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An in vitro study of drug displacement interactions: warfarin–salicylate and warfarin–phenylbutazone

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The binding interactions between warfarin–salicylate and warfarin–phenylbutazone in the presence of 4 g percent bovine serum albumin at 37 °C were studied using equilibrium dialysis. Methods of representing and analysing drug binding interactions are discussed. Scatchard plots, double reciprocal plots and the like are shown to be of no use in representing drug displacement interactions since they display only one drug and they can be potentially misleading. It is argued that a preferable method of analysing drug displacement interaction data is in terms of a stepwise multiple equilibria model. The numerical problems associated with fitting this kind of model to the data are discussed. A three-dimensional representation of the binding surface is proposed as a superior means of visualizing drug displacement interactions.

Clinically the phrase 'drug–drug interaction' refers to the significant modification of the pharmacological effect of one drug by one or more other drugs. This definition precludes interactions which may alter pharmacokinetic or physiological parameters but do not alter the pharmacological response, and usually only adverse interactions are considered. The mechanisms of drug interactions are diverse (see for example Morselli et al 1974) but one of the most widely studied is the interaction thought to be caused by displacement from plasma proteins. Many drugs bind strongly to plasma proteins and the effect of administration of a second drug which also binds strongly to the same proteins is to displace the first drug so as to increase its free or unbound concentration. As the pharmacological effect is thought to be related to the unbound concentration of the drug, the effect of displacement is to augment the response and perhaps produce adverse side effects. Displacement from tissue binding sites is obviously also important but these interactions are less amenable to study.

Many drugs interfere with coumarin anticoagulant therapy (Koch-Weser & Sellers 1971 a,b) and many of these interactions have been ascribed to displacement from plasma proteins. The effect of displacement is to potentiate the hypoprothrombinaemic action of the drug and so increase the danger of haemorrhage. Salicylate has been shown to displace warfarin in vitro (Muirden et al 1974) but the interaction with the clotting mechanism seems more complicated than the simple displacement of warfarin as salicylate also effects platelet aggregation by itself (O'Reilly et al 1971). Phenylbutazone has also been shown to displace warfarin in vitro and again this was thought to be the mechanism of the in vivo interaction (Aggeler et al 1967; O'Reilly 1973). However, Lewis et al (1974) have shown that phenylbutazone also interferes with the metabolism of warfarin, increasing the metabolism of the (+)-isomer and decreasing the metabolism of the more potent (−)-isomer and thus potentiation of the hypoprothrombinaemic effect is probably more complicated than originally reported.

The purpose of the present work was to study in greater detail the binding interaction between warfarin and salicylate and between warfarin and phenylbutazone. In the clinical situation, the salicylate and phenylbutazone concentrations are much greater than the warfarin concentration and hence warfarin will have little effect on the binding of these drugs. However, in an in vitro study a wider range of concentrations can be studied so that the effect of warfarin on the binding of the other drug can be investigated. In order to properly characterize the binding interaction it is necessary to measure the unbound and total concentrations of both drugs and assays were developed to do this. As most methods of representing and analysing binding data, for example the Scatchard plot, deal with only one drug, we investigated a different approach that allowed us to simultaneously represent both drugs. Our concern was that the binding interaction data in the literature did not allow one to predict the unbound concentration of *both* drugs given the total concentrations of the drugs.

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METHOD

Sample preparation

Stock solutions of warfarin, phenylbutazone and sodium salicylate were prepared in a solution of 4 g percent bovine serum albumin (BSA-Fraction V, Sigma Chemical Co.) in Krebs-Ringer buffer (Dawson et al 1969). All chemicals were of Analar grade. For the single drug studies twenty samples were used and for interaction experiments eighty samples were prepared. The salicylate concentration was varied between 0 and 4×10^{-3}M, while both the warfarin and phenylbutazone concentrations were varied between 0 and 8×10^{-4}M. Each sample was prepared by weighing out the appropriate amounts of the drug solutions and making up to volume with 4 g percent BSA solution. In the case of the warfarin–salicylate experiment, a trace (less than 1 μg ml^{-1}) of [^{14}C]warfarin (the Radiochemical Centre, Amersham —specific activity 76·4 μCi mg^{-1}) was added to each sample, whereas for the warfarin–phenylbutazone experiment each sample was divided into two, and to one a trace of [^{14}C]warfarin added and to the other a trace ($\sim$3 μg ml^{-1}) of [^{14}C]phenylbutazone (gift from Geigy Pharmaceuticals, U.K.—specific activity 11·0 μCi mg^{-1}) was added. Warfarin and phenylbutazone concentrations were corrected for the added radiochemicals. The radiochemicals were purified by high pressure liquid chromatography (h.p.l.c.).

