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COOPERATIVE INTERACTION OF WARFARIN AND PHENYLBUTAZONE WITH HUMAN SERUM ALBUMIN

BARRY W. MADSEN and GILLIAN M. ELLIS
Department of Pharmacology, University of Western Australia, W.A., 6009 Australia

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Abstract—Phenylbutazone is commonly thought to displace warfarin from human serum albumin (HSA) by direct competition, and the aim of the present study was to confirm or deny this mechanism using standard equilibrium dialysis. A number of titration curves for [^{14}C]warfarin (4–1000 μM) in the presence of fixed concentrations of phenylbutazone (0–2000 μM) were obtained at 25° in 0.1 M, pH 7.0 phosphate buffer. The first two site dissociation constants (K_1 and K_2) for each of these curves were estimated according to Adair's equation. Comparison of K_1 with that predicted for a single site directly competitive model showed large discrepancies, indicating unacceptability of the model beyond experimental error. The data for the first warfarin site could be partly explained by a dual interaction mechanism where at lower phenylbutazone concentration (< 100 μM) the interaction is negatively cooperative, and at higher levels (100–1000 μM) further displacement is caused by direct competition. Phenylbutazone also affects the second warfarin site resulting in an apparent positive cooperativity between the two warfarin sites. The results of this study suggest caution in assigning drugs to common binding sites on the basis of simple displacement data.

For the last decade it has been thought that phenylbutazone and warfarin compete directly for binding sites on human serum albumin (HSA), following experimental evidence produced by Solomon and Schrogie [1]. In the period since that original work, an increasing number of cooperative binding phenomena have been reported, particularly in the biochemical literature, and Weber [2] has speculated that non-competitive mechanisms may more often be the rule than the exception in ligand–protein interactions. Recently, when the warfarin–phenylbutazone–HSA interaction was closely examined using a fluorescence enhancement assay, it was found to manifest as a directly competitive system if a sub-optimal range of phenylbutazone concentrations was used, when in fact a negatively cooperative mechanism was operating [3]. The interpretation from this work was qualitative, and given other limitations inherent in fluorometric titration data, the aim of the present study was to examine whether cooperativity could be quantitatively demonstrated using standard equilibrium dialysis. The existence of such an alternative mechanism to simple competition has relevance to much recent work concerned with specific drug binding sites on serum albumin [4, 5].

MATERIALS AND METHODS

Experimental. [^{14}C]warfarin, stated to be greater than 98% pure, was purchased from The Radiochemical Centre, Amersham, U.K., unlabelled warfarin, phenylbutazone and HSA (fraction V) were obtained from Sigma, St. Louis, Mo., and all other reagents were of analytical reagent grade. Pyrex centrifuge tubes (25 ml) were used for the equilibrium dialysis with 10 ml 0.1 M pH 7.0 phosphate buffer on each side of the Visking membrane. Warfarin (4–1000 μM), HSA (10 μM) and phenylbutazone (0–2000 μM) were all placed initially on the inside of the bag. A constant amount of [^{14}C]warfarin, sufficient to enable reliable liquid scintillation counting (< 1% coefficient of variation), was used in all experiments and total warfarin concentration was varied with unlabelled drug. Equilibration time in a tumbling water bath (25°) was 18 hr; the adequacy of this period was verified by initially placing drug on one or other side of the bag and obtaining the same results. Bound and free drug concentrations were calculated by taking aliquots from each side of the membrane, counting radioactivity (Packard, model B2450) and conversion to molar concentration from known specific activities. The binding of unlabelled phenylbutazone alone to HSA under the same conditions was determined using a HPLC assay [6].

Data analysis. Warfarin binding data, up to a molar ratio of drug bound to HSA concentration ($\bar{n}_x$) of 1.5, were fitted by non-linear regression [7] to the following general model [2] originally given by Adair [8]:

$$\bar{n}_x = \frac{\sum_{j=0}^{N} j \binom{N}{j} \frac{X^j}{K_0 \ldots K_j}}{\sum_{j=0}^{N} \binom{N}{j} \frac{X^j}{K_0 \ldots K_j}}; \qquad K_0 = 1 \quad (1)$$

where X = molar free warfarin concentration, K_j = site equilibrium (intrinsic) dissociation constant and N = number of binding sites (=2).

