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COMPARISON OF GRAPHICAL PROCEDURES FOR ESTIMATING THE INTRINSIC MOLAR FLUORESCENCE OF PROTEIN-BOUND DRUGS FOR DRUG-BINDING STUDIES

A REEVALUATION OF EXISTING PLOTS AND INTRODUCTION OF TWO INVERSE HYPERBOLIC PLOTS

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Abstract—A theoretical evaluation of existing and new graphical procedures for estimating the intrinsic molar fluorescence (ψ_B) of a protein-bound drug has been undertaken. The results do not concord with a recent proposition that ψ_B should be obtained by direct reading from a graph of emitted fluorescence intensity (I) against the logarithm of the binding protein concentration ($[P]$) rather than by extrapolation of a double reciprocal plot. The calculated errors in estimates of ψ_B obtained by direct reading or by extrapolation of standard plots and of three new inverse hyperbolic plots showed that, independently of binding affinity:

1. Direct reading from the logarithmic plot gave the least accurate estimates.
2. The single reciprocal plot gave more accurate estimates than the double reciprocal plot providing the (constant) drug concentration was similar to or greater than its dissociation constant in the binding system. At lower drug concentrations the double reciprocal plot gave more accurate estimates.
3. Extrapolation of an inverse hyperbolic sine plot ($\sinh^{-1}(1/I)$ against $1/[P]$) did not give more accurate estimates than the standard reciprocal plots.
4. If the drug concentration was close to its dissociation constant the most accurate estimates were obtained with an inverse hyperbolic cosine plot of $\cosh^{-1}(I + 1)$ against $1/[P]$.

For a low affinity binding system in which non-specific binding is significant an inverse hyperbolic sine plot of $1/\sinh^{-1}I$ against $1/[P]$ gave the most accurate estimates at low drug concentrations. An experimental and theoretical procedure for optimizing the estimation of ψ_B is proposed on this basis.

The determination, from fluorescence or fluorescence polarization measurements, of a protein's binding parameters for a fluorescing drug requires knowledge of the protein-bound drug's intrinsic molar fluorescence (ψ_B) [1]. This cannot be obtained directly because the protein-bound drug cannot exist in the total absence of free drug under equilibrium conditions. There exist several graphical procedures for estimating ψ_B from fluorescence intensity (I) measurements with a constant drug concentration and increasing (excess) protein concentrations ($[P]$). Panjehshahin *et al.* [2] have compared two such methods and concluded that estimation of the plateau value reached on a direct plot of I against log $[P]$ was preferable to extrapolating a double reciprocal plot ($1/I$ against $1/[P]$) to the ordinate ($[P] = \infty$). However, their evidence is insufficient to support this conclusion, once the pitfall of assuming overall linearity of the double reciprocal plot is avoided, because the only valid criterion for choosing a particular graphical procedure is that it yields a value close to the true value of ψ_B. But ψ_B is an instrument-dependent constant and a true value cannot be obtained from the literature and must remain an unknown in any experimental comparison of the graphical procedures. A different approach to answering the problem of which graphical procedure to use has thus been taken here.

From a protein's known binding parameters for a drug one can calculate the free and bound drug distribution for a fixed total drug concentration and a range of protein concentrations. Then, from *predefined* ("true") intrinsic molar fluorescences of free and bound drug, one can calculate the total fluorescence intensity emitted at each protein concentration. These pseudo-experimental intensities can then be submitted to the different graphical transformations to find which one yields a value for ψ_B closest to that predefined.

The comparison requires exact estimation of the value of ψ_B given by each plot, so the insensitivity inherent in manual data plotting was circumvented by using calculation throughout. For the graphical procedures involving extrapolation, such as the double reciprocal plot, in which the plot is assumed to be virtually linear from a group of data points to the ordinate, calculation of the intercept requires knowledge of the binding curve slope in the region extrapolated. Further, although the regions extrapolated are those in which the protein is largely in excess, non-specific binding effects cannot realistically be neglected, particularly with lower affinity binding proteins such as albumin. The equations for these curve slopes have therefore been derived from the binding equations including non-specific binding.