Binding measurements

The binding was measured by equilibrium dialysis using a Dianorm apparatus (Weder et al 1971). All experiments were performed in a 37 °C water bath and dialysis lasted for 4 h. In earlier trials it was established that equilibrium was essentially reached in 4 h. At the end of the run aliquots were taken from the protein side of the membrane for the determination of both total drug (bound and unbound) concentration and protein concentration and an aliquot was taken from the buffer side of the membrane for the determination of the unbound concentration.

Assays

Both the unbound and total concentrations of salicylate were measured fluorimetrically using a Perkin Elmer spectrofluorometer 1203 with λ excitation 308 nm and λ emission 420 nm (uncorrected). The samples were made alkaline with sodium hydroxide until albumin had negligible fluorescence. Warfarin exhibited a slight fluorescence at the wavelengths used for the assay. The warfarin concentration having been determined, the residual fluorescence, due to warfarin, was subtracted from the total fluorescence using a previously determined calibration curve.

Before dialysis the hot (i.e. ^{14}C) to cold ratio of warfarin and phenylbutazone was determined. After dialysis the concentration of the total and unbound drug could be determined by counting and using the hot to cold ratio assuming that the ^{14}C-labelled drug distributed identically as the cold drug. A Packard Tri-Carb Scintillation Counter was used to measure all radioactivity.

H.p.l.c. assays of warfarin, salicylate and phenylbutazone post dialysis established that there was no detectable breakdown of any of these chemicals during the run.

The protein concentration was determined using the Biuret method (Gornall et al 1949) and a Unicam SP1750 Spectrometer (λ_{max} 550 nm) to read the solutions.

THEORY

Linear plots

The most common method for presenting the results of an in vitro binding study is in terms of one of the linearization procedures such as the Scatchard plot (Scatchard 1949) or the double reciprocal plot (Klotz 1946). These plots have the advantage that they give a convenient representation of the data and, when linear, have a simple interpretation. When these plots are curved interpretation is more complicated (De Meyts & Roth 1975). Further, they have limited use for representing drug–drug interactions, as they only display the binding of one of the drugs. The case in which the 'displacing' drug is always in excess presents no particular problems as this drug is not displaced by the original drug to any significant extent. However, when both drugs are present at approximately the same concentration and both occupy a substantial fraction of the available binding sites, then the mutual displacement of each drug has to be considered. The following simple example illustrates the possible danger of misinterpretation that results when this point is ignored.

Consider the case of two drugs, A and B, that bind competitively to a single class of non-interacting binding sites. The binding isotherms are assumed to be the following:

$$C_T^A = C_u^A + \frac{n\, k_A C_u^A P_T}{1 + k_A C_u^A + k_B C_u^B} \quad \ldots\ \ldots\ \ldots \text{(1a)}$$

$$C_T^B = C_u^B + \frac{n\, k_B C_u^B P_T}{1 + k_A C_u^A + k_B C_u^B} \quad \ldots\ \ldots\ \ldots \text{(1b)}$$

where C_T^X and C_U^X are respectively the total and unbound concentrations of drug (X = A or B); P_T is the total protein concentration; n is the number of binding sites; and k_X is the equilibrium constant. These equations can be cast into Scatchard form in the usual manner by transforming to the variables r/c (mol drug bound per mol of protein per mol of drug unbound) and r (mol of drug bound per mol of protein). The Scatchard plots for drug A in the presence of varying total concentrations of drug B are shown in Fig. 1. These plots are hyperbolic having a common intercept n on the r axis (Schary et al 1978). If, as is commonly implicitly assumed, the unbound concentration of the displacing drug (C_U^B) remains constant, then these plots become linear with the same intercepts on the r/c and r axis. The marked non-linearity of these plots arises because only the total concentration of drug B, and not its unbound concentration, remains constant: drug B displaces and is displaced by drug A. The possibility of misinterpretation can be seen if the experiments are not continued to low enough r/c values. It would be then argued that the apparent intercept on the r axis is lower than in the absence of the displacing drug and hence the effect of the displacing drug is to reduce the number of binding sites, which would be incorrect.

