of a *hysteron proteron*, i.e. the investigator must initially assume what he (she) purports to establish with regard to the numbers of classes of sites. One might assume, for example, based on a preliminary Scatchard plot (Scatchard, 1949), there are two classes of binding sites. The appropriate equations are employed, and internal consistency is established. This procedure is analogous to that regularly employed in pharmacokinetics. A model is assumed, equations are derived, and these are fitted to the data. Unlike most pharmacokinetics analyses, however, there exists no experience to guide the investigator through this procedure.

Addendum

It was recently pointed out by Dr. Gerhard Levy (personal communication) that protein leakage can virtually be eliminated from protein binding dialysis experiments by fitting dialysis cells with extra clamps. A technical resolution of the protein leakage problem is obviously preferable to the approach developed here.

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