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Short Communication

Simultaneous correction for volume shifts and protein leakage in equilibrium dialysis protein binding experiments

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A number of recent papers have dealt with the problem of plasma volume changes during equilibrium dialysis and their influence on the determination of free drug fractions (Behm and Wagner, 1979; Lockwood and Wagner, 1983; Huang, 1983; Tozer et al., 1983; Lima et al., 1983; Boudinot and Jusko, 1984). With the exception of Bowers et al. (1984), however, little attention has focused on protein leakage from plasma to dialysate and its influence on free fraction estimation. For a drug that is 90% bound, for example, a 1% leakage of binding protein will cause a 9% overestimate of free drug concentration (Bowers et al., 1984).

Since in our experience protein leakage from plasma to dialysate invariably occurs ($\cong 0.1\%$ with a 12–14,000 molecular weight cutoff membrane), the real issue becomes one of determining its extent and making appropriate corrections where deemed necessary. In general, for drugs that are highly protein-bound ($> 99\%$), corrections for protein leakage will be required for the accurate estimations of free fraction.

Consider the simplest case in which protein molecule P contains n equivalent and independent groups capable of attaching ligand D. We further assume: (1) the activities of the components equal their concentrations; (2) protein affinity and total binding capacity remain constant and equal in plasma and dialysate environment; (3) non-specific adsorption, Donnan equilibria and hindered passage of free drug are absent; and (4) pH fluctuations, anticoagulant effects and other experimental variables that affect binding are also absent. Let:

X = total protein amount in plasma compartment after equilibrium dialysis;
Y = total protein amount in buffer dialysis compartment after equilibrium dialysis;
U = total drug amount in both compartments before and after equilibrium dialysis (assumed equal);

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V_1 = volume of plasma compartment before equilibrium dialysis;
V_2 = volume of buffer compartment before equilibrium dialysis;
V_3 = volume of plasma compartment after equilibrium dialysis;
V_4 = volume of buffer compartment after equilibrium dialysis;
P^0 = unbound protein concentration in plasma before equilibrium dialysis;
P = unbound protein concentration in plasma after equilibrium dialysis;
DP^0 = drug-protein complex concentration in plasma before equilibrium dialysis;
DP = drug-protein complex concentration in plasma after equilibrium dialysis;
D^0 = unbound drug concentration in plasma before equilibrium dialysis;
D = unbound drug concentration in plasma and buffer after equilibrium dialysis;
P′ = unbound protein concentration in buffer after equilibrium dialysis;
DP′ = drug-protein complex concentration in buffer after equilibrium dialysis;
Z = uncorrected or apparent free drug fraction in plasma after equilibrium dialysis; i.e., $[(D) + (DP')]/[(D) + (DP)]$;
K_a = association constant;
fu^0 = unbound fraction of drug in plasma before equilibrium dialysis.
Mass balance requires the following:

$$X = (P)(V_3) + (DP)(V_3) \tag{1}$$

$$Y = (P')(V_4) + (DP')(V_4) \tag{2}$$

$$U = V_3[(D) + (DP)] + V_4[(D) + (DP')] \tag{3}$$

By definition, the uncorrected or apparent free fraction of drug in plasma at equilibrium is given by:

$$Z = [(D) + (DP')]/[(D) + (DP)] \tag{4}$$

The association constant, K_a, is:

$$K_a = (DP)/[(P)(D)] = (DP')/[(D)(P')] \tag{5}$$

Eqns. 1–5 may be combined and solved for DP, D and P:

$$DP = [(X)(U)(1 - Z)(V_4)]/[(V_3 + V_4Z)(XV_4 - YV_3)] \tag{6}$$

$$D = [U(XZV_4 - YV_3)]/[(V_3 + V_4Z)(XV_4 - YV_3)] \tag{7}$$

$$P = \frac{[(X)(V_3 + V_4Z)(XV_4 - YV_3)] - [(XUV_3V_4)(1 - Z)]}{(V_3)(V_3 + V_4Z)(XV_4 - YV_3)} \tag{8}$$

