Comparison of Graphical and Computerized Methods for Calculating Binding Parameters for Two Strongly Bound Drugs to Human Serum Albumin

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Abstract □ The determination of drug–protein binding parameters (n's and K's) can lead to important information on the required therapeutic dosage regimen and possible clinical complications associated with competitive displacement of one drug by a concurrently administered agent. Graphical and computer estimates of the data are often incorrectly formulated, and seldom are adequate data obtained at low binding ratios. Commonly used graphical procedures, inadequately formulated computer methods, and a statistically correct computer method were used to compare results obtained from a circular dichroic examination of dicumarol–human serum albumin and fenoprofen–human serum albumin interactions. Literature binding constants for dicumarol–albumin range from 1×10^5 to 30 times that figure, and it is shown here that a wide range in parameter estimates may be obtained depending on the method of data analysis. The parameter estimates in the case of fenoprofen–albumin are even more variable.

Keyphrases □ Binding parameters—dicumarol and fenoprofen to human serum albumin, comparison of graphical and computerized methods of calculation □ Dicumarol—binding to human serum albumin, comparison of graphical and computerized methods of calculation of binding parameters □ Fenoprofen—binding to human serum albumin, comparison of graphical and computerized methods of calculation of binding parameters □ Albumin, human serum—binding of dicumarol and fenoprofen, comparison of graphical and computerized methods of calculation of binding parameters

The importance of the determination of small molecule–macromolecule binding parameters is seen from the voluminous literature on the subject (1–5). The determination of numbers of the binding sites (n) and the corresponding association constants (K) led to information concerning the conformational nature of the active sites of enzymes, the nature of the binding forces, and possible clinical complications associated with competitive displacement of one drug by a concurrently administered second drug.

Protein binding data can be included in the pharmacokinetic compartmentalized description of a drug's distribution and action in the body (6, 7) and, consequently, used to determine therapeutic dosage regimens.

Most work done to elucidate binding parameters (n's and K's) for drug–albumin or ligand–macromolecule interactions has used graphical representation of the data. However, several shortcomings exist with this approach:

1. Measured quantities, *i.e.*, terms with significant errors, are almost always plotted against each other.
2. The slopes and intercepts are frequently incorrectly designated and represented mathematically.
3. Low binding ratios (*i.e.*, small r values) are seldom used, causing hazardous extrapolation to the various axes.
4. More weight is often (unknowingly) given to data obtained at high binding ratios, thus negating the possibility for clinically significant primary binding constants to be obtained.

Some of these obvious deficiencies were discussed recently (8, 9).

Computerized treatment of binding data also has become prevalent in recent years; however, it suffers from some of the same drawbacks as graphical representations. Frequently the data existing in the Scatchard or Klotz (double-reciprocal) format, which is subject to the plotting of error-containing terms against one another, are simply programmed into a computer to give the best curve through the points. This approach gives few improvements over the graphical method of determination by eye.

An attempt is made here to unify some graphical and computer methods for the analysis of binding data; often the relationship of graphical parameters to binding constants is not what seems to be taken for granted. Recently, a new computerized treatment of drug–albumin binding data for a noncooperative process appeared (10), and this method is used to compare the results for dicumarol–human serum albumin and fenoprofen–albumin interactions with the more standard graphical and computer techniques.

EXPERIMENTAL

Graphical Procedures—It has long been known that the primary step in a pharmacological action is the combination of one or several small molecules with a macromolecule. The equilibrium aspects of such interactions have been correlated through the mass law to yield the familiar equation:

$$r = \frac{nK[A]}{1 + K[A]} \qquad \text{(Eq. 1)}$$

where r represents the moles of ligand bound per mole of macromolecule, n is the number of similar binding sites available on the macromolecule, K is the association (binding) constant, and $[A]$ is the concentration of unbound (free) ligand. Equation 1 applies when the binding site is a single site or is a single class of sites with the assumed same intrinsic binding constant. When multiple classes of sites exist on the protein, the representation becomes:

$$r = \sum_{i=1}^{m} \frac{n_i K_i [A]}{1 + K_i [A]} \qquad \text{(Eq. 2)}$$

where m represents the number of classes of independent sites such that each class, i, has n_i sites with binding affinity K_i.

