J. Pharm. Pharmac., 1978, 30, 69–74 Received August 16, 1977

Problems associated with analysis and interpretation of small molecule/macromolecule binding data

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In the analysis of binding data, arbitrary transformations such as the Scatchard plot, may give misleading estimates of the binding parameters. The statistically correct approach is to determine values of K and n by non-linear regression of the actual dependent variable against the actual independent variable. In the case of the spectrophotometric titration method the dependent variable is the absorbance and the independent variable is the composition of the drug/macromolecule mixture. The method relies on an accurate estimate of the extinction coefficient of the bound drug and this is best treated as a parameter to be estimated in the regression analysis. In testing models by data fits alone it is emphasized that whilst a model may be rejected if it does not fit the data, a good fit does not ensure uniqueness and confirmatory, independent evidence must be sought.

For an interaction of the following type:

Drug + Macromolecule ⇌ Drug/Macromolecule

the concentration of the bound drug may be related to the concentration of unbound drug by the Law of Mass Action:

$$K = \frac{r}{c(n-r)} \quad .. \quad .. \quad (1)$$

where K is the association (or affinity) constant; r is the concentration of bound drug/concentration of macromolecule; c is the concentration of unbound drug; n is the number of binding sites for drug per macromolecule.

To determine K and n, a study of the degree of binding at various ratios of total drug to macromolecule is performed. The total concentration of drug (bound + unbound) and concentration of macromolecule are known for each sample of the drug/macromolecule mixture and it is usual to determine the concentration of unbound drug (c). The value r can then be calculated. In order to calculate K and n, equation (1) is usually rearranged to a linear form, most commonly using the method of Scatchard (Scatchard, 1949):

$$r/c = -Kr + Kn \quad .. \quad (2)$$

The Scatchard plot of r/c *vs* r gives a line of slope −K and an intercept Kn on the ordinate. A linear regression analysis is then performed to determine the slope of the line and the intercept. The advantage of a plot of this nature is that it gives a visual representation of the results. However, curvature is often found since the plot will only be linear if there is a single type of non-interacting site. Curvature in a Scatchard plot is difficult to interpret and any interpretation is dependent on the method of analysis used (Weder, Schildknecht & others, 1974). A second and major objection to the Scatchard plot is that even if a straight line is found, the method is still inappropriate since the conditions for use of linear regression analysis are not fulfilled (Draper & Smith, 1966). If y is regressed against x then the error in the variable x should be much less than that in y and the error about y should be normally distributed and independent of x and y. Clearly it is statistically incorrect to use a linear regression on the Scatchard plot of r/c *vs* r since r and c are both functions of the same variable, namely bound drug concentration. This criticism also applies to the plot of c/r *vs* c (Klotz & Urquhart, 1949) and to the double reciprocal plot (1/r *vs* 1/c) commonly employed in enzyme kinetics (Lineweaver & Burk, 1934). The limitations of linearization of binding data have in fact long been recognized in the field of enzyme kinetics (Wilkinson, 1961) where for example the non-parametric direct linear plot (Cornish-Bowden & Eisenthal, 1974) has been shown to give more reliable parameter estimates than linear transformations of the data (Atkins & Nimmo, 1975). Similarly, criticisms can also be made of the non-linear double log plot (log r *vs* log c: Thompson & Klotz, 1971) and semi-log plot (r *vs* log c: Bjerrum, 1941) and of the step-wise model (Fletcher, Ashbrook & Spector, 1973; Klotz & Hunston, 1975). The correct way to analyse binding data is to derive the constants K and n directly from the binding isotherm. However, although methods are available for non-linear regression of binding isotherms (Perrin, Vallner & Wold, 1974; Madsen & Robertson, 1974)

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the Scatchard plot and other similar transformations are still commonly used.