The directly competitive model for the first site of each of warfarin and phenylbutazone on HSA was

tested as follows: equation (3)

$$\bar{n}_x = \frac{X}{X + K_x\{1 + Y/K_y\}} \quad (2)$$

$$\bar{n}_y = \frac{Y}{Y + K_y\{1 + X/K_x\}} \quad (3)$$

where Y = molar free phenylbutazone concentration, was solved for Y to give equation (4)

$$Y^2 + \left(K_y + \frac{K_y X}{K_x} + P_t - Y_t\right) Y - \left(K_y Y_t + \frac{Y_t K_y X}{K_x}\right) = 0 \quad (4)$$

where P_t = total concentration of HSA (liganded and unliganded) and Y_t = total (bound plus free) molar phenylbutazone concentration. Solution of this quadratic equation gave Y as a function of Y_t and X. For each warfarin titration curve at a fixed total phenylbutazone concentration, Y was then calculated for experimental values of X, and a corresponding series of $\bar{n}_x (< 0.5)$ generated according to equation 2. The dissociation constants of these theoretical data ($\bar{n}_x$, X) were then estimated by non-linear regression.

A similar technique was used to determine whether the shift in the first dissociation constant of warfarin in the presence of increasing concentrations of phenylbutazone could be explained by a negatively cooperative interaction with the first site of phenylbutazone. The equation for such an interaction is [2]:

$$\bar{n}_x = \frac{X}{X + K_x K_{xy}\{\varepsilon + 1\}/\{\varepsilon + K_{xy}\}} \quad (5)$$

where

$$K_{xy} = \frac{YPX \cdot P}{PX \cdot YP} \quad (6)$$

in the reaction

$$YP + PX = YPX + P \quad (7)$$

and $\varepsilon = Y/K_y$, YPX = concentration of HSA with the first sites for warfarin and phenylbutazone occupied, P = concentration of HSA with neither of the first sites occupied, and YP and PX = concentrations of HSA with the first site for phenylbutazone and warfarin occupied respectively. Using an analogous form of equation 5 for $\bar{n}_y$, free phenylbutazone concentrations (Y) were estimated for given values of X, Y_t and K_{xy} by the Newton–Raphson method. These values of Y were then entered into equation 5 to give a series of predicted $\bar{n}_x$ (< 0.5) for the experimental X values, and K_{xy} was optimized [7] to give the best least squares fit between experimental and predicted values of $\bar{n}_x$ for both the 10 and 100 μM phenylbutazone data sets simultaneously.

In all non-linear regressions, each data point was weighted as the reciprocal of the value of the observation ($w_i = 1/\bar{n}_{x_i}$).

RESULTS

Figure 1 presents the warfarin binding data as Bjerrum plots. For a directly competitive interaction

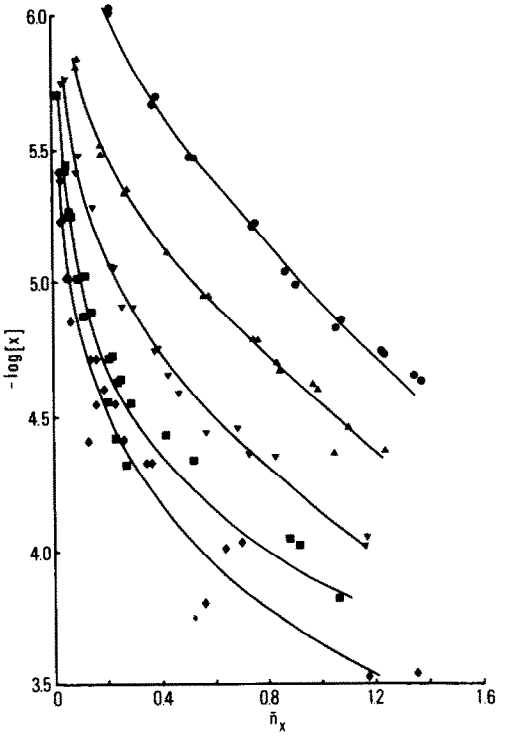

Fig. 1. Bjerrum plots of warfarin binding to HSA in the presence of varying concentrations of phenylbutazone; the points are experimental and the curves are predicted according to equation 1. Data for each phenylbutazone concentration are from two separate experiments. ●—control warfarin (zero phenylbutazone), ▲—10 μM, ▼—100 μM, ■—500 μM and ◆—1000 μM phenylbutazone (total concentration) present. HSA concentration was 10 μM. $\bar{n}_x$—molar ratio of bound warfarin concentration to HSA concentration, X—molar free warfarin concentration.