Scatchard (11), Klotz (12), and Scott (13) have all been responsible for transformation of these equations to equations suitable for graphical representation of binding data. The Scatchard plot results from graphing the data in the form:

$$\frac{r}{A} = Kn - Kr \qquad \text{(Eq. 3)}$$

The Klotz or double-reciprocal plot results from a representation given as:

$$\frac{1}{r} = \frac{1}{nK}\frac{1}{A} + \frac{1}{n} \qquad \text{(Eq. 4)}$$

and the Scott or half-reciprocal plot is given by:

$$\frac{A}{r} = \frac{1}{n}A + \frac{1}{nK} \qquad \text{(Eq. 5)}$$

All of these plots are linear equations and yield straight lines when only one single class of site exists on the macromolecule; however, when multiple classes are present, marked curvature of the plot is observed. Since one class of sites existing for the small molecule–macromolecule interaction occurs only rarely and since such data are easily handled, giving unequivocal values of n and K in this instance, only the determination of binding parameters when two or more classes of sites are present is considered here.

To obtain values for n and K from a set of binding data, appropriate extrapolations to the axes must be made for all plots according to Eqs. 3–5. However, such extrapolations frequently tend to be hazardous since, as in the case of the Scatchard and Klotz plots, r/A or $1/A$ approach zero as A gets very large; in the Scott plot, the intercept A/r when A is very small is a difficult experimental quantity to approach since r is small when A is small. When such circumstances exist, the error in r increases greatly since r is usually obtained from the difference between the initial concentration of A and that of free A, in most cases the difference between two large numbers.

Both the double-reciprocal and Scott representations of binding data suffer from the fact that an inverse function of A is being used, which results in a great compression of the concentration range as the concentration of A is increased. In practice, experimental data covering a range of A more than 10-fold is uncommon. Therefore, uncertainty exists as to whether a linear representation is exhibited with larger ranges of A values. Extrapolations to various intercepts are usually quite uncertain unless broad ranges in concentration are used or unless saturation of the protein is approached. Moreover, since r occurs on both the ordinate and abscissa of Scatchard plots and A occurs on both the ordinate and abscissa of the Scott representation, extrapolations should be viewed with some skepsis.

The graphical representations of the Klotz, Scatchard, and Scott plots are shown in Fig. 1 when multiple independent classes of sites exist on the macromolecule. In Table I, the limiting slope and intercept values are given for the situation in which two independent binding classes are displayed by the protein, each having one or more sites involved (14, 15). In the case of the much abused Scatchard plot, it can be seen from the table that slope 1 and intercept 3 are not equivalent to the frequently assumed values of K_1 and n_1, respectively. The situation is somewhat improved if K_1 is much larger than K_2, in which case the equations simplify to K_1 and n_1, respectively. If the binding constant of the second class of sites is not separated from the first by at least a power of 10, or if the number of binding sites in the second class far outweighs the number in the first, then, at best, only a hazardous prediction of the parameters can be made. The commonly used values for the slopes and intercepts are shown in parentheses in Table I.

Other graphical treatments have been suggested (16–19) but, in all instances, error terms are again plotted against one another. With the semilogarithmic plot (r *versus* log A), one can quite reliably determine the total number of sites on the macromolecule provided that saturation can be reached (19). Although the total number of sites (Σn_i) displayed by a protein for a particular ligand may be of interest from a physical standpoint, it is not an important quantity clinically or in pharmacokinetics. In other graphical methods, the fraction bound (usually given the symbol β) is used on the ordinate axis; this approach is fruitful when more than one binding protein exists in the mixture (16) but is of little value in the determination of quantitative binding constants.

Computer Procedures—Computerized treatment of protein binding data results in parameter fitting procedures generally regarded as more quantitative than graphical techniques. However, most of these analyses determine the parameters through a least-squares fit of the data based on the Scatchard model. In effect then, minimization of the square of the deviations from the "best" curve is carried out on data plotted in such a manner that substantial errors exist in both the ordinate and abscissa.