The binding system simulated was that of warfarin binding by albumin [2, 3] though higher and lower affinity systems were also tested in order to ensure that the results were not specific to warfarin. The three classical data transformations—the direct log

plot, the double reciprocal plot and the single reciprocal plot (I against $1/[P]$)—were compared. Moreover, since binding curves are generally considered to be hyperbolic, three inverse hyperbolic reciprocal plots which have not, to my knowledge, previously been used were also tested.

THEORY

If ψ_B and ψ_F are the intrinsic molar fluorescences of protein-bound and free drug, at concentrations $[D_B]$ and $[D_F]$ respectively, then the total fluorescence intensity emitted (I) is:

$$I = \psi_B[D_B] + \psi_F[D_F]. \quad (1)$$

The total drug concentration $[D_T]$ is:

$$[D_T] = [D_B] + [D_F]. \quad (2)$$

Thus:

$$I = \psi_F[D_T] + (\psi_B - \psi_F)[D_B]. \quad (3)$$

For a binding protein, at concentration $[P]$, with n equivalent sites of apparent intrinsic dissociation constant k, the equilibrium binding equation is:

$$[D_B] = \frac{n[P][D_F]}{k + [D_F]} + S[P][D_F] \quad (4)$$

where S is the non-specific binding, equivalent to n/k for a low affinity site class.

Using Eqn (2) to substitute for $[D_F]$ in Eqn (4), and solving the resulting quadratic in $[D_B]$ gives an expression for $[D_B]$ which can be substituted into Eqn (3) to yield:

$$I = \psi_F[D_T] + \frac{(\psi_B - \psi_F)}{2(1 + S[P])} \times (Q + 2S[P][D_T] - \{Q^2 - 4n[P][D_T]\}^{1/2}) \quad (5)$$

where:

$$Q = k(1 + S[P]) + n[P] + [D_T]$$

which can be used to obtain the slopes of the various plots involving extrapolation. For the two classical reciprocal plots:

$$\frac{dI}{d(1/[P])} = -[P]^2\frac{dI}{d[P]} \text{ and } \frac{d(1/I)}{d(1/[P])} = \frac{[P]^2}{I^2}\frac{dI}{d[P]} \quad (6a \text{ and } b)$$

where I is given by Eqn (5) and:

$$\frac{dI}{d[P]} = \frac{(\psi_B - \psi_F)}{2(1 + S[P])^2}\left[n + S[D_T] + \frac{\{Q(S[D_T] - n) + 2n[D_T](1 - S[P])\}}{(Q^2 - 4n[P][D_T])^{1/2}}\right].$$

The three inverse hyperbolic reciprocal plots tested are $\cosh^{-1}(I + 1)$ (the form $I + 1$ is used because $\cosh^{-1}I$ is meaningless when $I \leq 1$), $\sinh^{-1}(1/I)$ and $1/(\sinh^{-1}I)$ against $1/[P]$. The logarithmic forms of the inverse hyperbolic functions are:

$$\sinh^{-1}x = \log_e(x + (x^2 + 1)^{1/2})$$

$$\cosh^{-1}x = \log_e(x + (x^2 - 1)^{1/2}).$$

Thus the slopes of the three curves are:

$$\frac{d(\cosh^{-1}(I + 1))}{d(1/[P])} = \frac{-[P]^2}{(I^2 + 2I)^{1/2}}\frac{dI}{d[P]}. \quad (7a)$$

$$\frac{d(\sinh^{-1}(1/I))}{d(1/[P])} = \frac{[P]^2}{I(I^2 + 1)^{1/2}}\frac{dI}{d[P]}. \quad (7b)$$

$$\frac{d(1/\sinh^{-1}I)}{d(1/[P])} = \frac{[P]^2}{(I^2 + 1)^{1/2}(\log_e\{I + (I^2 + 1)^{1/2}\})^2} \times \frac{dI}{d[P]}. \quad (7c)$$

Once the intercepts on the ordinates of these three plots have been found they can be reconverted into fluorescence intensity units by:

$$I = \frac{1 + e^{2a}}{2e^a} - 1, I = \frac{2e^b}{e^{2b} - 1} \text{ and } I = \frac{e^{2/c} - 1}{2e^{1/c}}$$

where a, b and c are the intercepts for the curves described by Eqns (7a–c).

