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Investigations into the binding of phenprocoumon to albumin using fluorescence spectroscopy

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The fluorescence of phenprocoumon is enhanced following interaction with bovine and human serum albumin, allowing the binding parameters for two binding sites to be estimated by a modified Bjerrum method and the Scatchard method. The fluorescence is increased as the pH is raised to 9·0 and also on addition of the drugs ibuprofen and fenoprofen. Chloride ions, phenylbutazone and acenocoumarin seem to displace phenprocoumon from its primary binding site on human serum albumin. Investigations with the enantiomers of phenprocoumon show that the binding sites have some stereospecificity.

The high affinity of the coumarins for human serum albumin (HSA) has received much attention, particularly since it was realized that their anticoagulant action was frequently potentiated by the presence of other drugs in the plasma. More recently pharmacokinetic and pharmacodynamic investigations of the enantiomers of warfarin have shown different metabolic fates for the two forms (Breckenridge et al 1974) whereas the enantiomers of phenprocoumon show differences in volumes of distribution (Jahnchen et al 1976). The more potent *S*(−)-phenprocoumon is more strongly bound to HSA than is the *R*(+)-enantiomer. This observation has been confirmed by a recent limited dialysis investigation (Brown et al 1977). In the present investigation, the enhanced fluorescence of phenprocoumon following interaction with HSA is used to investigate the binding characteristics of the enantiomers to HSA at low drug to protein ratios, and to investigate the possibility of displacement of the drug from its primary binding site by other commonly prescribed drugs.

MATERIALS AND METHODS

HSA (lot NO. 126C–8070) and BSA (lot NO. 17C–8145), crystallized and lyophyllized, and essentially fatty acid free HSA (lot NO. 76C–7480) were obtained from Sigma Chemical Co., St. Louis, MO. Racemic phenprocoumon (Oraganon, Inc., W. Orange, N.J.), *S*(−)- and *R*(+)-phenprocoumon (Hoffman-LaRoche, Basel, Switzerland), phenylbutazone and acenocoumarin (Ciba-Geigy Company, Summit, N.J.), sodium fenoprofen (Eli Lilly Company, Indianapolis, IN) and sodium 2 (*p*-chlorophenoxy)-2-methylpropionate (CPIB) (ICI Ltd., Macclesfield Cheshire, U.K.) were used as supplied by the manufacturer. All other materials were reagent grade and all solutions were prepared in deionized water.

All solutions were prepared in 0·1 M phosphate buffer of pH 7·4 at 22 ± 1 °C. HSA concentrations of $0{\cdot}1\text{–}5{\cdot}0 \times 10^{-5}$ M (mol. wt 69 000) were used. Fluorescence measurements were made using a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The fluorometric titrations were as follows: 2·0 ml of the protein solution of an appropriate concentration in a 10 mm path length cell were titrated by the successive additions of 2·0 μl volumes of phenprocoumon solution to give a final drug concentration of $0{\cdot}1\text{–}20 \times 10^{-6}$ M in the cell. The fluorescence intensity was measured at 382 nm following excitation at 340 nm. For the experiments in the presence of antagonists, the fluorescence of phenoprocoumon bound to albumin was measured before and after the addition of the antagonist. The ratio of phenprocoumon to albumin was 0·1 and the ratio of competitor to protein ranged from 0·1 to 3·0.

RESULTS

The fluorescence intensity of phenprocoumon was enhanced when bound to crystalline, essentially fatty acid free HSA, and to bovine serum albumin (BSA). The fluorescence peak was also shifted from 386 nm to 382 nm following interaction with the albumin and excitation at 340 nm. Fluorometric titrations were made by altering the drug concentration at a fixed albumin concentration as shown in Fig. 1. For curve a, determined at high protein to drug ratios, all the drug is bound and this is a measure of the fluorescence of the drug-albumin complex. At lower albumin concentrations a curve is obtained as shown in Fig. 1, curve b. The fractions of the drug