Fletcher and coworkers (20–22) described a computer technique designed to determine stepwise association constants in ligand–macromolecule interactions which may undergo cooperative binding and conformational changes. Their model is not constrained in that sites do not have to bind independently. Again, however, a least-squares fit of the data in the Scatchard model is used.

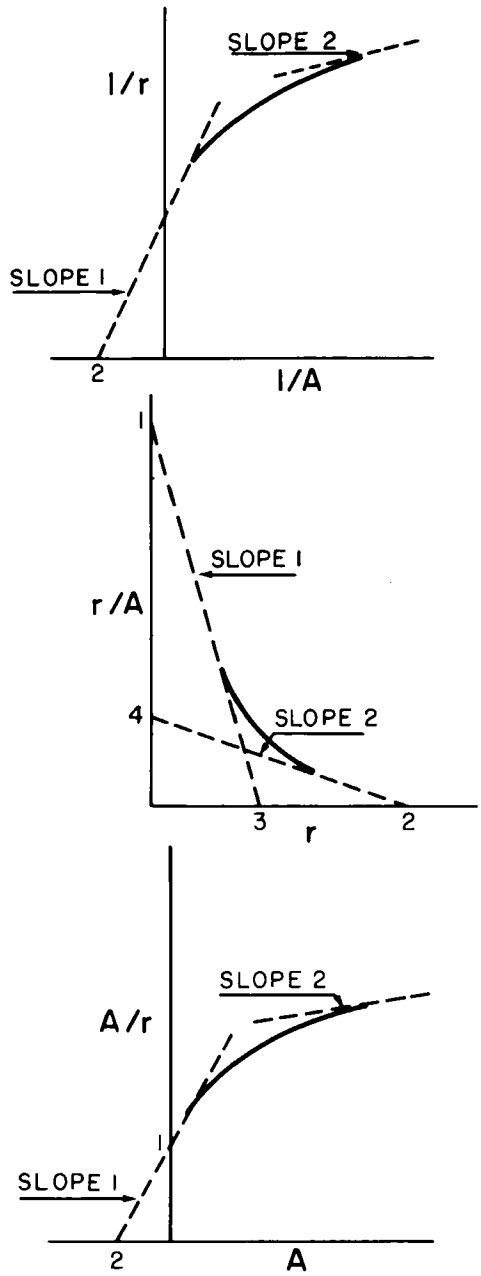

Figure 1—*Graphical representation of the Klotz, Scatchard, and Scott plots (in descending order) when multiple independent classes of sites exist on the macromolecule.*

Other computerized methods have been suggested (23–25), and parameter estimations are also based on either the minimization of Σ (errors)^2, which corresponds to the assumption that the free concentration is measured accurately compared to the bound concentration, or the assumption that errors in the bound concentration are not correlated with the free. When r/A is plotted against r (Scatchard plot), the assumption is adopted that the errors in r/A are much larger than the errors in r and independent of r.

Recently, a new method of binding data analysis appeared which circumvents the biased results obtained with other techniques (10). This method is based on a number of well-known statistical assumptions (26), the violation of which can cause serious errors in the parameter values estimated.

DISCUSSION

To illustrate more clearly some problems involved with the various procedures for handling drug binding data, the results of a dicumarol–human serum albumin and fenoprofen–albumin study will be

Table I—Values for Limiting Slopes and Intercepts when Two Independent Binding Classes, Each with One or More Sites, Exist on the Macromolecule[a]