In this paper we examine some of the errors involved in using the Scatchard plot and advocate the use of a non-linear regression method. The interaction of daunomycin with DNA has been chosen as the example since the nature of this interaction can be predicted with confidence (Di Marco & Arcamone, 1975). At low ratios of daunomycin to DNA, the drug intercalates into the helix (Pigram, Fuller & Hamilton, 1972) then, as the ratio of drug to DNA increases, additional drug molecules bind to the exterior of the helix (Zunino, Gambetta & others, 1972). We have attempted to negate this latter mode of binding by carrying out the studies in buffer containing 0·3 M sodium chloride. Under these conditions there should be a single intercalative mode of binding (Zunino, 1971).

MATERIALS AND METHODS

A spectrophotometric titration was performed by sequential addition of a solution of DNA in 0·008 M tris-Cl, 0·3 M NaCl buffer at pH 7·0 to 3 × 3 ml aliquots of a solution of daunomycin hydrochloride ($5{\cdot}833 \times 10^{-5}$ M) in the same buffer, at room temperature (20°). After allowing time for equilibration the absorbance was measured at 480 nm (the λmax of unbound daunomycin) after each addition of DNA. The apparent extinction was calculated allowing for the change in volume. The DNA solution used was $2{\cdot}538 \times 10^{-3}$ M Sigma Type I calf thymus DNA, assayed at 260 nm using the figure $\epsilon(P)_{260} = 6600$. The protocol for the sequential addition of this DNA solution was as follows: 2 aliquots each of 40 μl, 16 aliquots each of 20 μl, 7 aliquots each of 100 μl; giving a total addition of 1·10 ml to each 3 ml sample of drug solution, in 25 aliquots.

RESULTS

The data have been evaluated by the Scatchard plot, by non-linear regression of r *vs* c and by non-linear regression of the fraction of drug bound *vs* the cumulative volume of DNA added. It will be shown that only the latter method of interpretation is valid.

Scatchard plot

In order to evaluate data by the Scatchard plot, r/c and r must be calculated. On binding to DNA daunomycin shows a bathochromic shift and a decrease in extinction, and an isosbestic point is observed (Di Marco & Arcamone, 1975). The fraction of drug bound after each addition of DNA can therefore be calculated using equation (3) (Peacocke & Skerret, 1956).

$$\alpha = \frac{\epsilon_f - \epsilon_{obs}}{\epsilon_f - \epsilon_b} \quad \cdot\cdot \quad \cdot\cdot \quad (3)$$

where ϵ_f is the extinction of the unbound (free) drug, ϵ_{obs} is the apparent extinction and ϵ_b is the extincton of the drug when bound to DNA. Knowing α, r/c and r can now be calculated since c is equal to $(1-\alpha)$Ct and r is equal to αCt/DNAP, where Ct is the total concentration of drug and DNAP is the concentration of DNA expressed in terms of phosphorus. The parameters K_1 and n_1 were calculated by linear regression of the initial linear segment of the Scatchard plot of r/c *vs* r. These values of K_1 and n_1 are given in Table 1.

It is evident that there must be a reliable estimate of ϵ_b, and several methods are available (Bontemps & Fredericq, 1974). In the present study ϵ_b was found from a plot of ϵ_{obs} *vs* DNAP/Ct (Double & Brown, 1975). As DNAP/Ct increases ϵ_{obs} decreases until the drug is fully bound; the plot then plateaus and ϵ_b was found to be $6{\cdot}172 \times 10^3$ by linear regression in this region. By averaging the initial values for the three samples (containing no DNA) ϵ_f was found to be $9{\cdot}991 \times 10^3$.

Non-linear regression of r vs *c*

The values of r and c were next fitted to the equation for a one site model:

$$r = \frac{Kcn}{1 + Kc} \quad \cdot\cdot \quad \cdot\cdot \quad (4)$$

using the non-linear regression program NONLIN (Metzler, Elfringe & McEwan, 1974) with r treated as the dependent variable and c as the independent variable. The value of K (Table 1) estimated by this procedure is lower than that predicted by the Scatchard plot since, unlike the Scatchard plot, all the points have been fitted in this non-linear treatment. To allow for the curvature in the Scatchard plot, equation (4) can be modified to accommodate the possibility of a second site as shown in equation (5):

$$r = \frac{K_1 n_1 c}{1 + K_1 c} + \frac{K_2 n_2 c}{1 + K_2 c} \quad \cdot\cdot \quad (5)$$

The data were fitted to equation (5) using the NONLIN program and a closer agreement was noted between the values of the parameters K_1 and n_1 determined in this manner and those derived from the Scatchard plot (Table 1). However, the plot of

residuals (calculated r − observed r *vs* calculated r) shows a systematic trend (Fig. 1a), undoubtedly due to the fact that both r and c are subject to error. In order to correct this, it is necessary to regress the data against the true independent variable.