Stepwise multiple equilibria model

In practice the situation is more complicated than depicted in the example above, in that competitive and non-competitive interactions, as well as the possibility of multiple binding sites have to be catered for. We have examined a more general approach to representing drug displacement interactions, based on Klotz's stoichiometric formulation (Klotz 1953; Spector & Ashbrook 1970). Consider the following equilibria between protein P, drug A and drug B.

$$A + P \underset{}{\overset{K_{1,0}}{\rightleftharpoons}} AP \qquad B + P \overset{K_{0,1}}{\rightleftharpoons} BP$$

$$A + AP \overset{K_{2,0}}{\rightleftharpoons} A_2P \qquad B + BP \overset{K_{0,2}}{\rightleftharpoons} B_2P$$

$$B + AP \overset{K_{1,1}}{\rightleftharpoons} BAP$$

where $K_{i,j}$ is the equilibrium constant for the species containing i molecules of A and j molecules of B. The resulting binding isotherm for drug A is

$$C_T^A = C_u^A + \frac{(K_{1,0}C_u^A + 2K_{1,0}K_{2,0}(C_u^A)^2 + K_{1,0}K_{1,1}C_u^AC_u^B + \ldots)P_T}{(1+K_{1,0}C_u^A+K_{1,0}K_{2,0}(C_u^A)^2+K_{0,1}C_u^B+K_{0,1}K_{0,2}(C_u^B)^2+K_{1,0}K_{1,1}C_u^AC_u^B+\ldots)} \quad (2)$$

with a corresponding equation for drug B. It should be noted that AP, for example, represents the sum of all species of that empirical formula so that the equilibrium constants $K_{i,j}$ are really averages. In this context the species ABP is identical to the species BAP. Thus, although this model is much more general than the independent-site model (the basis of the Scatchard plot), in that no a priori assumptions are made about the existence and nature of the binding sites, the amount of physical insight that can be obtained from the $K_{i,j}$'s is limited. This approach, then, is more one of representation rather than interpretation.

The independent-site model (equation 1) can be shown to be a special case of the more general model (Fletcher et al 1973) if we assume that the n sites pre-exist and that all n sites have the same microscopic association constant, k_A, towards A ligands and the same constant, k_B, towards B ligands. The relationship between the macroscopic constants and the microscopic constants is the following

$$K_{i,j}^A = \frac{n-(i+j-1)}{i} k_A$$

$$K_{k,l}^B = \frac{n-(k+l-1)}{l} k_B$$

Equation 2 then reduces to

$$C_T^A = C_u^A + \frac{\left\{nk_AC_u^A \left[\sum_{j=0}^{n-1}\sum_{j=1}^{n-j} \frac{i}{n} \cdot \frac{n!\left[k_AC_u^A\right]^{i-1}\left[k_BC_u^B\right]^{j}}{(n-i-j)!i!j!}\right]\right\}P_T}{(1 + k_AC_u^A + k_BC_u^B)\left[\sum_{j=0}^{n-1}\sum_{j=1}^{n-j} \frac{i}{n} \frac{n!\left[k_AC_u^A\right]^{i-1}\left[k_BC_u^B\right]^{j}}{(n-i-j)!i!j!}\right]}$$

and the result follows after cancellation.

Computational details

The computational problem involved is that of fitting two sets of observations simultaneously to three independent variables. Firstly, the dependent and independent variables that enter the regression must be chosen. This is not as simple as it seems. One

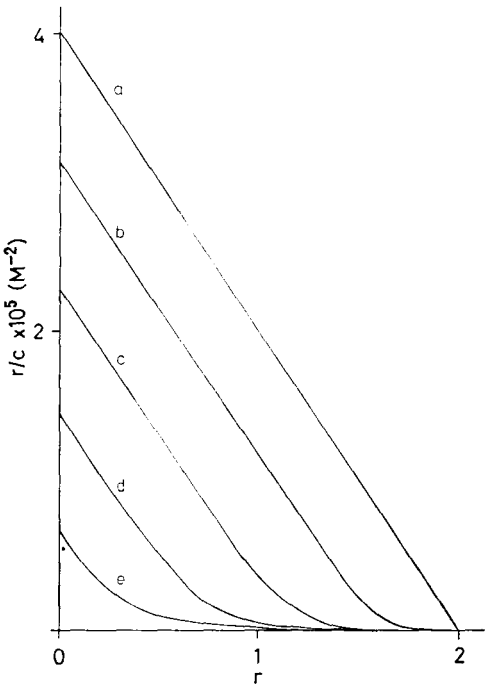

FIG. 1. Scatchard plots for the competitive interaction between two drugs. The value of the parameters used in the simulations were: $n = 2$; $k_A = k_B = 2 \times 10^5\ M^{-1}$; $P_T = 5{\cdot}8 \times 10^{-4}$ M. The total concentration of the second drug was: (a) 0; (b) $2{\cdot}5 \times 10^{-4}$ M; (c) $5{\cdot}0 \times 10^{-4}$ M; (d) $7{\cdot}5 \times 10^{-4}$ M; (e) $1{\cdot}0 \times 10^{-3}$ M.