Plot	Slope 1	Slope 2	Intercept 1	Intercept 2	Intercept 3	Intercept 4
$1/r$ *versus* $1/A$ (Klotz plot)	$\frac{\frac{n_1}{K_1}+\frac{n_2}{K_2}}{(n_1+n_2)^2}$	$\frac{1}{n_1K_1+n_2K_2}\left(\frac{1}{n_1K_1}\right)$	$\frac{1}{n_1+n_2}$	$\frac{2K_1K_2}{K_1+K_2}$	—	—
r/A *versus* r (Scatchard plot)	$\frac{n_1K_1^2+n_2K_2^2}{n_1K_1+n_2K_2}(-K_1)$	$\frac{n_1+n_2}{\frac{n_1}{K_1}+\frac{n_2}{K_2}}(-K_2)$	$n_1K_1+n_2K_2(n_1K_1)$	n_1+n_2	$\frac{(n_1K_1+n_2K_2)^2}{n_1K_1^2+n_2K_2^2}(n_1)$	$\frac{(n_1+n_2)^2}{\frac{n_2}{K_2}+\frac{n_2}{K_2}}$
A/r *versus* A (Scott plot)	$\frac{n_1K_1^2+n_2K_2^2}{(n_1K_1+n_2K_2)^2}\left(\frac{1}{n_1}\right)$	$\frac{1}{n_1+n_2}$	$\frac{1}{n_1K_1+n_2K_2}\left(\frac{1}{n_1K_1}\right)$	$\frac{n_1K_1+n_2K_2}{n_1K_1^2+n_2K_2^2}\left(\frac{1}{K_1}\right)$	—	—

[a] Values in parentheses result when K_1 is much larger than K_2 and n_2 is of the same order as n_1.

Table II—Summary of Literature Binding Affinities and Number of Sites for the Dicumarol–Albumin Interaction

Reference	n_1	K_1	n_2	K_2
23	2.1	$2.1 \times 10^6\ M^{-1}$	7.3	$1.4 \times 10^4\ M^{-1}$
27	1.0	$2.9 \pm 10\% \times 10^6\ M^{-1}$	1.0	$1.8 \pm 5\% \times 10^5\ M^{-1}$
28	2	$1.0 \times 10^5\ M^{-1}$	—	—
29	1	$2 \times 10^5\ M^{-1}$	—	—
30	3	$2–5.2 \times 10^5\ M^{-1}$	—	—

examined (15, 27). The dicumarol–albumin data presented (27) are considered precise quantitative information, so the variation in binding parameters, depending on the method of analysis, should not be overly significant. However, the fenoprofen–albumin interaction has led to less precise data (15). Since many techniques for studying drug–albumin interactions may lead to even less precision and quality of data, the variation in the parameters estimated can be of an extremely significant magnitude.

Dicumarol–Albumin Interaction—Table II summarizes literature binding parameter results for the anticoagulant–albumin interaction. These results indicate that various inadequacies must exist in either the experimental design or the compilation of data. A possibility for the results for K_1 being in the 10^5 range is that most experimental techniques used to measure free and bound drugs do not allow the investigator to obtain results at low binding ratios (*i.e.*, $r < 1$), particularly with this drug of very limited solubility. Therefore, obtaining a clinically significant binding constant becomes hazardous because the tangent (Scatchard plot, slope 1) drawn is usually less steep, giving a smaller binding constant, and intersects the r axis at larger r values, giving a greater number of equivalent binding sites than exists.

Examination of the data of Ref. 27 by the three graphical methods (invoking the assumptions that $K_1 > K_2$ and $n_1 \approx n_2$ allows these parameters to be obtained) and the newly developed computer technique (10) as well as the common Σ (errors)^2 gives the results shown in Figs. 2–5 and Table III. These data were considered to be accurate and reproducible and contained 66 data points obtained by a circular dichroic technique, which allowed for the accumulation of a large amount of data at low binding ratios. The accumulation of sufficient data at low binding ratios (*i.e.*, r values <1) is extremely important to the determination of a primary binding constant having clinical and pharmacokinetic application. Only these low ratios are usually reached with therapeutic dosages of a drug.

Figure 2 presents the Klotz plot, and it can be determined that two binding sites exist. Heterogeneity in the binding process is clearly indicated, so logic implies two classes of binding sites, each containing a single site. Making this assumption allows the binding constants for each class to be determined (Table III). The data show that slope 2 can, at best, be drawn only with large inaccuracy, and the calculation of binding parameters from it would be meaningless. The value of K_1 from the Klotz plot is approximately 50% higher than the value estimated by the newly developed computer technique; however, such discrepancies are common and inherent to binding analyses.