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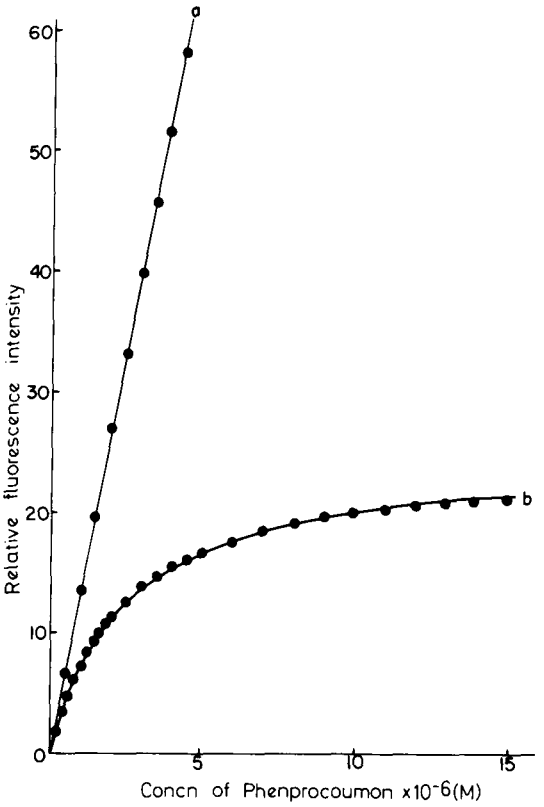

FIG. 1. Plots of relative fluorescence intensities as a function of phenprocoumon concentration for the phenprocoumon—HSA interaction. a = high, b = lower concentration.

bound, X, can be determined by using the equation:

$$X = \frac{F_p - F_o}{F_b - F_o} \quad (1)$$

where F_p and F_o are the fluorescence intensities of a given concentration of drug in a solution of low albumin concentration and in solution without albumin. F_b is the fluorescence of the same concentration of fully bound drug. For this treatment to be valid, the fluorescence intensity of the bound drug must be a linear function of concentration. This is the case only when the absorbance of the complex at the exciting wavelength is low. A correction for this absorption can be made by the method already described (Otagiri et al 1979) and is made for all the data when the absorbance at 340 nm is greater than 0·02.

This method of calculating the fraction bound gives the expected binding constant for 1:1 complexes, and for higher complexes if the quantum yields associated with the various binding sites are identical. Analysis of the data of Fig. 1, curve b, by an iterative least squares technique, assuming 1:1 complex formation, were unsuccessful. Apparently the binding of more than one ligand molecule to the albumin accounts for the change in fluorescence. This observation is confirmed by the Job plot shown in Fig. 2. Although the peak for the two HSA samples is near 0·5, the value expected for 1:1

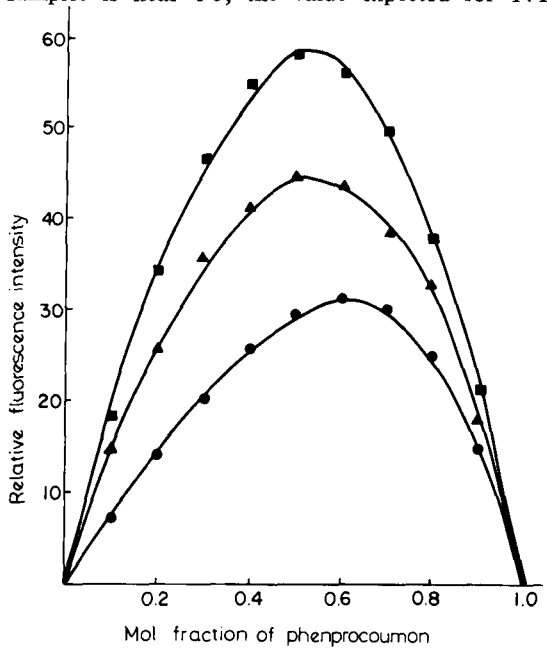

FIG. 2. A Job plot of relative fluorescence intensities for the phenprocoumon-serum albumin interaction ■: HSA ▲: FFA ●: BSA.