METHOD

The binding model for testing the various plots was that of warfarin binding by rat serum albumin. The known binding parameters are: $n = 1$, $k = 1.2\,\mu M$ and $S = 4.3 \times 10^4\,M^{-1}$ [3]. The intrinsic molar fluorescences of free and bound drug were predefined to be: $\psi_F = 0.1\,\mu M^{-1}$ and $\psi_B = 1.0\,\mu M^{-1}$. The total warfarin concentration was fixed at $1\,\mu M$; of the same order as the dissociation constant.

The total fluorescence intensities emitted by the warfarin for a range of albumin concentrations (1–25 μM) were calculated using Eqn (5). These intensities were then used to find the values of ψ_B which would be obtained if one assumed, at each different protein/drug molar ratio, that the direct logarithmic plot was close to its plateau or that the reciprocal plots could be considered linear from that point to the ordinate. In the latter cases, the value of the intercept was calculated by substituting the coordinates of the corresponding curve at that molar ratio and its slope there (given by Eqns (6a–7c)) into the equation for a straight line. The percentage error in each value of ψ_B, relative to the predefined value, for each protein/drug molar ratio on each curve was then found.

In order to ensure that the results were not specific to the binding system chosen the same calculations were carried out for drugs bound at lower and higher affinities by (human) albumin: the binding parameters for 7-anilinocoumarin-4-acetic acid (ACAA) [4] ($n = 1$, $k = 9.4\,\mu M$ and $S = 1.8 \times 10^4\,M^{-1}$) and for iophenoxate (IPX) [5] ($n = 1$, $k = 0.013\,\mu M$ and $S = 3.8 \times 10^5\,M^{-1}$) were used. For the sake of comparison, both these ligands were arbitrarily assigned the same fluorescence properties (ψ_F and ψ_B) as warfarin. The ligand concentrations were again of the same order as the dissociation constants (10 and $0.01\,\mu M$ for the lower and higher affinity systems, respectively). The molar excesses of albumin were the same as for the warfarin/albumin system.

Finally, all the calculations were repeated with (constant) drug concentrations higher and lower than

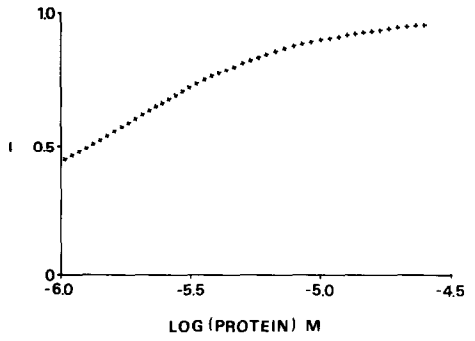

Fig. 1. The direct logarithmic plot of calculated fluorescence intensities emitted by warfarin (1 μM) in the presence of increasing albumin concentrations (1–25 μM).