Figure 3 contains the data plotted in the Scatchard format. The

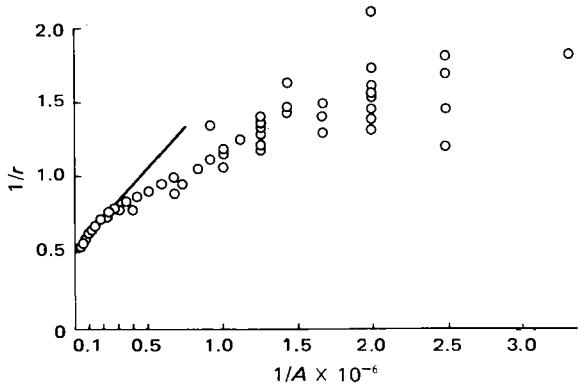

Figure 2—*Klotz (double-reciprocal) plot for the dicumarol–albumin interaction. (More points exist at low 1/A values than can be shown in the figure.)*

Table III—Dicumarol–Albumin Data[a]

Parameter	Graphical Value	Parameter Estimates			
		K_1	n_1	K_2	n_2
1/*r versus* 1/*A* (Klotz)					
Intercept 1	0.50				
Slope 1	1.14×10^{-6}				
Intercept 2	-4.38×10^{5}	5.4×10^{6}	—[b]	2.3×10^{5}	—[b]
Slope 2	1.79×10^{-7}				
r/A versus r (Scatchard)					
Intercept 1	3.36×10^{6}				
Slope 1	-3.30×10^{6}				
Intercept 3	1.00				
Intercept 4	4.37×10^{5}	3.61×10^{6}	1.0	4.1×10^{5}	1.0
Slope 2	-2.15×10^{5}				
Intercept 2	2.00				
A/r versus A (Scott)					
Intercept 1	1.11×10^{-7}				
Slope 1	1.12				
Intercept 2	-0.99×10^{-7}	4.45×10^{6}	—[c]	3.1×10^{5}	—[c]
Slope 2	—				
Σ(errors)^2 (computer)		1.4×10^{6}	0.53	3.3×10^{5}	1.45
New computer (Ref. 10)		$2.9 \pm (10\%) \times 10^{6}$	1.0	$1.8 \pm (5\%) \times 10^{5}$	1.0

[a] Values are for the slopes and intercepts of the three graphical procedures for binding estimation as well as the common and newly developed (10) computer techniques. Parameter estimates are given from all five techniques using the data from Ref. 27. [b] It was determined unequivocally that two classes of sites existed, but the number in each cannot be determined by this method. [c] Separation of terms in order to determine n_1 and n_2 is not possible with this method.

slopes and intercept values are presented in Table III. Good agreement for the numbers of sites present in each of the two classes from the Scatchard graphical procedure and the new computer treatment is seen. Moreover, the primary binding affinity is in excellent agreement with that of the unbiased computer method. It should be recalled, however, that these data for the dicumarol–albumin data are of a high quality, in direct contrast to those to be presented for the fenoprofen–albumin interaction.

Figure 4 represents the low free dicumarol data plotted in the manner of Scott. In common with the Klotz procedure, the n's cannot be determined; slope 2 is at best only grossly obtained.

Figure 5 presents the data plotted in the Scatchard manner but analyzed according to the newly developed computer technique (10, 27). The solid line in the figure is the result of holding $n_1 = n_2 = 1$ in the program; the dashed line allows n_1 and n_2, as well as K_1 and K_2, to be free floating. As can be seen from the figure, little difference results from fixed or free-floating n values, probably due to the quality of the data in this instance.

As seen from Table III, the results for K_1 and K_2 contain 95% confidence intervals, a feature of few other methods of binding analysis. Weights were incorporated in the program to allow for differing regions of error in the circular dichroic method. The fact that most methods of measuring free and bound ligand concentrations vary widely over the amount of ligand present in the system makes this an attractive inclusion in the minimization. Consequently, the parameters were minimized using the weighted form (26):

$$U = \sum_k W_k (G/G^1)^2 \qquad \text{(Eq. 6)}$$

The weights are inversely proportional to the variance of $Y_{obs,k}$. That is, when little drug exists as free drug or as saturation of the protein is approached, differences in the accuracy of measurement are inherent in almost all methods. In this case, relative error (*i.e.*, weight) of R_k is 4% if $0.9 \leq C_k \leq 1.3$, 11% if $C_k < 0.8$ or $1.5 < C_k$, and 7% if $0.8 \leq C_k < 0.9$ or $1.3 < C_k \leq 1.5$. For the 66 data points of the dicumarol–albumin study, the residuals ranged from -0.15×10^{-7} to 0.45×10^{-8} when the method of Ref. 10 was employed.