complex formation, the tangents at the origins are not equal in magnitude and opposite in sign as is necessary for 1:1 complex formation (Job 1928). The quantum yield associated with the lower affinity sites appears to be much lower than that associated with the first site. This means that equation (1) does not strictly apply to the data obtained at high drug to alumin ratios, and the second binding constant obtained by this method is only an estimate of the method. This problem is shared by other spectroscopic techniques and is not peculiar to the fluorescence technique (Perrin et al 1975). It was found that the data fitted a modified Bjerrum model for two independent sites (Naik et al 1975) is:

$$P + D \rightleftharpoons PD \quad (2)$$

$$K_1 = \frac{[PD]}{[P]\,[D]},\ K_2 = \frac{[PD_2]}{[PD]\,[D]} \quad (3)$$

$$\bar{n} = \frac{C_D - [D]}{C_p} = \frac{K_1[D] + 2K_1K_2[D]^2}{1 + K_1[D] + K_1K_2[D]^2} \quad (4)$$

using the following equation

$$\frac{\bar{n}}{(2-\bar{n})[D]^2} = \frac{1-\bar{n}}{(2-\bar{n})[D]} K_1 + K_1K_2 \qquad (5)$$

where P, D and PD are the concentrations of unbound albumin, unbound drug, and the complex respectively. C and C_p are the total concentrations of drug and albumin and $\bar{n}$ is the moles of drug bound per mole of albumin. A least squares analysis of the linear equation 5 gives the binding data shown in Table 1 for the phenprocoumon—HSA and BSA interactions, and in Table 2 for the enantiomers to HSA interactions. Table 1 also shows the binding

Table 1. Binding parameters for phenprocoumon-serum albumin complexes. K_1 and K_2 are binding constants for two binding sites and r is the correlation coefficient for data points.

Bjerrum method	pH 6·5	r	pH 7·4	r	pH 8·5	r
BSA						
$K_1(n_1 = 1)$	..	..	$0·9 \times 10^6$	0·997	—	—
$K_2(n_2 = 1)$	—	—	$7·0 \times 10^4$	—	—	—
HSA						
$K_1(n_1 = 1)$	$1·0 \times 10^6$	0·994	$1·1 \times 10^6$	0·999	$1·3 \times 10^6$	0·997
$K_2(n_2 = 1)$	$2·2 \times 10^4$	—	$1·6 \times 10^4$	—	$2·0 \times 10^4$	—
FFA						
$K_1(n_1 = 1)$	$1·0 \times 10^6$	0·998	$1·0 \times 10^6$	0·998	$1·4 \times 10^6$	0·998
$K_2(n_2 = 1)$	$1·9 \times 10^4$	—	$3·2 \times 10^4$	—	$5·2 \times 10^4$	—
Scatchard method						
BSA						
$K_1(n_1 = 1)$	—	—	$8·0 \times 10^5$	—	—	—
$K_2(n_2 = 1)$	—	—	$1·2 \times 10^5$	—	—	—
HSA						
$K_1(n_1 = 1)$	$1·0 \times 10^6$	—	$9·5 \times 10^5$	—	$1·6 \times 10^6$	—
$K_2(n_2 = 1)$	$2·1 \times 10^4$	—	$5·0 \times 10^4$	—	$1·0 \times 10^5$	
FFA						
$K_1(n_1 = 1)$	$9·8 \times 10^5$	—	$1·9 \times 10^6$	—	$1·9 \times 10^6$	—
$K_2(n_2 = 1)$	$7·4 \times 10^4$	—	$3·8 \times 10^4$	—	$2·8 \times 10^4$	—

parameters calculated by the Scatchard method (Otagiri et al 1979) assuming two independent binding sites, enough material was not available to obtain the data points at the high drug to albumin ratios necessary for reliable estimates of the binding parameters of the enantiomers by the Scatchard method.

The fluorescence intensity of the phenprocoumon-HSA and -BSA complexes increases as the pH is increased from 6–9 (Fig. 3) and is decreased upon the

Table 2. Binding parameters of binding of phenprocoumon and its enantiomers to HSA at pH 7·4. K_1 and K_2 are binding constants for two binding sites and r is the correlation coefficient for data points.