of the independent variables is obviously the protein concentration but the other two can be either the unbound concentrations or the total concentrations of the two drugs: the dependent variables are then automatically selected. Since the drug concentration on either side of the membrane was measured, both the unbound and total concentrations are subject to error. Further, in the case of warfarin and phenylbutazone, as both the unbound and total concentrations are derived from the hot/cold ratio method, there is a certain amount of correlation between the errors in the unbound and total concentrations. Thus we have violated the primary postulate of least-squares—that the independent variables should be error free and that the errors in the dependent variables should be normally distributed with constant variance (Draper & Smith 1966; p. 17)—before starting. Having noted this, there is little we can do about it. From a statistical point of view perhaps ultracentrifugation is preferable to dialysis as the total concentration is known with much greater precision than the unbound concentration and so may be chosen as the independent variable. However, the large number of samples needed to define the system and the desire to perform as many experiments within a run as possible caused us to choose equilibrium dialysis. Thus, the parameter values obtained in the fitting and their associated tolerances must be viewed in the light of the experiment. Nevertheless, the overall data fit was certainly adequate to describe the binding surface and, in view of our earlier comments on the physical significance of the macroscopic association constants, this is probably more relevant.

In the main, for computational ease, the total concentrations (observed variables) were regressed against the unbound concentrations (independent variables). To do the inverse problem of regressing the unbound concentrations against the total concentrations requires firstly that equation 2 be inverted to the form

$$C_u^A = f(C_T^A, C_T^B, P_T)$$

$$C_u^B = g(C_T^A, C_T^B, P_T)$$

Since there is no analytical expression for these functions this was achieved numerically using a Newton-Raphson method. If A_n and B_n are the n^{th} estimates of C_U^A and C_U^B respectively, then the $n + 1^{th}$ estimates are

$$A_{n+1} = A_n - \frac{\begin{vmatrix} F & F_B \\ G & G_B \end{vmatrix}}{\begin{vmatrix} F_A & G_A \\ F_B & G_B \end{vmatrix}}$$

$$B_{n+1} = B_n + \frac{\begin{vmatrix} F & F_A \\ G & G_A \end{vmatrix}}{\begin{vmatrix} F_A & G_A \\ F_B & G_B \end{vmatrix}}$$

where

$$F = \frac{A_n + (K_{1,0}A_n + 2K_{1,0}K_{2,0}A_n^2 + K_{1,0}K_{1,1}A_nB_n + \ldots)\,P_T}{(1 + K_{1,0}A_n + K_{1,0}K_{2,0}A_n^2 + K_{0,1}B_n + K_{0,1}K_{0,2}B_n^2 + K_{1,0}K_{1,1}A_nB_n + \ldots)} - C_T^A$$

A similar expression exists for G, the corresponding function for species B. F_A denotes the partial derivative of F with respect to A evaluated with the n^{th} estimates and G_A represents the corresponding derivative of G. The process is repeated until successive estimates agree to within a preset tolerance (0·001%). For the initial estimates of C_U^A and C_U^B the experimentally determined values were used.

It was found that the protein concentration was fairly constant from sample to sample and since it made little difference whether the protein concentration was explicitly included either as an independent variable or a constant protein concentration equal to the average value was used, only the average protein concentration was employed subsequently.

The general protocol for the fitting procedure was as follows. The parameters were admitted to the model one by one, starting with the lowest order and working up. Thus, initially, the first order terms, PA and PB were regressed, then the second order terms, PAA, PBB and PAB, were added and so on. Within any particular order the term that reduced the total sum of squares the most was added first, and then similarly from the remaining terms, the one that reduced the total sum of squares the most was added and so on until any remaining term did not reduce the total sum of squares significantly as adjudged by an F test at the 5% level of significance (Boxenbaum et al 1974). The model was then either deemed complete or terms from a higher order were included and the process repeated. This method does not necessarily produce the 'minimal' set of parameters that describe the data and actually the parameters should be added one by one regardless of order. It was felt, however, that the present approach was physically more logical.

The computer program used employed a modified Gauss-Newton procedure (Metzler et al 1974). Due to the several assays used it was difficult to assess the variance of the observed variables and so no attempt at weighting the observations was made. This was partially vindicated by the fact that the final residual (difference between observed and calculated value) plots showed a reasonably normal distribution about the fitted surfaces (Draper & Smith 1966; Ch. 3).

