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Interaction of substituted phenoxazine chemosensitizers with bovine serum albumin

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Abstract

The binding of 10-[3′-[*N*-bis(hydroxyethyl)amino]propyl]phenoxazine [BPP], 10-[3′-[*N*-bis(hydroxyethyl)amino]-propyl]-2-chlorophenoxazine [BPCP], 10-[3′-[*N*-bis-(hydroxyethyl)amino]propyl]-2-trifluoromethylphenoxazine [BPFP], 10-(3′-*N*-pyrrolidino propyl)-2-chlorophenoxazine [PPCP] or 10-(3′-*N*-pyrrolidinopropyl)-2-trifluoromethyl-phenoxazine [PPFP] to bovine serum albumin (BSA) has been measured by gel filtration and equilibrium dialysis methods. The binding of these modulators to bovine serum albumin based on dialysis experiments has been characterized by the following parameters: percentage (β) of bound drug, the association constant 'K_1', the apparent binding constant 'k' and the free energy $\Delta F°$. The binding of phenoxazine derivatives to bovine serum albumin is correlated with their octanol–water partition coefficient, $\log_{10} P$. In addition, the displacing activity of hydroxyzine and acetylsalicylic acid on the binding of phenoxazines to albumin has been studied. The results of the displacing experiments showed that the phenoxazine benzene rings and the tertiary amines attached to the side chain of the phenoxazine moiety are bound to a hydrophobic area on the albumin molecule.

Keywords: Phenoxazine; Hydrophobic interaction; Bovine serum albumin

1. Introduction

The study involving the binding of drugs to albumin has been described in numerous papers because of their pharmacokinetic significance [1]. But, the drug–albumin complex may be considered as a model for gaining general fundamental insights into drug–protein binding. General rules of protein binding gained from this model could apply at least partially to the drug–receptor complex provided that the receptor has a protein structure. The determination of albumin binding of several structurally related compounds is a valuable tool for identifying the groups of a drug molecule which are involved in binding and for

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characterizing the binding forces concerned with the interaction of drugs with protein.

The binding of analogous phenothiazine derivatives to BSA has been studied [1]. Although, most of the authors obtained total binding constants of the same order of magnitude, the number of binding sites varied considerably. It has been found [2] that the number of binding sites on BSA for promazine and chlorpromazine changed with the concentration of drugs, higher numbers being obtained at higher drug concentrations. They suggested that phenothiazine derivatives are bound by hydrophobic interaction with the aromatic amino acids of the BSA molecule and that under the influence of high drug concentrations, the number of available sites increased by swelling and unfolding of the BSA molecules in solution. Glasser and Krieglstein [3] correlated the $\log_{10} P$ octanol (at pH 7.4) of some phenothiazine drugs and related compounds with their $\log(\beta/\alpha)$ values, 'β' and 'α' being the fractions of bound drug and free drug respectively. They obtained a fairly good linear correlation ($r = 0.969$) for five 10-dimethylaminopropyl derivatives of phenothiazine, but when other drugs were included the correlation deteriorated.