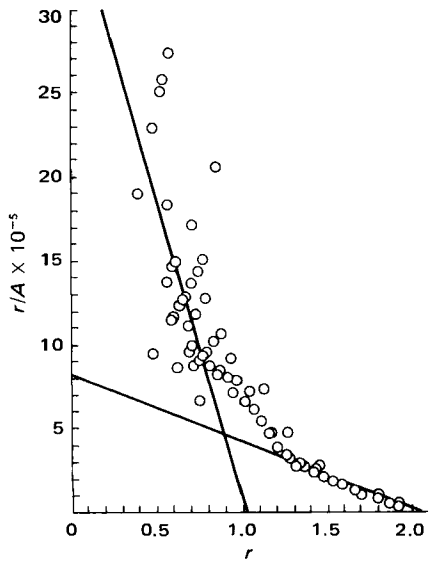

Figure 3—*Scatchard plot of the 66 data points obtained for the dicumarol–albumin interaction. Good agreement is seen between the graphical procedure and the newly developed computer method.*

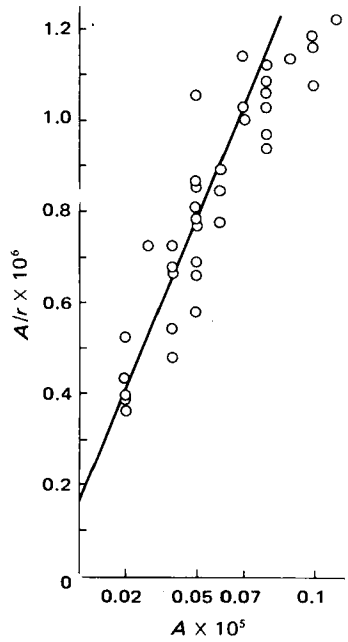

Figure 4—*Scott (half-reciprocal) plot of the data at low concentrations of unbound dicumarol (i.e., slope 1 of the Scott plot). When more free drug is present, the points become more separated and do not allow for slope 2 to be drawn with any degree of accuracy.*

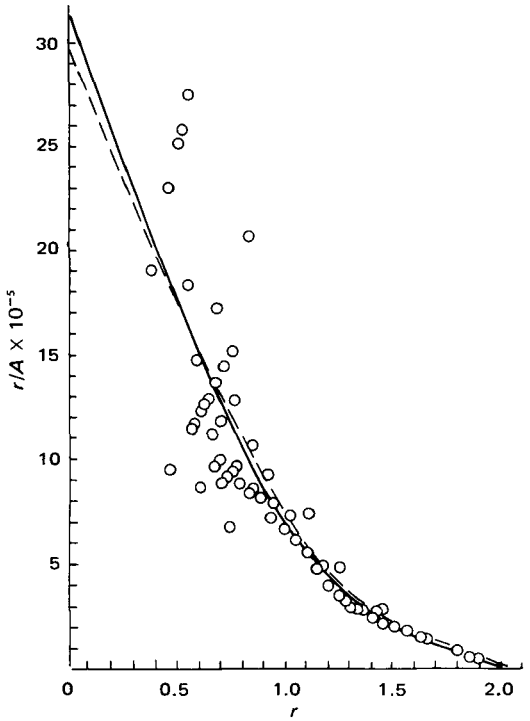

Figure 5—*Data analyzed according to the newly developed computer technique (10) and the resultant estimates for* n_1, n_2, K_1, *and* K_2 *represented in the Scatchard manner. (See text for explanation of solid and dashed curves.)*

Table III also contains parameter estimates for n_1, n_2, K_1, and K_2 of the dicumarol–albumin interaction based on minimization of the sum of squares of the errors, a common technique of computerized analyses. This procedure gives values for K_1 and n_1 far different from graphical analysis or the newly developed computer method. Perhaps the implied assumptions in such a technique, that the free concentration is measured accurately compared to the bound concentration and that the errors in the bound concentration are not correlated with the free (10), are somewhat less than adequate.