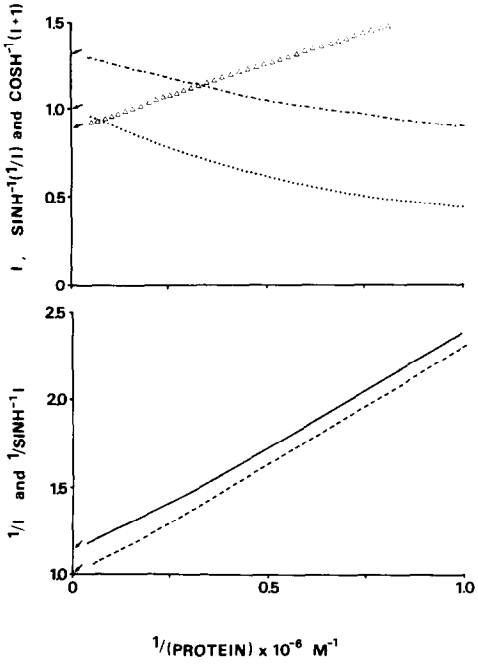

Fig. 2. Reciprocal plots of the calculated fluorescence intensities emitted by warfarin (1 μM) in the presence of increasing albumin concentrations (1–25 μM). Arrows indicate the intercepts corresponding to the predefined value of ψ_B. Single reciprocal plot (I vs $1/[P]$) (. . . .), double reciprocal plot ($1/I$ vs $1/[P]$) (----), inverse hyperbolic cosine reciprocal plot ($\cosh^{-1}(I + 1)$ vs $1/[P]$) (-·-·-·), inverse hyperbolic sine reciprocal plot ($\sinh^{-1}(1/I)$ vs $1/[P]$) (△△△△) and inverse hyperbolic sine double reciprocal plot ($1/\sinh^{-1}I$ vs $1/[P]$) (——).

the corresponding dissociation constant in order to see whether this affected the choice of graphical transformation.

RESULTS

The six plots of the calculated fluorescence intensities of warfarin are shown in Figs 1 and 2. None of these plots are linear. The percentage differences

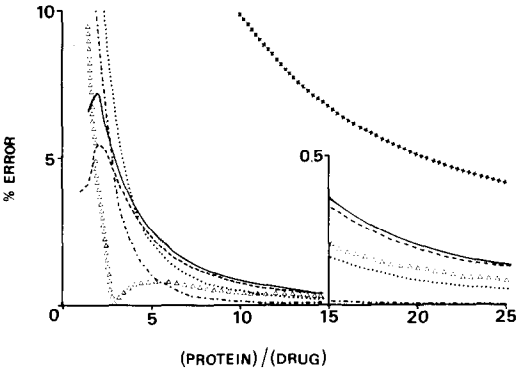

Fig. 3. Absolute percentage errors in the estimates of ψ_B read directly from the logarithmic plot or obtained by extrapolation of the reciprocal plots. Errors are shown as a function of the albumin:warfarin molar ratio at which estimates were obtained. A 10-fold scale expansion is shown for errors from reciprocal plots at albumin:warfarin ratios greater than 15:1. Logarithmic plot (I vs $\log_{10}[P]$) (+++++), single reciprocal plot (1 vs $1/[P]$) (. . . . . .), double reciprocal plot ($1/I$ vs $1/[P]$) (----), inverse hyperbolic cosine reciprocal plot ($\cosh^{-1}(I + 1)$ vs $1/[P]$) (-·-·-·), inverse hyperbolic sine reciprocal plot ($\sinh^{-1}(1/I)$ vs $1/[P]$) (△△△△) and inverse hyperbolic sine double reciprocal plot ($1/\sinh^{-1}I$ vs $1/[P]$) (——).

between the estimated values of ψ_B and the predefined value are shown in Fig. 3 as a function of the part of the corresponding binding curve extrapolated to zero. Only the absolute values of the errors are shown. While the direct logarithmic and single reciprocal plots gave underestimates of ψ_B, the double reciprocal plots of $1/I$ and $1/\sinh^{-1}I$ against $1/[P]$ gave overestimates. The plots of $\sinh^{-1}(1/I)$ and $\cosh^{-1}(I + 1)$ against $1/[P]$ gave underestimates when regions up to albumin:warfarin molar ratios of 3:1 and 20:1, respectively, were extrapolated and thence gave slight overestimates. Calculations for the higher and lower affinity binding systems showed that the molar ratios at which these inflexions occurred increased as the binding affinity increased.

Figure 3 shows that the direct reading from the logarithmic plot never gave errors less than 4% (i.e. fractional drug binding never exceeded 96%) up to an albumin:warfarin molar ratio of 25:1. For the lower and higher affinity systems these errors were 3 and 5%, respectively. For warfarin, at least a 100-fold molar excess (0.1 mM albumin) was required for this plot to give a value of ψ_B within 1% of the predefined value. Only in the case of the lower affinity system did non-specific binding contribute significantly to the fractional binding at high albumin concentrations: in its absence the minimum error on the logarithmic plot was increased from 3 to 4%.