Table 1. Comparison between using the unbound concentration and the total concentration as independent variable in the fitting of the stepwise model to the salicylate binding data.[a,b]

c	C_T vs C_u	C_u vs C_T
$K_{1,0}$	22 500 (3700)	10 100 (6 800)
$K_{2,0}$	9 800 (1700)	19 600 (10 000)
$K_{3,0}$	810 (180)	440 (170)
$K_{4,0}$	1 550 (290)	2 500 (880)
Correlation	1·000	1·000
Probit plot	nonlinear	linear

[a] BSA concentration = 5·6 × 10^{-4}M.
[b] Figures in brackets are standard deviations.
[c] Molar^{-1}.

RESULTS AND DISCUSSION

Single drug studies

A comparison was made between regressing the unbound concentration (C_u) as independent variable and the total concentration (C_T) as independent variable using the salicylate binding data. The results are shown in Table 1. In both cases convergence was very rapid and the fits appear to be equally as good when the correlations between the observed and calculated vectors are compared. However, the parameter estimates are very different. The only difference between the fits can be seen in the residual plots. Whereas the residuals from the regression in which C_T was chosen as the independent variable are randomly distributed about the fitted curve, as demonstrated by a linear probit plot of the residuals (Atkins 1976), the corresponding residuals from the alternate regression were not randomly distributed. This is what one would suspect given that one has more confidence in the larger C_T values. However,

Table 2. Summary of the results of fitting the stepwise model and the Scatchard model to the binding data of warfarin, salicylate and phenylbutazone.

Warfarin[a]			
Stepwise model		Scatchard model	
$K_{1,0}$	140 000 (12 000)	n_1	2·0 (3·5)
$K_{2,0}$	33 390 (6 400)	k_1	65 000 (75 000)
$K_{3,0}$	5 700 (3 400)	n_2	1·9 (400)
		k_2	1 600 (400 000)
Correlation 0·997		Correlation 0·996	

Salicylate[b]			
Stepwise model		Scatchard model	
$K_{1,0}$	22 500 (3 700)	n_1	2·5 (0·3)
$K_{2,0}$	9 800 (1 700)	k_1	9 400 (2 100)
$K_{3,0}$	810 (180)	n_2	19 (120)
$K_{4,0}$	1 550 (290)	k_2	36 (260)
Correlation 1·000		Correlation 1·000	

Phenylbutazone[c]			
Stepwise model		Scatchard model	
$K_{1,0}$	312 400 (6 400)	n_1	0·75 (0·11)
$K_{2,0}$	7 110 (790)	k_1	437 000 (100 000)
$K_{3,0}$	23 700 (3 800)	n_2	3·0 (1·5)
		k_2	8 300 (6 900)
Correlation 1·000		Correlation 0·999	

BSA concentrations: (a) 5·7 × 10^{-4}M; (b ×) 5·6 10^{-4}M; (c) 5·5 × 10^{-4}M.

some doubt must be cast on the parameter values obtained from either method. For example, even the numerical ordering of the parameters may be an artifact of the method of fitting and as pointed out by Fletcher & Spector (1977) this ordering can be affected by including more parameters beyond those needed to satisfactorily describe the data.

The results of fitting the stepwise model to the warfarin, salicylate and phenylbutazone data, together with the corresponding two-site independent binding site model fits are summarized in Table 2. The independent binding site model parameters are comparable to literature values (Jusko & Gretch 1976). The only relevant study in which the stepwise model has been used is that of Fletcher & Spector (1977) who fitted the salicylate data of Mais et al (1974) but the concentration of protein used was only 0·3 g % and so the results are not comparable. We chose 4 g % albumin as this is its average concentration in plasma.

It is interesting to note the way in which the parameters enter the two models. In the stepwise model they enter one by one but in the independent binding site model they enter two at a time. This is very relevant when considering the minimum number of parameters required to fit a particular data set. Thus, for warfarin, although four parameters are required to fit the independent binding site model, only three are required to fit the stepwise model. The low confidence in the parameter estimates from the independent binding site model is explained by the fact that, for this particular data, a two-site fit is only just significantly better than a one-site fit and that for the two-site fit the n and k parameters are highly intercorrelated. Of course, if the data set was extended, better estimates of the n and k parameters could be obtained and perhaps the stepwise model could be extended to a fourth parameter. In practise the most economical method—in terms of number of parameters—of binding representation is the stepwise model.