Regression of fraction bound (α) vs *cumulative volume of DNA added* (x)

If x_i represents the volume of the i th aliquot of DNA solution added to the drug solution then the cumulative volume Σx_i is the true independent variable. Although this variable is subject to some error, this is much less than the error incurred in α which is therefore treated as the dependent variable. Substitution of αCt/DNAP for r and (1-α)Ct for c in the equation for a one-site model, equation (4), yields:

$$\frac{\alpha}{DNAP} = \frac{nK(1-\alpha)}{1 + K(1-\alpha)Ct}$$

$$\alpha = \frac{a - \sqrt{a^2 - 4nK^2CtDNAP}}{2KCt} \quad .. \quad (6)$$

where $Ct = (C_D V)/(V + x)$, $DNAP = (C_A x)/(V + x)$; $a = (1 + KCt + nKDNAP)$ x is the cumulative volume of DNA added and the constants C_D, V and C_A are the initial concentration of drug solution, the initial volume of drug solution and the concentration of master DNA solution respectively. A similar treatment for a two site model gives a cubic equation in α which also has a closed analytical solution.

The data were fitted to both the one site and the two site models (Table 1), the two site model giving the best fit to the data as expected. Examination of the residual plots (Fig. 1b) shows a lessening of the systematic trend seen for the regression of r *vs* c (Fig. 1a) but it is evident that there are three distinct groups, parallel to the residual axis. This can only be due to combining the data points from all three samples of daunomycin solution to obtain a common ϵ_f and ϵ_b which were then used to calculate the values of α. Consequently an individual ϵ_f and ϵ_b was determined for each sample of daunomycin solution and the values of α recalculated using the appropriate values of ϵ_f and ϵ_b. The three data sets were fitted simultaneouslv to the two site model, the results showing a distinct improvement (Table 1). This underlines the value of analysis of residuals in regression analysis. In all subsequent analyses the cells were treated separately in this manner.

Comparison of the α vs *x and r* vs *c regression methods*

To assess the effect on the parameter estimates of transformation of the data, a perfect set of data was generated for α and x with a normally distributed random noise added to α. The parameters n and K for a two site model were then determined by the α *vs* x method and, after transformation of the data, by the r *vs* c method (Table 2). Both methods gave broadly similar results for the data with least scatter ($\sigma = 0{\cdot}02$), but as the scatter was increased ($\sigma = 0{\cdot}04$) the parameter estimates from the r *vs* c method were found to diverge dramatically from the theoretical values. Also, the residual plots for the r *vs* c regression (Fig. 2a) show the same systematic trend seen when regression of r *vs* c was used to analyse the

Table 1. *Binding parameters for the titration of daunomycin with DNA.*

Method of evaluation	$K_1 \times 10^{-6}$	n_1	$\omega \times 10^{-3}$	$K_2 \times 10^{-3}$	n_2	r	r^2	SSE × 10^2†
Scatchard plot	1·734	0·153	—	—	—	0·774	—	—
r *vs* c (1 site)	0·786 (0·424)*	0·156 (0·002)	—	—	—	0·899	0·804	1·46
r *vs* c (2 site)	1·605 (0·278)	0·116 (0·005)	—	56.504 (19·042)	0·060 (0·013)	0·914	0·836	1·23
α *vs* x (1 site)	0·627 (0·040)	0·157 (0·001)	—	—	—	0·998	0·995	2·63
α *vs* x (2 site)	0·855 (0·100)	0·146 (0·001)	—	0·431 (2·580)	0·773 (4·600)	0·998	0·996	2·44
α *vs* x (2 site) (cells treated separately)	1·329 (0·124)	0·143 (0·003)	—	0·611 (7·643)	0·749 (9·060)	1·000	1·000	0·24
Non-cooperative binding model (cells treated separately)	6·482 (1·690)	0·171 (0·003)	4·253 (0·570)	—	—	1·000	1·000	0·27

* Values in parentheses are standard deviations.
† Sum of squared deviations due to error.