With the classical reciprocal plots, the single reciprocal plot gave more accurate estimates of ψ_B than the double reciprocal plot except when regions at low albumin:warfarin molar ratios were extrapolated. Extrapolations of the plots of $\sinh^{-1}(1/I)$ and $1/\sinh^{-1}I$ against $1/[P]$ gave similar errors to those of the classical reciprocal plots but extrapolation of the plot of $\cosh^{-1}(I + 1)$ against $1/[P]$ gave the most

Table 1.

Plot	Drug	Protein/drug molar ratio giving ψ_B with error: <10%	<5%	<1%	<0.1%
I vs log[P]	ACAA	8	15	>25	—
	Warfarin	10	20	>25	—
	IPX	12	23	>25	—
I vs 1/[P]	ACAA	2	3	5	12
	Warfarin	3	4	7	19
	IPX	3	4	8	22
$1/I$ vs 1/[P]	ACAA	<1	2	6	22
	Warfarin	<1	3	9	>25
	IPX	2	3	10	>25
$1/\sinh^{-1}I$ vs 1/[P]	ACAA	<1	<1	6	19
	Warfarin	<1	4	9	>25
	IPX	2	2	10	>25
$\sinh^{-1}(1/I)$ vs 1/[P]	ACAA	<1	2	7	22
	Warfarin	2	2	3	22
	IPX	2	3	5	7
$\cosh^{-1}(I+1)$ vs 1/[P]	ACAA	2	2	4	11
	Warfarin	2	3	5	10
	IPX	3	4	7	15

Comparison of graphical procedures for estimating ψ_B in terms of the minimum molar ratio of protein to drug required to obtain an estimate within a given error range of the predefined value. Calculations are for albumin binding of warfarin and fluorescent drugs bound with the same affinities as 7-anilinocoumarin-4-acetic acid (ACAA) and iophenoxate (IPX).

accurate estimates of ψ_B over most of the range of protein concentrations tested. With the higher and lower affinity systems the errors were of the same order for the different plots. The essential features of Fig. 3 and the corresponding results with the other binding systems are shown in Table 1.

This order of usefulness of the plots did not, however, remain the same whatever the (constant) drug concentration. Figure 4 shows the minimum albumin concentrations giving data which, extrapolated on reciprocal plots, gave estimates of ψ_B within ± 1% of the predefined value as a function of the drug concentration. The inverse hyperbolic sine plot of $\sinh^{-1}(1/I)$ against 1/[P] is not included in this figure since it did not give better estimates than the other plots. As described above, the inverse hyperbolic cosine plot gave optimum results when the drug was at approximately the same concentration as its dissociation constant (k)—the optimum experimental situation. At lower drug concentrations ($<k/2$) the double reciprocal plot gave more accurate estimates and at higher concentrations ($>3k/2$) the single reciprocal plot was preferable. Similar results were found with the high affinity binding system (not shown). For the lower affinity system the plot of $1/\sinh^{-1}$ against 1/[P] gave better estimates of ψ_B than the double reciprocal plot at low drug concentrations and the inverse hyperbolic cosine plot remained optimum to higher drug concentrations ($<5k/2$) (Fig. 5). These differences corresponded to the significant presence of non-specific binding in this system; when this was set to zero the results were similar to those for the other two binding systems.