between the two drugs, the shift of the warfarin titration curves would be expected to continue without limit as phenylbutazone concentration increased, whereas for a negatively cooperative interaction the shape of the curves would be indistinquishable from the competitive case but the degree of shift would be finite. Visual inspection of Fig. 1 would more likely support the former mechanism of displacement than the latter because there does not seem to be any clear evidence of saturation. This is in contrast to saturation seen at 500 μM phenylbutazone for the same interaction when binding was measured by fluorescence enhancement [3].

The first two site dissociation constants for warfarin and HSA from the data of Fig. 1 are given in Table 1. Between experiment variation was minimal, particularly at the lower phenylbutazone levels, and this is reflected in the relatively small estimated dissociation constant errors (e.g. 7 amd 11% coefficient of variation for control warfarin). The uncertainty in the control phenylbutazone dissociation constants was greater, $K_1 = 3.44 \pm 30\%$ (μM) and $K_2 = 29.3 \pm 51\%$, mainly because of decreased precision in the HPLC assay. As expected from Fig. 1, the shift in K_1 did not appear to show any evidence of saturation. Affinity for warfarin at the second site (K_2) was not independent of phenylbutazone con-

Table 1. Effect of phenylbutazone on site dissociation equilibrium constants for the first two warfarin sites on HSA

Total inhibitor concentration (phenylbutazone, μM)	Observed K_1 (μM)	Observed K_2 (μM)	Predicted K_1 for direct competitive model (μM)	Ratio of relative shift
0	7.85 ± 7%	16.3 ± 11%	7.85	
10	28.5 ± 6%	29.0 ± 11%	24.2	1.18
100	74.9 ± 5%	66.1 ± 13%	227	0.28
500	195 ± 13%	119 ± 37%	1140	0.52
1000	294 ± 14%	215 ± 37%	2280	0.75

Dissociation constant errors given as coefficient of variation.

Ratio of relative shift computed as change in K_1 progressing from one phenylbutazone concentration to the next, divided by the corresponding change in predicted K_1, e.g. (74.9/28.5)/(227/24.2) = 0.28.

centration. Discrepancies between observed K_1 and that predicted according to a single site competitive model were large and consistent (e.g. 74.9 compared to 227 μM), indicating inadequacy of the model beyond experimental error. The (relative) incremental change in K_1 for a given increase in phenylbutazone concentration was biphasic, suggesting a change of interaction mechanism at or about 100 μM phenylbutazone. A value of $K_{xy} = 0.33 \pm 12\%$ ($s_{n_x} = 0.17$, $r = 0.8$) was obtained assuming that the interaction between the first sites of warfarin and phenylbutazone on HSA was cooperative. However, this model did not explain all variation in the data, as residuals between the 10 μM and 100 μM phenylbutazone data sets were non-random.

DISCUSSION

It is ironical that serum albumins were extensively used during the 1940's in the development of experimental methodology and theory of protein binding, yet despite numerous studies concerned with extent and variation in binding of many different ligands and enormous advances in knowledge at the molecular level in more exotic proteins, comparatively little is still known about mechanisms of binding in HSA [9].