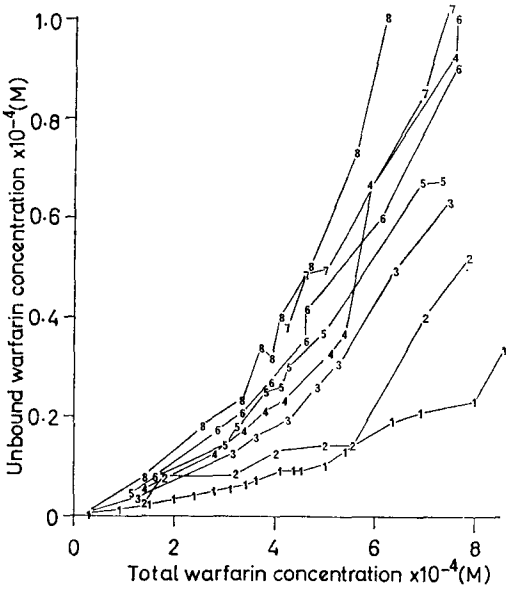

FIG. 3. Experimental binding data for warfarin, by itself and in the presence of salicylate. The approximate total concentrations of salicylate are: (1) 0; (2) $5{\cdot}5 \times 10^{-4}$ M; (3) $1{\cdot}1 \times 10^{-3}$ M; (4) $1{\cdot}9 \times 10^{-3}$ M; (5) $2{\cdot}2 \times 10^{-3}$ M; (6) $2{\cdot}7 \times 10^{-3}$ M; (7) $3{\cdot}4 \times 10^{-3}$ M; (8) $3{\cdot}1 \times 10^{-3}$ M.

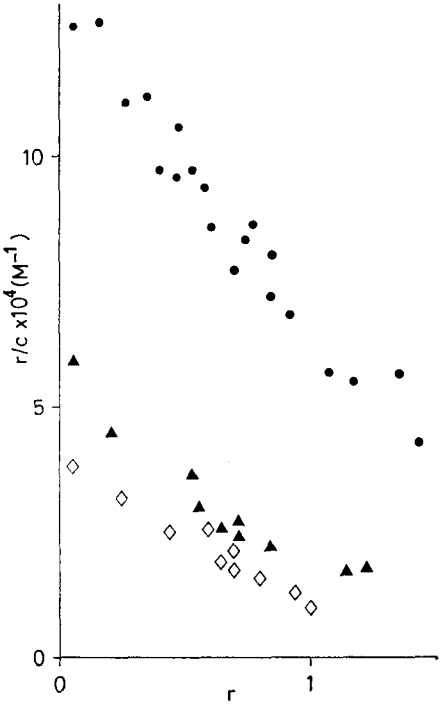

FIG. 2. Scatchard plots for the warfarin–salicylate interaction. ● Warfarin alone; ▲ warfarin + $2{\cdot}2 \times 10^{-3}$ M salicylate; ◇ warfarin + $3{\cdot}1 \times 10^{-3}$ M salicylate.

Warfarin–salicylate interaction

Some of the data from the warfarin–salicylate interaction study are shown, plotted in Scatchard form, in Fig. 2. Three data sets are displayed: one in which there is no added salicylate and two which have differing amounts of total salicylate present. It appears that the intercept on the r-axis for

warfarin in the presence of salicylate is less than that for warfarin alone. Although one is tempted to assign this change to a decrease in the number of available binding sites on albumin, in view of earlier comments, this could be an erroneous conclusion due to insufficient experiments being performed at low values of r/c. It could not be said, from these data alone, that salicylate decreases the number of binding sites for warfarin on albumin.

The binding data for warfarin, by itself and in the presence of salicylate, is presented in Fig. 3. Each curve represents an experiment in which the total warfarin concentration was varied while the salicylate concentration was maintained approximately constant. Much of the scatter in these plots is due to the fact that, far from being constant, the salicylate concentration varies significantly on any particular curve. Although the same total salicylate concentration was added to the protein side of the equilibrium dialysis membrane, with water fluxes, displacement and drug movement across the membrane, the final salicylate concentration varied from sample to sample. The need for an alternate representation of the binding surface is discussed at the end of this section.

The results of fitting the stepwise model to the data are summarized in Table 3. Seven parameters were needed to describe the binding and even then the fit cannot be described as particularly good. The residuals were not randomly distributed about the fitted plane and the confidence limits on the parameters were poor. The main reason for this is the difficulty of trying to analyse so much data simultaneously. Poorer data sets adversely affect the overall fit, whereas individual sets could be fitted quite well. Selective data sets could be removed so as to improve the overall fit, but in this instance we decided to use all the data in an effort to gain a complete picture of the binding surface at the expense of precise estimates of the parameters.