Compounds of pharmacological interest have been found among phenoxazine derivatives and they have been claimed to be nervous system depressants in particular with sedative, antiepileptic, tranquillising activity [4–9], spasmolytic activity [10], antitubercular activity [11] and anthelmintic activity [12]. Multidrug resistance [MDR] has become a major obstacle for the clinical treatment of cancer as well as microbial diseases. A variety of small molecules capable of modulating MDR have been prepared and examined. While a number of pharmacological agents have been shown to reverse MDR in vitro, there remains a need to identify more potent, more specific and less toxic chemosensitizers for clinical use. In an attempt to search for more potent and less toxic chemosensitizers, Thimmaiah et al. [13] have reported that the parent phenoxazine potentiated the uptake of vincristine (VCR) and vinblastine (VLB) in MDR GC_3/cl and $KBCh^R$-8-5 cells to a greater extent than verapamil, a standard modulator. However, it was less effective in sensitizing MDR cells, in part, due to its instability in culture medium. In a subsequent study [14,15], 20 N^{10}-substituted phenoxazines were synthesised and examined for their ability to enhance the uptake of VLB and VCR in GC_3/cl and $KBCh^R$-8-5 cells. Recently, Thimmaiah et al. [16] have demonstrated that 2-chlorophenoxazines were able to partially reverse VLB resistance in MDR colon carcinoma cell line GC_3/cl and completely reversed the 86-fold VLB resistance in the MDR1 overexpressing breast carcinoma cell line BC 19/3. The same agents could partially sensitize BC 19/3 cells to taxol and doxorubicin suggesting that the chlorophenoxazines show some specificity for modulating VLB resistance. The results revealed that substitution on the phenoxazine ring at position N^{10} was associated with an increase in antiproliferative and anti-MDR activities. Nevertheless, the exact mechanism of pharmacological action of phenoxazines remains unknown. Since these molecules are found to be useful as potential anti-MDR agents, studies have been undertaken to elucidate the nature of interactions with serum transporter protein (BSA).

2. Experimental

The synthesis and chemical characterization of BPP, BPCP, BPFP, PPCP and PPFP have been carried out according to published methods [14,17,18]. The structural formulas of the compounds are given in Table 1.

Acetylsalicylic acid, bovine serum albumin and hydroxyzine were purchased from Sigma (St Louis, MO). All other chemicals were of reagent grade. All binding measurements were made in the presence of 0.02 M phosphate buffer, pH 6.8 containing 0.15 M NaCl and 3 mM sodium thiosulphate [1]. Just before protein binding of a drug was determined, the pH of the sample solution was measured and where necessary adjusted to pH 6.8 with 0.1 N HCl or 0.1 N NaOH.

2.1. Separation of phenoxazine–albumin complex by gel-filtration experiment

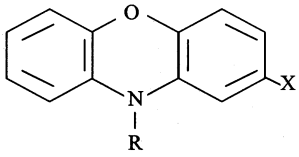
The binding of phenoxazine derivatives to albumin was studied with the aid of gel filtration

experiments. Gel filtration experiments were performed on a 20×1.3 cm column of Sephadex® G-50 fine (Pharmacia) at 22°C, equilibrated with standard buffer solution, the flow rate being maintained at 25 ml/h. BSA solution (20 ml, 1%) containing 1.0×10^{-4} M phenoxazine modulator, after incubation at 37°C for 6 h, was loaded onto the column and washed with standard buffer. The effluent from the column was collected into fractions of 3 ml. A 1.5-ml aliquot of each fraction was used to determine the protein concentration and the remaining 1.5 ml for the estimation of phenoxazine modulator. The albumin content was determined by the 'biuret' method and phenoxazine modulator estimated after extracting with *n*-heptane or chloroform colorimetrically in 50% sulfuric acid containing 10 mg% $FeCl_3$.

2.2. Study of interaction of phenoxazines with BSA by equilibrium dialysis

Sample solution (20 ml) containing BSA (1%) and one of the modulators [BPP, BPCP, BPFP, PPCP or PPFP] in the concentration range $0.1–5 \times 10^{-4}$ M was taken in a 50-ml centrifuge tube and incubated at 37°C for 6 h in a shaking

Table 1
Name, molecular structure, molecular weight and $\log_{10} P$ values of the phenoxazine derivatives