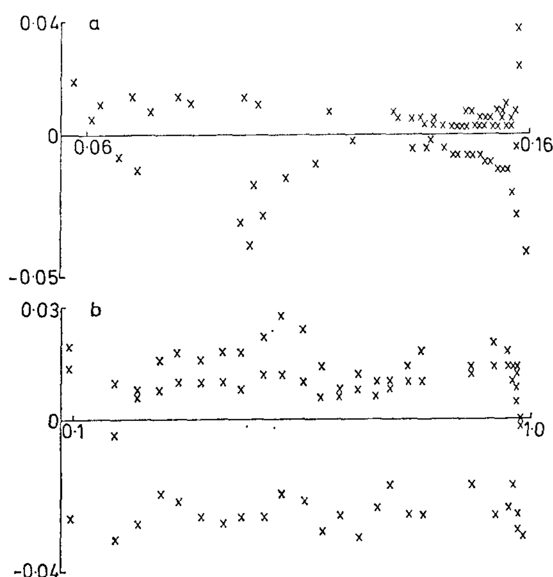

FIG. 1. Residuals plot for non-linear regression of (a) r *vs* c. (b) α *vs* x using the two site model. Ordinate: Residuals. Abscissa: Y_{calc}.

Table 2. *Non-linear regression analysis of perfect data with scatter.*

	Set values	σ = 0·02		σ = 0·04	
		r *vs* c	α *vs* x	r *vs* c	α *vs* x
$K_1 \times 10^{-6}$	1	1·111 (0·2137)	0·8555 (0·1518)	99·98 (264·48)	0·6647 (0·2971)
n_1	0·2	0·186 (0·017)	0·209 (0·015)	0·090 (0·014)	0·236 (0·052)
$K_2 \times 10^{-4}$	1	1·0527 (0·4896)	0·7238 (0·4650)	3·390 (1·4892)	0·5978 (1·6801)
n_2	0·8	0·842 (0·234)	0·986 (0·454)	0·611 (0·122)	1·000 (2·111)
r		0·990	0·999	0·933	0·996
r^2		0·980	0·999	0·854	0·991
SSE × 10^2*		1·97	0·55	9·20	1·83

* Sum of squared deviations due to error.

experimental data (Fig. 1a), whereas the residual plot for regression of α *vs* x shows random scatter (Fig. 2b). This confirms that transformation from α *vs* x variables to r *vs* c variables before analysis will give erroneous results. Most experimental data will probably have a variance greater than that in the second set of data where σ = 0·04.

A non-cooperative binding model

Since the data fit a two site model when α is regressed against x, there is now a temptation to conclude that there are two types of binding site for daunomycin on DNA under the conditions of the assay. Indeed, it is known that there are two possible modes of binding of daunomycin to DNA, intercalation and external

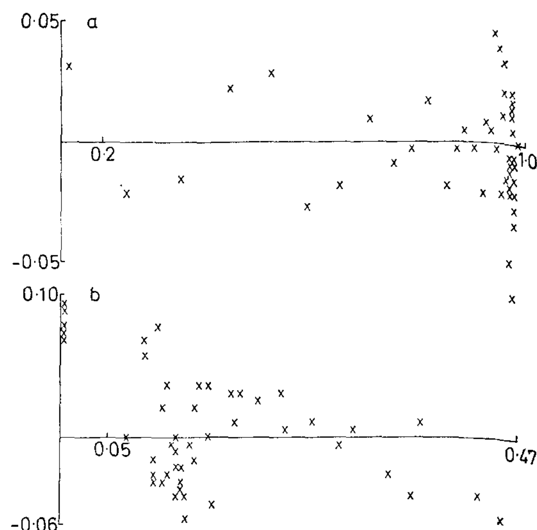

FIG. 2. Residuals plot for non-linear regression of (a) r *vs* c. (b) α *vs* x using the two site model and perfect data with scatter (σ = 0·04). Ordinate: Residuals. Abscissa: Y_{calc}.