Fenoprofen–Albumin Interaction—Fenoprofen[1] [2-(3-phenoxyphenyl)propionic acid] is a nonsteroidal anti-inflammatory and analgesic drug. It has been shown to exhibit extrinsic optical activity following the binding to protein (31). Again the circular dichroic technique was chosen to assess the binding constants and numbers of sites displayed by albumin for this drug (15).

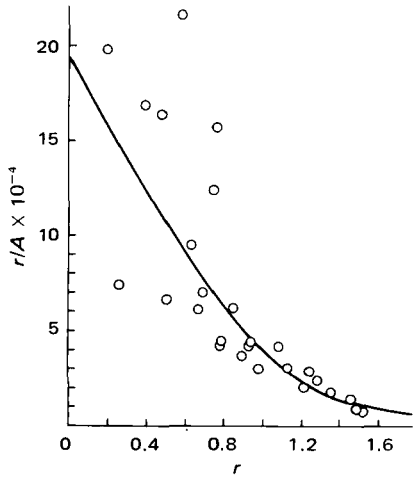

Figure 6—*Data for the fenoprofen–albumin interaction plotted according to the Scatchard technique, showing that considerable variability is present. The curve drawn is the result of the parameter estimates obtained according to the newly developed computer technique. If slopes 1 and 2 were drawn through the data, it would be expected that slope 1 would be much greater, resulting in an overestimated primary binding constant.*

[1] Eli Lilly and Co., Indianapolis, Ind.

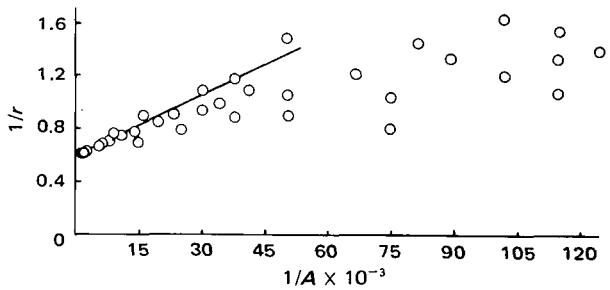

Figure 7—*Klotz plot of the fenoprofen–albumin data.*

Considerable variation in the fenoprofen binding data existed because of low signal-to-noise ratios (approximately 10:1) with this drug; it exhibited a rather small circular dichroic signal and had a relatively high absorbance. The circular dichroic peak was at 282 nm, a wavelength at which albumin has significant absorption, making quantitative measurements more difficult. Nevertheless, considerable data were obtained at low r values, certainly more than are possible with other more classical techniques. Overall, a total of 43 data points was obtained. However, the quality of the data and the reproducibility were not nearly so great as with the dicumarol data.

Table IV presents the results of the fenoprofen–albumin interactions when the graphical procedures and the unbiased computer techniques were used for data analysis. The graphical procedures (Figs. 6–8) overemphasize somewhat the slope used in the determination of the K_1 value; this fact can be seen readily upon examination of the Scatchard results in that the best slope through the points (using a linear least-squares fitting procedure) results in a large value for K_1 and an n_1 of 0.68. The significance of two-thirds of a binding site is somewhat obscure.

In applying the new computer technique, the values of all parameters (n_1, K_1, n_2, and K_2) were allowed to be free floating. Furthermore, in an attempt to assess the validity of the program to arrive at sensible estimates, the values for n_1 and n_2 were constrained to certain values. The results in Table IV for the computer technique are given when $n_1 = 1$ and $n_2 = 1$, which seemingly were the best estimates obtainable. In constraining n_1 from values of 0.6–2.0 (by increments of 0.1) while fixing n_2 at 1.0, it was observed that K_2 was negligible if K_1 was estimated with n_1 greater than 1.0. Also, when n_1 was greater than 1.0, the standard error for the K_1 value was high and the variance was large.