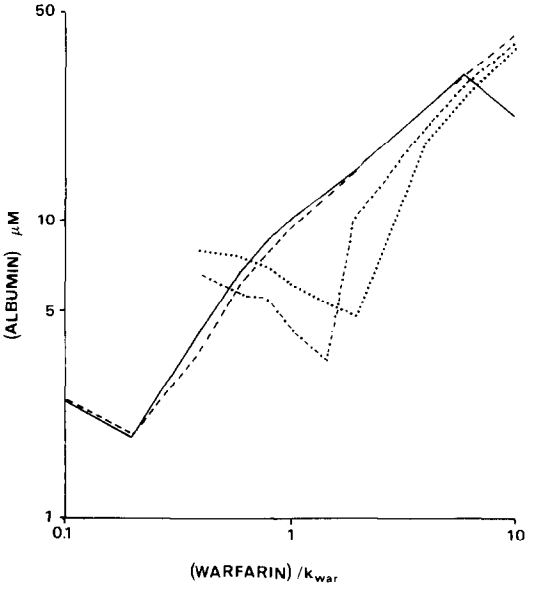

Fig. 4. The effect of the constant drug concentration on the minimum albumin concentrations giving data which, extrapolated to the ordinates of the reciprocal plots, yields estimates of ψ_B to within an error of ± 1% for the warfarin/albumin binding system. The warfarin concentration is given relative to its dissociation constant (k_{war} = 1.2 μM). Single reciprocal plot (I vs 1/[P]) (. . . . . .), double reciprocal plot ($1/I$ vs 1/[P]) (– – – –), inverse hyperbolic cosine reciprocal plot ($\cosh^{-1}(I+1)$ vs 1/[P]) (–·–·–·) and inverse hyperbolic sine double reciprocal plot ($1/\sinh^{-1}I$ vs 1/[P]) (——).

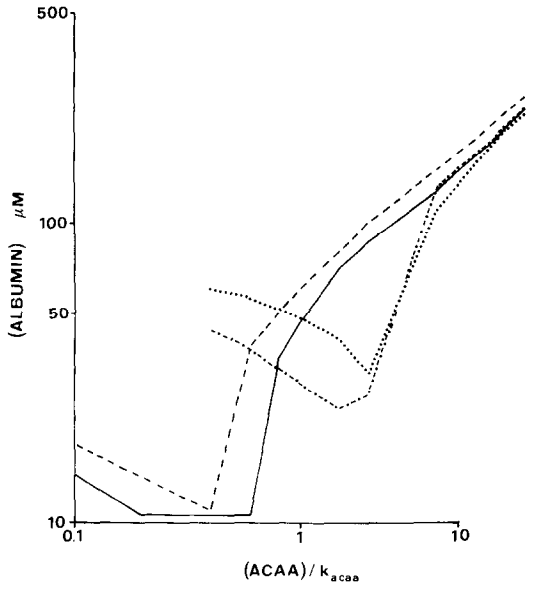

Fig. 5. The effect of the constant drug concentration on the minimum albumin concentrations giving data which, extrapolated to the ordinates of the reciprocal plots, yields estimates of ψ_B to within an error of ±1% for the low affinity binding system. The drug concentration is given relative to its dissociation constant for albumin binding ($k_{acaa} = 9.4\,\mu M$). Single reciprocal plot (I vs $1/[P]$) (. . . . . .), double reciprocal plot ($1/I$ vs $1/[P]$) (– – – –), inverse hyperbolic cosine reciprocal plot ($\cosh^{-1}(I + 1)$ vs $1/[P]$) (–·–·–) and inverse hyperbolic sine double reciprocal plot ($1/\sinh^{-1}I$ vs $1/[P]$) (——).

DISCUSSION

The method of investigation used here is analogous to that used by Zierler [6] in his study of the errors caused by the use of the double reciprocal plot. One major difference is that non-specific binding effects have been included in this study because, even though estimation of ψ_B involves the use of high molar excesses of binding protein, these effects may remain significant and because albumin has a number of secondary, low affinity sites for drugs [7] which may be assimilated to non-specific sites. The other major difference is that a wider range of classical and new graphical methods have been covered here.

One result of this investigation is that all the reciprocal plots gave more accurate estimates of ψ_B than the direct logarithmic plot. This does not agree with the conclusion reached by Panjehshahin *et al.* [2], though the true values of ψ_B remained unknown in their comparison with the double reciprocal plot. Our results show that Zierler's statement, that the intercept of the extrapolated double reciprocal plot will not give the true value of ψ_B unless the extrapolated data is on the plateau of the logarithmic plot; should not be taken to imply that the error due to extrapolation is greater than the distance of the data from the logarithmic plot's plateau (which is only ever reached asymptotically). Further, in Pajehshahin's study the quasi-linear part of the double reciprocal plot was used for extrapolation and not just the data at high protein concentration, as is normally the case. With the warfarin/albumin system the results here show that only points at protein:drug molar ratios of over 10:1 should be extrapolated if an error less than 1%, excluding experimental error, is required (the warfarin concentration being approximately the dissociation constant). To achieve this accuracy with the logarithmic plot, for which prolongation of the curvature to higher protein concentrations than used cannot be justified, would require albumin concentrations at which protein interference in fluorescence determinations may be excessive.