binding (Di Marco & Arcamone, 1975), however, only the former mode of binding should occur in the buffer containing 0·3 M sodium chloride. It is possible that as daunomycin intercalates into the helix, the binding of subsequent molecules becomes increasingly hindered due to a non-cooperative binding process. In fact a 'nearest neighbour exclusion' model has been proposed for intercalating drugs, the intercalation only being allowed at alternate base pairs (Crothers, 1971). To investigate this model, an analysis was performed in which the parameter K was varied by inclusion of an interaction parameter ω in the manner of the Deybe-Huckel theory in solution kinetics (Chambron, Daune & Sadron, 1966). The resulting equation is

$$r = \frac{Ke^{-2\omega r/nRT}cn}{1+Ke^{-2\omega r/nRT}c} \quad .. \quad (7)$$

where R and T are the gas constants and absolute temperature respectively. After substituting α and x for r and c, equation (7) can be arranged to a form $f(\alpha_1,x) = 0$.

It is not possible to express α in terms of x in a closed analytical fashion and so a numerical method must be used. The simplest method is the Newton-Raphson method which requires an initial estimate of α (α_0) which is updated by the following means:

$$\alpha_1 = \alpha_0 - \frac{f(\alpha_1,x)}{\alpha f/\sigma\alpha}$$

The new (and hopefully improved) estimate of α (α_1) is used as a new input and the process repeated until self-consistency is obtained to the required degree of accuracy. Although this method sometimes runs into convergence problems we experienced no such difficulty here. The parameters obtained are included in Table 1. To test for significance between the various models, an analysis of variance was performed (Table 3) on the error sum of squares of the one site (α *vs* x) the two site (α *vs* x) and the non-cooperative binding model (one site model plus interaction), assuming they are nested (Boxenbaum, Riegelman & Elashoff, 1974). The analysis is not strictly correct as the non-cooperative binding model is not a subset of the two site model, nevertheless the analysis does provide a basis for comparison. The fit to the two site model is significantly better than the fit to the non-cooperative binding model (one site model plus interaction) which is significantly better than the one site model ($P < 0.05$).

Table 3. *Analysis of variance of the one site* (α vs x), *two site* (α vs x) *and non-cooperative binding models**.

Model	SSE	df†	F‡
1-Site	0·00671	73	62·5 (1-site *vs* 2-site)
2-Site	0·00243	71	8·5 (1-site + interaction *vs* 2-site)
Non-cooperative Binding (1-site + interaction)	0·00272	72	

* Boxenbaum & others (1974).
† Number of degrees of freedom.
‡ $F(5\%; 1, 70) = 3.98$; $F(5\%; 2, 70) = 3.13$.

Non-linear regression of absorbance vs *cumulative volume of DNA added (x) in which ϵ_b for each cell is treated as a parameter to be estimated*

Non-linear regression of α *vs* x using a two site model gives the best fit to the data but there is one further point to consider, namely that α is a function of both the absorbance and the 'constant' ϵ_b. One major drawback of the spectroscopic titration method of analysing drug binding is that the data evaluation is highly dependent on the value used for ϵ_b (Bontemps & Fredericq, 1974). To estimate the influence of ϵ_b on the binding parameters the data were fitted to the one site and the two site models with ϵ_b to be determined as a parameter (Table 4). Using the one site model there was a strong correlation between ϵ_b and K and this was reflected in the lowering of the value of K when the regression was repeated omitting the 18 points at highest concentrations of DNA. In the two site model the interaction between K_1 and ϵ_b was lessened mainly due to the fact that the two site model fits the data better than the one site model and hence the additional parameters (ϵ_{b1}, ϵ_{b2} and ϵ_{b3}) do little to improve the fit. The residual plot for this two site model showed a completely random scatter, and this method gave the optimum fit to the data (Table 4). It must be emphasized that in using this method (and indeed any other method) to evaluate data from spectroscopic titrations, enough data must be obtained at sufficiently high ratios of macromolecule to drug to give unbiased estimates of ϵ_b.