Compound	Name	*R*	*X*	MW	$\log_{10} P$
BPP	10-[3′-[*N*-bis(hydroxyethyl) amino]propyl]phenoxazine	$-CH_2-CH_2-CH_2-N(CH_2-CH_2-OH)(CH_2-CH_2-OH)$	–H	328.00	1.50
BPCP	10-[3′-[*N*-bis(hydroxyethyl) amino]propyl]-2-chlorophenoxazine	$-CH_2-CH_2-CH_2-N(CH_2-CH_2-OH)(CH_2-CH_2-OH)$	–Cl	362.00	2.20
BPFP	10-[3′-[*N*-bis(hydroxyethyl) amino] propyl]-2-trifluoromethylphenoxazine	$-CH_2-CH_2-CH_2-N(CH_2-CH_2-OH)(CH_2-CH_2-OH)$	$-CF_3$	396.00	2.30
PPCP	10-(3′-*N*-Pyrrolidinopropyl)-2-chlorophenoxazine	$-CH_2-CH_2-CH_2-N(CH_2-CH_2)(CH_2-CH_2)$	–Cl	365.21	2.40
PPFP	10-(3′-*N*-pyrrolidinopropyl)-2-trifluoromethylphenoxazine	$-CH_2-CH_2-CH_2-N(CH_2-CH_2)(CH_2-CH_2)$	$-CF_3$	398.50	2.80

water-bath incubator. For each of the four dialysis tubings (3/4″ diameter), 4 ml of the above reaction mixture was pipetted out. After closing, the dialysis tubing was immersed in a standard buffer solution taken in a measuring jar. The dialysis tubings were agitated up and down mechanically (12 h, 22°C). At the end of the dialysis experiment, the free phenoxazine modulator from the buffer medium was extracted into chloroform. After evaporating the organic layer, the phenoxazine concentration was measured colorimetrically using 4 ml of 50% sulfuric acid containing 10 mg% $FeCl_3$.

2.3. Displacement of phenoxazine modulator from BSA binding site by equilibrium dialysis

The displacement of phenoxazines by hydroxyzine or acetylsalicylic acid was studied by means of equilibrium dialysis. In these experiments, the binding of the phenoxazine modulator to BSA was determined after the displacing agents, hydroxyzine or acetylsalicylic acid were added to the incubation mixture.

2.4. Measurement of lipophilicity

The relative lipophilicity at pH 7.4, of each of the compounds used in this study, was assessed using an adaptation of the method of Zamora et al. [19]. This method involves measuring the partitioning of modulator between 1-octanol and PBS (pH 7.4). HPLC grade 1-octanol was pre-saturated with aqueous phase buffer and conversely buffered aqueous phase was pre-saturated with HPLC grade 1-octanol before use. The modulator was dissolved in aqueous phase buffer/octanol at a final concentration of 1×10^{-4} M, an equal volume of 1-octanal/buffer was added and the tubes were then continuously inverted for 15 min (experiments carried out over time intervals ranging from 5 to 60 min confirmed that equilibration was reached within 15 min). The final concentration of modulator in both aqueous and octanol fractions was assessed by measuring the UV absorbance of these experimental fractions. The partition coefficient, P, was determined by dividing the concentration of modulator in the 1-octanol by the concentration in the aqueous phase. $\mathrm{Log}_{10} P$ was used as a measure of lipophilicity.

3. Results and discussion

3.1. Evaluation of binding parameters

The binding of MDR modulators [BPP, BPCP, BPFP, PPCP and PPFP] to BSA based on dialysis experiments was characterized by the parameters, percentage (β) of bound modulator, the association constant, 'K_1', the apparent binding constant 'k' and the free energy $\Delta F°$ (Table 3). The symbols, dimensions and methods of analysis of the values used to characterize the protein binding and the hydrophobic character of the modulators are summarized in Table 2. The results of gel filtration experiment revealed that the bound phenoxazine modulator moves with the velocity of BSA. When experiments were performed in series for one substance, only the fractions after the protein zone have been assayed for phenoxazine modulator.