One of the problems in analysis of drug albumin binding data is the uncertainty of N, the total number of binding sites per protein molecule; this number is usually large and very dependent on how vigorously the total titration curve is pursued. With large N, application of more flexible binding models like equation 1 becomes increasingly difficult, and usually Scatchard-type models (site independence, equivalent affinity within a class of binding sites) are used. This dependency has caused problems in interpretation of data, e.g. the concept that drugs may have a single primary site of high affinity and a number of others of very much lower affinity. It is not often admitted that the discrepancy between class affinities is correlated with estimates of capacity and the assumption of site equivalency within a class. Hence in the second class, the larger the number of sites, the smaller the estimated affinity, and the greater the apparent differences in affinity between classes. When the control warfarin data in Fig. 1 were analysed using a two class binding model, the parameter estimates were: $n_1 = 0.8$, $K_1 = 3.0$ μM, $n_2 = 4.6$ and $K_2 = 140$ μM ($r = 0.996$ and $s_{\cdot n x} = 0.04$) suggesting a fifty-fold ratio of affinities rather than two-fold, as found with equation (1). The effect of rounding non-integral capacity estimates on affinity estimates is not often mentioned (in the analysis above it might be assumed that $n_1 = 1$, but K_1 is not adjusted from 3 μM). The site independence assumption in Scatchard-type analyses has come under increased scrutiny in recent years [10] and newer, more sophisticated models have been reported in the literature [11–13].

In the present study, equation 1 was used to summarize and interpret the primary binding data. Since there was no evidence of binding saturability, interest was confined to the most important segment of the titration curve, that involving the first two sites. By restricting experimental data to $\bar{n}_x < 1.5$ and setting $N = 2$, it was hoped that the contribution of the second site to total binding in the range $0 < \bar{n}_x < 1$ could be allowed for, and that consequently the estimate of K_1 would be unbiased, while accepting that uncertainty in K_2 would be greater (because of binding contributions from the third site) and that it would be biased to some extent. For this reason quantitative modelling was only done on affinity changes for the first site, and trends in K_2 were interpreted qualitatively. When the experimental binding data were restricted to $\bar{n}_x < 0.5$, the estimate for K_1 was 18% depressed, and based on the common observation that differences in affinity between sites decrease with increased binding, the bias in the estimated values of K_2 in Table 1 would be less than 18% too low.

The difference in saturability of the shift of titration curves established by fluorescence assay [3] and equilibrium dialysis in the present study was unexpected. While it is usually reasonable to assume that high affinity sites characterized by different techniques are the same, it is not always so; quantitation of binding by fluorescence enhancement may only be concerned with certain sites on the HSA molecule, while equilibrium dialysis monitors binding to all sites. No value judgement can be made on which technique gives the better results as it depends on the purpose of the study; in certain circumstances

the reduction in complexity with a technique that only 'sees' certain sites may enable a start to be made on a complicated problem.

Interpretation of the data in Table 1 is best done conservatively in a sequential manner, beginning with the simplest plausible model and only progressing to more complicated models when inadequacies are demonstrated. The simplest model for the interaction of warfarin and phenylbutazone with HSA is one with site independence where both drugs have a primary or high affinity site on HSA, and it is the same site for both. The data in Table 1 do not support this hypothesis. In the presence of 10 μM phenylbutazone, the observed dissociation constant (K_1) increased to a slightly greater extent than could be accounted for by direct competition (28.5 μM compared to 24.2 μM) given the estimated independent dissociation constants (7.85 and 3.44 μM for warfarin and phenylbutazone respectively). Above this concentration of phenylbutazone, the observed K_1 was less than that predicted by competition, and this difference increased in a regular manner and to an extent that leaves no doubt about the unacceptability of this model (74.9 μM compared to 227 μM, 195 to 1140 μM and 294 to 2280 μM).

The next least complicated model of the interaction would be to postulate that the primary sites of warfarin and phenylbutazone are separate but not independent. This model is compatible with changes in K_1 at 10 μM phenylbutazone, but does not seem to fit with changes at the highest levels (500–1000 μM) where there is no evident saturation. However, if the relative change in K_1 for a given increment in phenylbutazone concentration is compared with the corresponding change in the predicted competitive K_1 (Table 1), it appears that saturation in shift is being approached at 100 μM phenylbutazone (ratio = 0.28). At higher levels, the degree of shift increases again, up to a ratio of 0.75 for 1000 μM phenylbutazone. An explanation for this result could be that with phenylbutazone concentrations up to 100 μM, the affinity of warfarin for its primary site on HSA is reduced by a negatively cooperative mechanism, but at higher levels, the increased concentration of free phenylbutazone begins also to compete directly for the primary warfarin site and therefore causes the titration curve to continue to shift. A similar two stage mechanism has been described for the interaction of 1-anilino-8-naphthalene-sulphonate and 3,5-dihydroxybenzoate with bovine serum albumin [14]. In another study it has been found that medazepam marginally increases the binding of bilirubin to HSA by a cooperative mechanism at low concentration, but then decreases binding at higher levels by a second competitive mechanism [15].