We felt that the estimates of confidence in the parameters were worse than they should be. To test this we performed a Monte Carlo error analysis (Chandler et al 1972). Ten sets of data to which, random noise from a pseudo Gaussian distribution of mean zero and variance equal to the experimental variance as estimated from the original computer fit was added, were generated from the seven parameter model, using the parameters as obtained from fitting the experimental data (Table 3). The standard deviations of the parameters calculated from the ten individual fits are presented in Table 4. It can be seen that although the mean parameter estimates agree quite closely with the original parameter set (and would agree even more closely if further simulations

Table 3. Results of fitting the stepwise model to the warfarin–salicylate interaction data[a].

$K_{1,0}$[b]	$K_{2,0}$	$K_{0,1}$	$K_{0,2}$	$K_{0,3}$	$K_{1,1}$	$K_{1,2}$	Corr.
162 000 (59 000)	36 000 (17 000)	28 (3 000)	$1{\cdot}4 \times 10^6$ ($1{\cdot}5 \times 10^8$)	2400 (1000)	15 800 (8 600)	5400 (2100)	0·992

[a] BSA concentration = $5{\cdot}5 \times 10^{-4}$M.
[b] $K_{i,j}$: the first index refers to warfarin and the second to salicylate.

Table 4. Monte Carlo error analysis for the fit of the stepwise model to the warfarin–salicylate interaction data.

	Original value	Mean[a]	s.d.[b,c] (NONLIN)	s.d (Monte Carlo)
$K_{1,0}$	162 000	154 000	62 000	18 000
$K_{2,0}$	36 000	42 000	18 000	13 000
$K_{0,1}$	28	28	3 200	10
$K_{0,2}$	$1{\cdot}4 \times 10^6$	$1{\cdot}7 \times 10^6$	$1{\cdot}6 \times 10^8$	800 000
$K_{0,3}$	2 400	2 400	1 000	710
$K_{1,1}$	15 800	15 700	9 000	4 500
$K_{1,2}$	5 400	6 400	2 200	3 400

[a] Mean of ten simulations. Standard deviation of added random variation was $1{\cdot}4 \times 10^{-4}$.
[b] Standard deviation.
[c] The standard deviations in this column refer to the linearized error estimates calculated by the program for one of the simulated data sets.

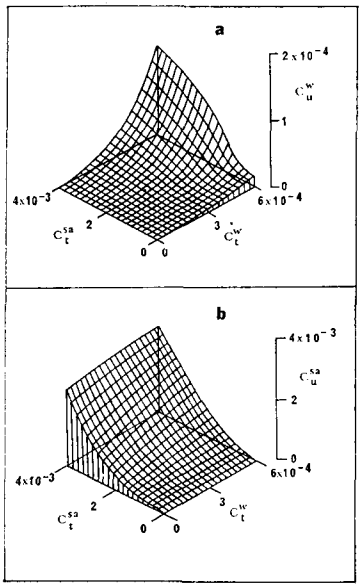

FIG. 4. Three-dimensional plots of the warfarin-salicylate interaction:
(a) The ordinate is the unbound concentration of warfarin (C_u^w);
(b) The ordinate is the unbound concentration of salicylate (C_u^{sa}). The symbols C_t^w, C_t^{sa} refer to the total concentration of warfarin and salicylate respectively. The units on all axes are molar (M).

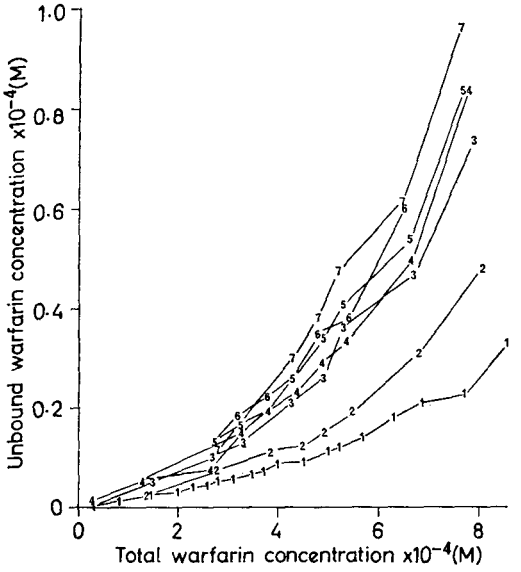

FIG. 5. Experimental binding data for warfarin in the presence of phenylbutazone. The approximate total concentrations of phenylbutazone are: (1) 0; (2) $1{\cdot}5 \times 10^{-4}$ M; (3) $2{\cdot}8 \times 10^{-4}$ M; (4) $3{\cdot}6 \times 10^{-4}$ M; (5) $4{\cdot}5 \times 10^{-4}$ M; (6) $5{\cdot}6 \times 10^{-4}$ M; (7) $7{\cdot}0 \times 10^{-4}$ M.

were included) the mean standard deviations—and particularly $K_{0,1}$ and $K_{0,2}$—are quite different from those estimated by the computer program, which parallel those shown in Table 3. The reason for this is that the standard deviations evaluated by the program are linearized error estimates and hence inaccurate for a non-linear model.