The effect of concentrations of modulators [BPP, BPCP, BPFP, PPCP and PPFP] on the binding to BSA was studied by dialysis experiments. In these studies, the concentrations of the modulator were varied in the range $0.1–5 \times 10^{-4}$ M and keeping the protein concentration constant (1%) and the data are shown in Fig. 1. Examination of the data has revealed that the binding increased with increasing concentration of phenoxazine at low modulator/protein ratios. The amount of free drug remains the same in spite of the fact that the concentration of the modulator was further increased (Fig. 1), suggesting a higher number of binding sites on BSA, a similar observation made in the case of binding of phenothiazines to BSA [1]. The apparent binding constant 'k' and regression coefficient 'm' were calculated by plotting concentration of free-phenoxazine modulator versus concentration of bound-phenoxazine modulator (Fig. 2). In order to calculate the total binding constant (association constant) K_1, a Scatchard plot for the binding of the phenoxazine modulators to BSA was done (Fig. 3). Comparison of the k and K_1 values within the compounds

Table 2
Symbols, dimensions and methods of analysis of the parameters used

Parameter	Symbol	Dimension	Method of analysis
Total concentration of phenoxazine derivative	c	M	By weight, colorimetry
Concentration of free phenoxazine derivative	c_f	M	Gel filtration, dialysis
Concentration of bound phenoxazine derivative	c_b	M	$c_b = c - c_f$
Concentration of albumin	c_a	g/100 ml	By weight, biuret method
Percentage of free phenoxazine derivative	α	%	Gel filtration, dialysis
Percentage of bound phenoxazine derivative	β	%	$\beta = 100 - \alpha$
Specific binding capacity	$\bar{r}$	M/M	$\bar{r} = c_b/c_a$ (mol)
Regression coefficient	m	–	Fig. 2
Apparent binding constant	k	$(10^{-5}\ M)^{1-m}$	$k = c_b/c_f$ (Fig. 2)
Association constant	K_1	$10^4\ M^{-1}$	Scatchard plot (Fig. 3)
Free binding energy	$\Delta F°$	Cal/M	$\Delta F° = -RT \ln K_1$
Partition coefficient	P	M/M	Partition between n-octanol and buffer solution, pH 7.4

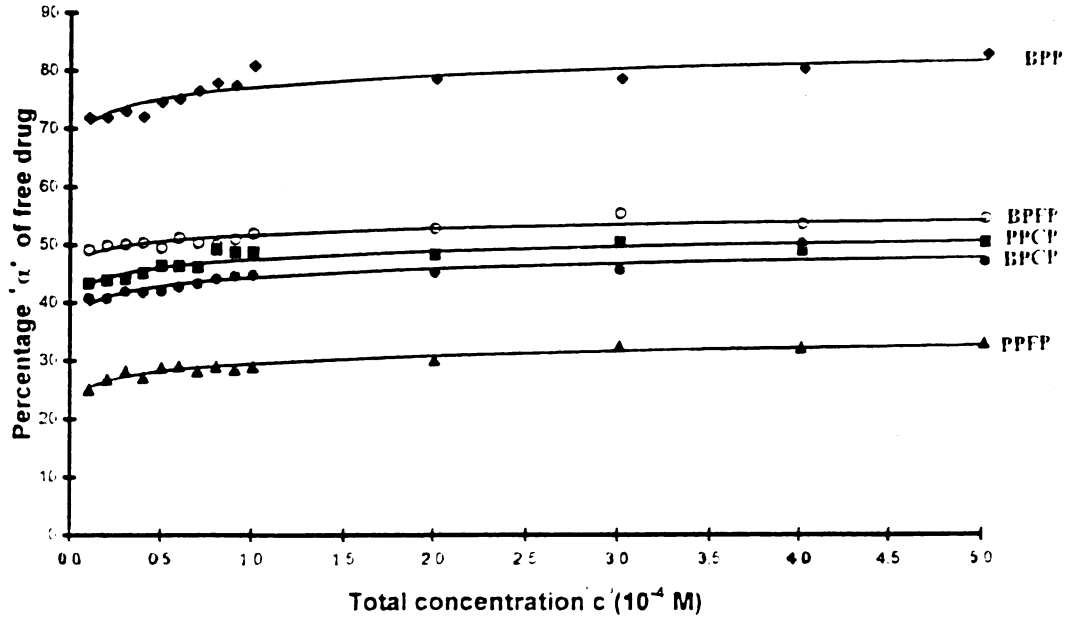


Fig. 1. Binding of concentrations of the phenoxazine modulators to bovine serum albumin. Ordinate: Percentage of free phenoxazine derivative. Abscissa: total concentration of phenoxazine derivative (10^{-4} M). Binding measurements were carried out in a 1% BSA solution (pH 6.8, 22°C, incubated at 37°C for 6 h). Each point represents the mean value of two experiments.