Eqns. 6–8 may be substituted into Equation 5 to solve for K_a:

$$K_a = [SQ]/[R(Q - US)] \tag{9}$$

where

$$Q = (V_3 + V_4Z)(XV_4 - YV_3) \tag{10}$$

$$R = XZV_4 - YV_3 \tag{11}$$

$$S = V_3V_4(1 - Z) \tag{12}$$

Mass balance requires the following:

$$\left[(P^0) + (DP^0)\right]V_1 = X + Y \tag{13}$$

$$\left[(D^0) + (DP^0)\right]V_1 = U \tag{14}$$

In plasma before dialysis:

$$K_a = (DP^0)/\left[(P^0)(D^0)\right] \tag{15}$$

Eqs. 13–15 may be combined and solved for P^0:

$$P^0 = \left[-b + (b^2 - 4ac)^{\frac{1}{2}}\right]/2a \tag{16}$$

where

$$a = K_aV_1 \tag{17}$$

$$b = K_a(U - X - Y) + V_1 \tag{18}$$

$$c = -(X + Y) \tag{19}$$

The unbound fraction of drug in plasma before dialysis is:

$$fu^0 = (D^0)/\left[(D^0) + (DP^0)\right] = \left(1 + K_aP^0\right)^{-1} \tag{20}$$

Therefore, substitution of the value of P^0 from Eqn. 16 into Eqn. 20 gives fu^0. The value of K_a has already been solved from Eqn. 9.

In summary, the following information is needed to solve for K_a and fu^0: V_1, V_3, V_4, total drug concentration in plasma after dialysis, (D) + (DP), total drug concentration in buffer after dialysis, (D) + (DP′), total protein concentration in plasma after dialysis, (P) + (DP), and total protein concentration in buffer after dialysis, (P′) + (DP′). This calculation solves for unbound fraction of drug in the original plasma specimen regardless of volume shifts and protein leakage. Moreover, if the investigator wishes, plasma and buffer may be mixed in any proportion prior to dialysis. For example, suppose assay methodology is insufficiently sensitive to measure protein concentration in buffer after dialysis with normal leakage. The

investigator could in this situation dialyze equal volumes of 9:1 plasma–buffer against 9:1 buffer–plasma. The calculated fu^0 still represents the free fraction in plasma before the mixing. An additional advantage to this method is that it makes no assumptions about linearity. Free fraction in plasma before dialysis (fu^0) is calculable regardless of the degree of non-linear binding. A disadvantage to the method, however, is the assumption of a single protein with equivalent and independent binding groups (models for more complicated binding systems are presently under study). Other difficulties may arise. Values for n and/or K_a may be plasma protein concentration dependent, e.g. as with some corticosteroids (Boudinot and Jusko, 1984). Also, lower molecular weight protein fragments with or without binding capacity may slip across the dialysis membrane. Finally, if protein leakage and/or volume shifts are continuous, equilibrium will never be reached. Therefore, a time independence of Z values should be demonstrated to at least establish a quasi-equilibrium condition.

As an example of the method, consider the binding of S-warfarin to plasma albumin (O'Reilly, 1973; Banfield et al., 1983). Assume the following hypothetical but realistic values:

$V_1 = 1.00$ ml
$V_2 = 1.00$ ml
$V_3 = 1.03$ ml
$V_4 = 0.97$ ml
Total warfarin concentration in plasma after equilibrium dialysis = 7.5 μg/ml;
Total warfarin concentration in buffer after equilibrium dialysis = 0.03225 μg/ml;
Total albumin concentration in plasma after equilibrium dialysis = 50,000 μg/ml;
Total albumin concentration in buffer after equilibrium dialysis = 70 μg/ml.

Using these values, the apparent free fraction (Z) of warfarin in plasma is 0.0043. However, the correct fu^0 is 0.0028. Using a Scatchard (Scatchard, 1949) or other type relationship, n may also be calculated as 1.00. The following variable and parameter estimates may be used to check each step of the calculation (these calculations have been programmed for the Texas Instrument TI-55 programmable calculator; a free magnetic card is available upon request):

$X = 51{,}500\ \mu g$	$D = 0.0217804927\ \mu g/ml$
$Y = 67.9\ \mu g$	$P = 49992.52178\ \mu g/ml$
$U = 7.7562825\ \mu g$	$K_a = 0.0068679237\ ml/\mu g$
$Z = 0.0043$	$a = 0.0068679237\ ml^2/\mu g$
$Q = 51589.68549\ \mu g \cdot ml^2$	$b = -353.1111355$ ml
$R = 144.8695\ \mu g \cdot ml$	$c = -51567.9\ \mu g$
$S = 0.99480387\ ml^2$	$P^0 = 51560.16556\ \mu g/ml$
$DP = 7.478219507\ \mu g/ml$	$fu^0 = 0.0028160186$

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