As we noted earlier, the advantage of the Scatchard plot and the like was that they gave a convenient representation of the data. These representations fail in the case of drug–drug interactions as they only display one drug. We experimented with a different kind of representation that allows one to visualise drug–drug displacements much more readily. This involves using a three-dimensional plot in which the abscissi are the total concentrations of the two drugs and the ordinate is the unbound concentration of either drug. A plot of the warfarin–salicylate binding isotherm represented in this manner is shown in Fig. 4. Only the fitted surface is shown. The displacing effect of one drug upon another is readily seen in this form of presentation. For instance, since salicylate is present in much higher concentrations than warfarin (which is typical of the clinical situation), salicylate is seen to displace warfarin to a much greater extent than warfarin displaces salicylate.

Table 5. Results of fitting the stepwise model to the warfarin-phenylbutazone data[a].

$K_{1,0}$[b]	$K_{2,0}$	$K_{0,1}$	$K_{0,2}$	$K_{1,1}$	$K_{1,2}$	Corr.
135 000 (11 000)	30 000 (2 900)	325 000 (21 000)	15 500 (1 700)	143 000 (16 000)	22 800 (2 800)	0·989

[a] BSA concentration = $5{\cdot}4 \times 10^{-4}$M.
[b] $K_{i,j}$: the first index refers to warfarin and the second to phenylbutazone.

Warfarin–phenylbutazone interaction

The overall fit of the model to the warfarin–phenylbutazone data set, as adjusted by the residual plots, proved to be better than the warfarin–salicylate study. Six parameters were needed for the model. These results are listed in Table 5. The experimental binding data and the corresponding three-dimensional fitted surfaces are shown in Fig. 5 and Fig. 6, respectively. Warfarin and phenylbutazone bind to albumin with about the same affinity, but phenylbutazone displaces warfarin to a greater extent than vice versa. As expected, phenylbutazone is a stronger displacer of warfarin than salicylate at the same molar concentration.

From the way that the stepwise model is built up, it seems probable that certain parameters which

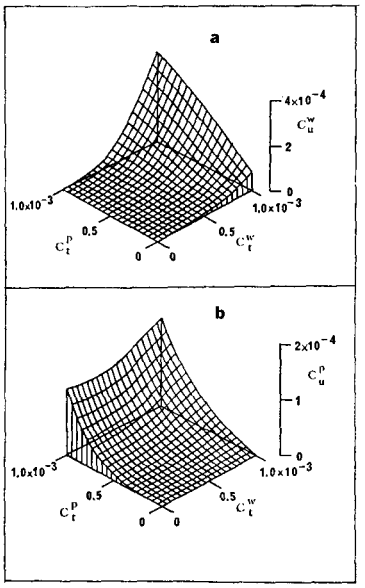

FIG. 6. Three-dimensional plots of the warfarin–phenylbutazone interaction: (a) The ordinate is the unbound warfarin concentration (C_u^w); (b) The ordinate is the unbound phenylbutazone concentration (C_u^p). The symbols C_t^w, C_t^p refer to the total concentration of warfarin and phenylbutazone respectively. The units on all axes are molar (M).

involve only one drug species, such as $K_{1,0}$, $K_{0,1}$, should be transferable. That is, since terms such as $K_{1,0}$ and $K_{0,1}$ are supposed to represent the interaction of a single drug with the protein, one would expect that the values would be the same, irrespective of whether they were determined in a single drug experiment or a drug–drug interaction experiment. In the case of warfarin and phenylbutazone, this is roughly correct. Thus, in the three studies, warfarin alone, warfarin–salicylate and warfarin–phenylbutazone, the parameters $K_{1,0}$ and $K_{2,0}$ remain fairly constant. However, the salicylate parameters are completely different between the two studies, salicylate alone and warfarin–salicylate, probably because the salicylate concentrations used in the studies were too high to properly characterise the low order parameters, i.e. $K_{0,1}$ and $K_{0,2}$. Further studies are needed before the transferability of these parameters can be demonstrated. An idea of the degree of displacement between two drugs may be estimated from the parameters obtained from single drug studies, bearing in mind that cross terms, e.g. $K_{1,1}$, can alter the picture completely.

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