The hypothesis of a negatively cooperative interaction between the two drugs and HSA up to a phenylbutazone concentration of 100 μM was quantitatively tested using equation 5 and the best estimate of K_{xy} was 0.33 ± 12%. This model considered only the first sites for each of warfarin and phenylbutazone, and for such a model $K_{xy} < 1$ infers a lower concentration of YPX than of PX or YP (equations 6 and 7), i.e. antagonism toward the doubly liganded protein, or negative cooperativity. $K_{xy} = 0.33$ corresponds to a thermodynamic coupling energy of 2.8 kJ/mole.

Even though the estimated uncertainty of K_{xy} was small (± 12%), suggesting a good model, there was some inconsistency between the two data sets (10 and 100 μM phenylbutazone) leading to residuals which were not randomly distributed. This could have been due to cooperative saturation being achieved at less than 100 μM phenylbutazone, and binding data at 100 μM already having some component of the second competitive mechanism; consideration of the phenylbutazone concentration to dissociation constant ratio (100/3.4 μM = 29.4) would support this interpretation. Alternatively, the single site model for each of warfarin and phenylbutazone may not be an entirely sufficient explanation for the displacement. Inspection of Table 1 shows that K_2 is affected by phenylbutazone concentration in a gradual manner, and more particularly, the magnitude of K_2 changes relative to K_1; in the control situation K_1 is less than K_2, at 10 μM phenylbutazone they are equivalent, and thereafter the magnitude of K_1 becomes increasingly greater than K_2. Weber [2] has described a similar pattern of results, and the simplest model is one where the effector (phenylbutazone) has a separate thermodynamic coupling to each of the two warfarin sites. In the present study the situation is slightly more complicated in that the two warfarin sites do not begin with identical affinity, but the development of an apparent positive cooperativity between them (K_2 becoming less than K_1 with increasing phenylbutazone concentration) when actually the interaction is negatively cooperative between phenylbutazone and warfarin, is identical to the earlier study [2]. Furthermore, preliminary work on the reciprocal experiment of the effect of warfarin on the first two phenylbutazone sites show similar cooperative behaviour, suggesting quite a complex interaction between the two ligands.

A number of reports have recently appeared in the literature concerned with characterisation of specific binding sites on HSA which are common for various drugs. The basic approach in these studies is to observe the effect of different drugs on binding of suitable markers, whereupon three results are possible—the binding may be reduced, unchanged or increased. Where the binding is unchanged, it may be valid to conclude that the binding sites are separate or independent, and if the binding is increased, then usually cooperative effects are suggested. However, when binding is decreased it is usually assumed, without further evidence, to mean that the test drug and the marker ligand are competing for the same site, and so lists are compiled of all the drugs binding to that particular site. The present work with phenylbutazone, warfarin and HSA, a system long thought of as a prototype of simple competitive displacement, should serve as a caution in too ready assignment of common sites based solely on a displacement result. Independently of this conclusion, there is debate over the assignment and discreteness of these sites. Initially, two distinct sites were proposed [16], but it has lately been suggested that they are not distinct but overlap [17], while other workers have described three spe-

cific sites and indicated that there may be more [5]. Sjoholm *et al.* [5] maintain that dicoumarol binds to the diazepam site on HSA, but Fehske *et al* [18] concluded that these two drugs have separate sites which are located close to one another. Sjoholm *et al.* [5] report indomethacin as binding to both the diazepam and warfarin sites, while Zini *et al.* [19] concluded that indomethacin had other completely separate sites. With these problems, it may be that a cooperative viewpoint of displacement phenomena with HSA will provide a more stable framework for subsequent understanding than continuing amendments to the common site model; direct competition in this framework is simply then a special case (with a degree of incidence to be established) of a more general phenomenon.

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