For the two classical reciprocal plots the results here show that, unless the drug concentration is much lower than the dissociation constant, the single reciprocal plot yields much more accurate estimates of ψ_B than the double reciprocal plot. If manual extrapolation is carried out this will be even more true when protein binding enhances drug fluorescence because of the scale contraction at higher fluorescence intensities on the double reciprocal plot. However, the corresponding scale expansion at lower intensities may prove useful if protein binding causes fluorescence quenching. Nevertheless intercept calculation by linear regression analysis is preferable to manual extrapolation, especially since the former also gives the standard deviation of the intercept.

Of the inverse hyperbolic plots, selected because visual inspection indicated that they might introduce linearity into the binding curves, none did so. Further, the inverse hyperbolic sine curve of $\sinh^{-1}(1/I)$ against $1/[P]$ did not improve the accuracy of ψ_B estimation except at very low molar excesses of albumin. On the other hand, as long as the constant drug concentration was optimal for binding studies, close to the dissociation constant, extrapolation of the plot of $\cosh^{-1}(I + 1)$ against $1/[P]$ gave significantly better estimates of ψ_B than any other method. This was found for all three binding systems tested and is thus independent of binding affinity. With this graphical transform the error was less than 1% when a region corresponding to only 80% drug binding was extrapolated. The increased accuracy of this plot may make its use worthwhile despite its increased complexity, particularly when large enough molar excesses of protein to give sufficient accuracy on the single reciprocal plot cannot be used because of poor protein solubility, protein fluorescence interference, self-absorption effects etc.

It remains, however, that the object of these experiments is usually to determine the dissociation constant of a binding system and not even an approximate value may be known prior to experimentation. Further, even if it is known, it may not be practical to use the drug at an equivalent concentration. On the one hand, drugs bound with high affinity may not fluoresce sufficiently at a low concentration and, on the other hand, the protein concentrations needed to determine ψ_B for drugs bound with low affinity and at the corresponding concentration may be such as to interfere with fluorescence determinations. Given the results shown in Figs 4 and 5, this means that the inverse hyperbolic cosine plot will not always be optimal. For drugs at concentrations higher than their dissociation constants the single reciprocal plot

will give more accurate estimates of ψ_B and for those at a low relative concentration the double reciprocal plot or, if the affinity is low enough for non-specific binding to be significant, the plot of $1/\sinh^{-1}I$ against $1/[P]$, will give better estimates. Moreover, for systems in which the protein interferes with fluorescence determinations, the fact that the latter plots need less protein to give estimates with the same accuracy as the inverse hyperbolic cosine plot providing the drug concentration is much lower than its dissociation constant may prove useful.

From these considerations a practical procedure for estimating ψ_B may be proposed for systems in which the binding affinity is not too low ($k <$ 0.1 mM). Experiments should be carried out with the lowest drug concentration compatible with its fluorescence in the presence of binding protein and the sensitivity of the fluorimeter. Then an approximate estimate of ψ_B can be obtained with, for example, the single reciprocal plot and this used to calculate an approximate dissociation constant. Comparison of the latter with the drug concentration will then permit selection of the appropriate reciprocal plot for obtaining the most accurate estimate of ψ_B.

While improving the accuracy of ψ_B estimation in fluorescence studies of drug binding, this selection of graphical transforms may be doubly important in fluorescence polarization studies. There, not only is ψ_B required to calculate the fractional drug binding but also the fluorescence polarization (or anisotropy) of protein-bound drug [8] which, although not instrument-dependent like ψ_B, must be estimated in a similar manner.

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