DISCUSSION

There are a number of points which must be considered when analysing data from binding studies:

1. Transformation of binding data before analysis, such as in the Scatchard method, is statistically incorrect. The errors in the dependent (r/c) and independent variable (r) are highly correlated.
2. It is important to examine the experimental method to identify the true dependent and independent variables. The parameter estimates should be obtained from the regression of the actual dependent variable against the actual independent variable. The errors involved in using the incorrect variables are clearly seen by comparing the results from regressing r against c and α against x for the same set of data (Table 2).
3. Data fitting alone cannot be used to gain information about the number of different types of binding site and their nature. If a particular model does not fit the data then it can be rejected; if, however, the

Table 4. *Estimation of K_1, n_1, K_2 and n_2 with ϵ_b fixed and as a parameter* (α vs x).

	ε_b estimated graphically* One site model	ε_b estimated graphically* Two site model	ε_b entered as a parameter One site model	ε_b entered as a parameter Two site model
$\varepsilon_{b1} \times 10^{-3}$	6·133	6·133	6·014 (0·017)	6·134 (0·017)
$\varepsilon_{b2} \times 10^{-3}$	6·267	6·267	6·159 (0·017)	6·280 (0·017)
$\varepsilon_{b3} \times 10^{-3}$	6·117	6·117	6·034 (0·017)	6·154 (0·016)
$K_1 \times 10^{-6}$	0·799 (0·049)	1·329 (0·124)	0·490 (0·003)	2·358 (0·874)
n_1	0·157 (0·001)	0·143 (0·003)	0·158 (0·001)	0·129 (0·006)
$K_2 \times 10^{-3}$	—	0·611 (7·643)	—	36·097 (16·719)
n_2	—	0·749 (9·060)	—	0·052 (0·003)
r	0·999	1·000	1·000	1·000
r^2	0·999	1·000	1·000	1·000
SSE $\times 10^3$	6·71	2·43	0·14	0·06

* ε_b was determined for each of the samples of daunomycin hydrochloride from a plot of DNAP/Ct *vs* ε_{obs} and linear regression of the final plateau region where drug is fully bound. The subscripts refer to the individual samples.

model does fit the data then it cannot be regarded as a unique solution. This point has not always been appreciated despite the fact that it has often been emphasised (for example Riggs, 1963; Berman, 1963) that the apparent suitability of one mathematical model over another, does not imply that the preferred model is descriptive of the binding system. The data obtained in this study can be described by a one site model with a non-cooperative interaction and if this model had been used as the sole model then it may have been concluded that daunomycin intercalates into DNA in a non-cooperative manner. In fact a two site model gives a significantly better fit to the data (Table 3). Further independent experimental results would be needed to confirm that the two site model is in fact correct.

Although estimation of the binding of a drug to DNA by spectrophotometric titration has been chosen as the example, the above conclusions are relevant to all studies of small molecule/macromolecule binding interactions including enzyme kinetics and the investigation of pharmacological response. In the spectroscopic titration method absorbance and cumulative volume added are the dependent and independent variables respectively and care must be taken to ensure the titration is continued until drug is fully bound. In equilibrium dialysis, perhaps the most common method of analysing drug binding, a choice of dependent variable has to be made since both total drug and free drug are assayed. For highly bound drugs the total drug concentration is usually more precisely determined and so would be considered to be the independent variable: this is a rational choice since the unbound drug concentration depends on the total concentration of drug. The unbound concentration can then be regarded as the dependent variable. In analysis of binding data, the parameters K and n only have significance within the chosen model. It is therefore fallacious to imply that there are two distinct binding sites if the data will fit a two site model unless there is strong corroborative evidence from independent studies. The values of K and n can only serve to compare drugs within the context of a chosen model, all the binding studies having been conducted in an identical manner. Finally we would point out that the Scatchard plot, although statistically incorrect, is still useful both as a means of graphical representation of the data and for providing parameter estimates for the input to a non-linear regression program.

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