The estimates of K_1 and K_2 when $n_1 = 1$ and $n_2 = 1$ seem to make sense both from the standpoint of sensibility of the K_2 value (the Scatchard plot was curved, indicating a heterogeneity in binding sites so the data were analyzed on the basis of two classes of sites) and the standard errors for each value. Lending credibility to the likelihood of the correctness of $n_1 = 1$ and $n_2 = 1$ are the parameter estimates when no constraints are imposed on the system: $n_1 = 0.98 \pm 0.02$, $K_1 = 2.12 \pm 0.29 \times 10^5$, $n_2 = 0.81 \pm 0.11$, and $K_2 = 8.24 \pm 1.24 \times 10^3$.

Figure 6 presents some data points plotted according to the Scatchard procedure. The line through these points is the result obtained

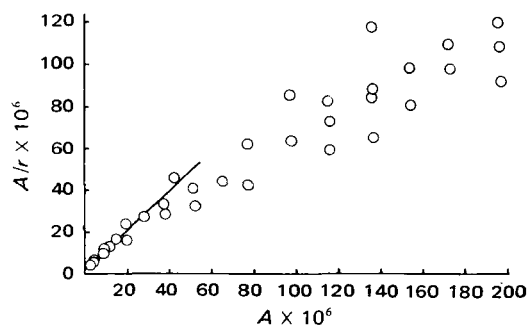

Figure 8—*Scott plot of the fenoprofen–albumin data.*

Table IV—Binding Parameters for the Fenoprofen–Albumin Interaction

Method of Analysis	K_1	K_2	n_1	n_2
Klotz	7.5×10^6	3.3×10^4	—	—
Scott	3.0×10^6	3.5×10^4	—	—
Scatchard	6.3×10^6	6.9×10^4	0.68	1.70
New computer	$1.86 \pm 0.21 \times 10^5$	$7.94 \pm 1.03 \times 10^3$	1.0	1.0

by the unbiased computer method ($K_1 = 1.8 \times 10^5$ and $K_2 = 7.94 \times 10^3$); the residuals ranged from -0.31×10^{-7} to 0.62×10^{-6}. It is readily apparent that had the data been analyzed by any of the graphical procedures alone, a much larger primary binding constant would have resulted.

Seemingly a good estimate of K_1 for the fenoprofen–albumin interaction was obtained. Supporting evidence for the assignment arises when one considers the only other work published (to date) measuring the binding of fenoprofen to albumin (32). In this work, the investigators stated that concentrations of 40 μg of fenoprofen/ml (which implies a 1.515×10^{-4} *M* solution) and 4.9 μg of albumin/ml (which is 7.10×10^{-5} *M*) were used in equilibrium dialysis experiments at pH 7.4. It was stated that 99% of the drug was bound at these concentrations and that the affinity constant was 3×10^4 at a saturation (total *n*) level of 4–5 moles of fenoprofen/mole of albumin. If one assumes that the binding affinity of all these sites is equal and applies simple mass law analysis, an affinity constant greater than 10^5 is obtained.

In the same work, data were presented on attempts to displace some drug from its albumin binding sites; however, in ratios greater than 3:1 of competitor to fenoprofen, various competitors were unable to act as antagonists. However, when phenylbutazone was used in a 10-fold excess [$K_1 = 2.37 \times 10^5$ (33)], it was able to displace fenoprofen. These data imply that either competitors do not compete for the same sites as fenoprofen or that fenoprofen has a binding constant greater than 3×10^4. It seems likely, therefore, that fenoprofen has a binding affinity for albumin greater than 10^5.

CONCLUSION

Obvious deficiencies are inherent in the various graphical methods of analyzing binding data. Computer techniques also exist; however, most fall into the same type of pitfalls; *i.e.*, the analysis is based on minimizing the error in terms of measured (error-containing) quantities. More statistically and, therefore, more clinically correct binding parameters may be obtained if one represents the problem in terms of independent and dependent variables. It can be seen from these analyses that merely fitting the data may result in parameter estimates widely different than those in which the situation is correctly treated by known statistical methods. It is also extremely important to have a fair number of data at low binding ratios ($r < 1$) if drug binding parameters with clinical significance are to be obtained.

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