Phenprocoumon (Bjerrum)	K_1	K_2	r
S(—)-	$1·3 \times 10^6$	$2·3 \times 10^4$	0·998
R(+)-	$0·8 \times 10^6$	$1·5 \times 10^4$	0·998
RS(±)-	$1·1 \times 10^6$	$1·6 \times 10^4$	0·997

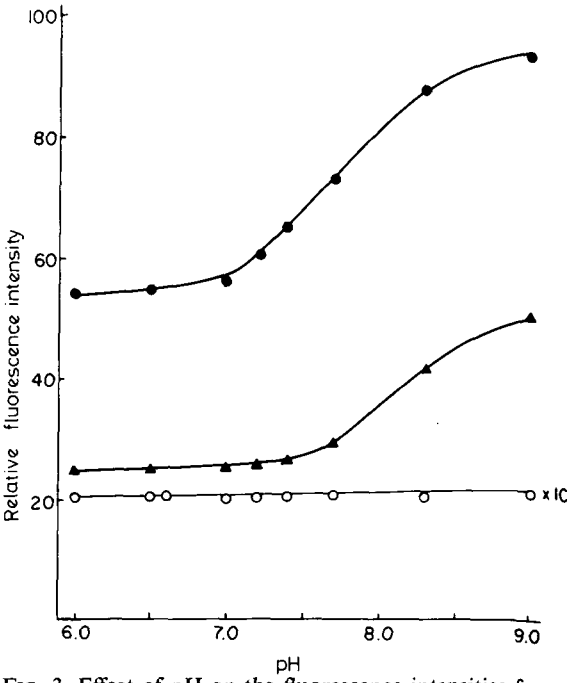

FIG. 3. Effect of pH on the fluorescence intensities for phenprocoumon-serum albumin interactions. ○: phenprocoumon alone ●: phenprocoumon-HSA system ▲: phenprocoumon-BSA system.

addition of sodium chloride (Fig. 4). The fluorescence intensity of complexes is lowered by phenylbutazone and acenocoumarin, but not significantly altered by clofibrate in Fig. 5. The fluorescence is slightly increased by ibuprofen and fenoprofen.

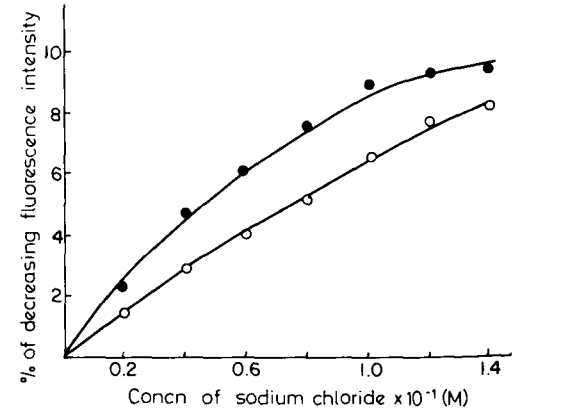

FIG. 4. Effect of sodium chloride on fluorescence intensities for phenprocoumon-serum albumin interactions at pH 7·4. ●: HSA ○: BSA Ordinate: decrease in fluorescence as a percentage of original fluorescence.

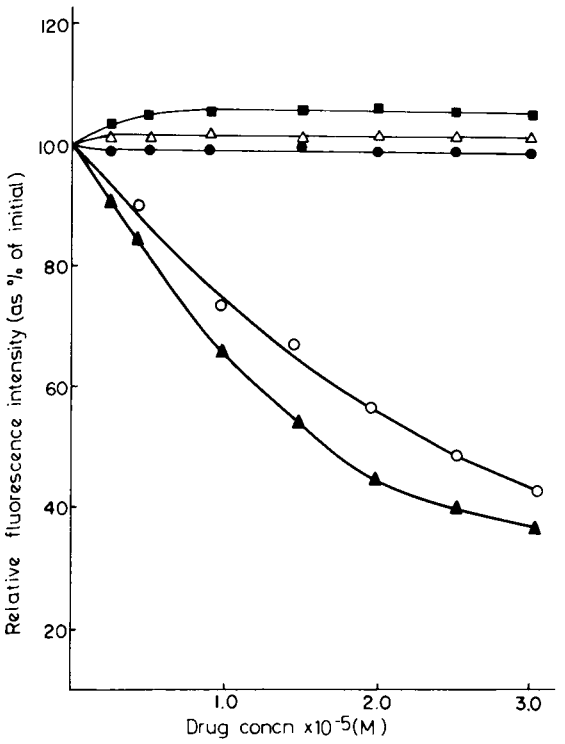

FIG. 5. Effect of various drugs on fluorescence intensity of phenprocoumon in the presence of serum albumin ○: acenocoumarin ●: CPIB △: fenoprofen ▲: phenylbutazone ■: ibuprofen.

DISCUSSION

The fluorescence of phenprocoumon is enhanced following the interaction with albumins, allowing a quantitative investigation of the binding phenomena. Although the enhanced fluorescence is mainly the result of the interaction at a single site, the data cannot be interpreted in terms of a 1 : 1 reaction but gave a better fit with a two site model. A similar situation has earlier been reported for the interaction of warfarin with HSA, also investigated by this fluorescence technique (Otagiri et al 1979). The binding constants given in Table 1 show that the affinity of phenprocoumon for albumin increases as the pH is increased. This increased binding is also reflected in the increased fluorescence of the complex as the pH is increased. For the binding at the first site there is little difference between crystalline and essentially fatty acid free HSA. However the binding at the second site of crystalline HSA appears to be pH independent, whereas with essentially fatty acid free HSA, the binding constant increases with pH. The N → B transition in HSA occurs over the pH region 6–9 (Leonard et al 1963; Harmsen et al 1971) and apparently the binding to the secondary site in the B form is enhanced following the treatment to remove the fatty acids. It should be noted that the binding at the first site is the phenomenon of clinical relevance and also that the affinity of warfarin for HSA also increases as the pH is increased. These changes as a function of pH must be due to changes in the protein rather than the changes in the degree on ionization of the coumarins as the pK_a of phenprocoumon is 4·30 and of warfarin is 5·10 (Otagiri et al 1978). Data of Table 2 show that the affinity for the *S*(−)-enantiomer is greater than the affinity for the *R*(+)-enantiomer for both the first and second site on crystalline HSA, the racemic form, the commercial form of the drug, being of intermediate behaviour. These observations are in agreement with those reported earlier, however, the binding constants reported here are significantly higher than those reported by Brown et al (1977). In the current investigations more data are available at low drug to protein ratios, and so greater reliability can be placed on the computed binding constants than those of Brown et al. Although the binding sites seem to be non-specific, in that a wide range of acidic drugs seem to share the same binding sites, the sites do show some stereospecificity. Sodium chloride diminishes the fluorescence of HSA and BSA complexes with phenprocoumon as shown in Fig. 4. Although chloride has a small effect on the N → B transition at the physiological pH (Harmsen et al 1971), the effect of chloride appears to be primarily one of displacement as was observed with warfarin (Wilting, Van der Giesen, Janssen, Weideman, Otagiri, submitted for publication). Fenoprofen and ibuprofen slightly increased the fluorescence of phenprocoumon-HSA complexes at pH 7·4; in an earlier study ibuprofen had been shown to increase the fluorescence of warfarin-HSA complexes (Otagiri et al 1979). When the enhancement caused by these non-steroidal anti-inflammatory agents is compared with that caused by pH changes (Fig. 3) it is possible to conclude that the two drugs cause an increase in the B conformation of albumin. Sudlow et al (1976) have indicated that warfarin and ibuprofen do not share the same primary binding site on HSA. Clofibrate did not significantly lower the fluorescence of the phenprocoumon complexes, whereas acenocoumarin and phenylbutazone did as shown in Fig. 5. These observations are in agreement with the classification of Sudlow et al. Binding constants of $8 \times 10^5\ M^{-1}$ for acenocoumarin and $1{\cdot}2 \times 10^5\ M^{-1}$ for phenylbutazone can be estimated from these data (Perrin et

al 1975), however, unpublished observations in these laboratories, suggest that phenylbutazone may have a small effect on the N → B transition.

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