examined, showed that the modulators bind to BSA in the order: PPFP > BPCP > PPCP > BPFP > BPP indicating that phenoxazines containing $-CF_3$ or –Cl in position C-2 have greater affinity to the protein than those phenoxazines containing –H in position C-2. The binding of

Table 3
Binding of several phenoxazine derivatives to bovine serum albumin

Phenoxazine derivative	% of bound drug (β)[a]	Regression coefficient (m)[b]	Apparent binding constant (k)	Association constant (K_1)[c] [10^4 M^{-1}]	Free binding energy ($-\Delta F°$)[d]	$\text{Log}_{10}(\beta/\alpha)$
10-[3′-[*N*-bis(hydroxyethyl) amino]propyl]phenoxazine (BPP)	19.43	0.226	2.5	0.8	5285	−0.6177
10-[3′-[*N*-bis(hydroxyethyl) amino]propyl]-2-chlorophenoxazine (BPCP)	55.31	1.125	3.2	2.7	6004	0.0926
10-[3′-[*N*-bis(hydroxyethyl) amino] propyl]-2-trifluoromethyl phenoxazine (BPFP)	48.12	0.836	2.0	1.8	5762	−0.0327
10-(3′-*N*-pyrrolidinopropyl)-2-chloro-phenoxazine (PPCP)	51.46	0.997	2.5	2.5	5955	0.0253
10-(3′-*N*-pyrrolidinopropyl)-2-trifluoro-methyl-phenoxazine (PPFP)	71.00	2.021	4.2	5.2	6385	0.3889

[a] β is the percentage of bound drug in a 1% BSA solution with a total concentration $c = 10^{-4}$ M of phenoxazine modulator.
[b] m, the regression coefficient and k, the apparent binding constant, were obtained from Fig. 2, see also Table 2.
[c] K_1, is the association constant obtained from the Scatchard plot (Fig. 3).
[d] $\Delta F°$ is the free binding energy calculated from $-RT \ln K$.

these compounds to BSA was supported by $\Delta F°$ values (Table 3). Further, the compounds at IC_{10} were examined for their ability to modulate the cytotoxicity of VLB in $KBCh^R$-8-5 cells and the data revealed that the anti-MDR activity increased with respect to control in the order PPFP (20-fold) > BPCP (18-fold) > PPCP (13-fold) > BPFP (12-fold) > BPP (sixfold) [14–18]. This rank order is the same as for the binding efficiency of these compounds with BSA.

3.2. Relationship between bovine serum albumin binding and hydrophobic character of phenoxazine modulators

Several authors have demonstrated a correlation between the hydrophobic character and protein binding of low molecular weight substances [9,20–24], if the hydrophobic character of the substances was characterized between *n*-octanol and buffer. These results suggest that hydrophobic interactions play an important role in protein binding of organic compounds. However, a good correlation between protein binding and partition coefficients can be shown only for substances of structurally related groups. Hence, not only hydrophobic interactions can be present in protein binding of organic molecules, but also other binding mechanisms such as ionic binding, hydrogen binding or steric effects, etc. must also be involved [25].

The hydrophobic character of phenoxazine modulators ($\log_{10} P$ values) was determined (Table 1) to look for a possible quantitative correlation between the lipid solubility of these compounds and their protein binding ability. Analysis of the relationship between lipid solubility of modulators used in this study and the fraction, $\log(\beta/\alpha)$, where 'β' is the percentage of phenoxazine bound and 'α' is the percentage of free phenoxazine modulator (total concentration was 10^{-4} M) showed a good correlation (Fig. 4).

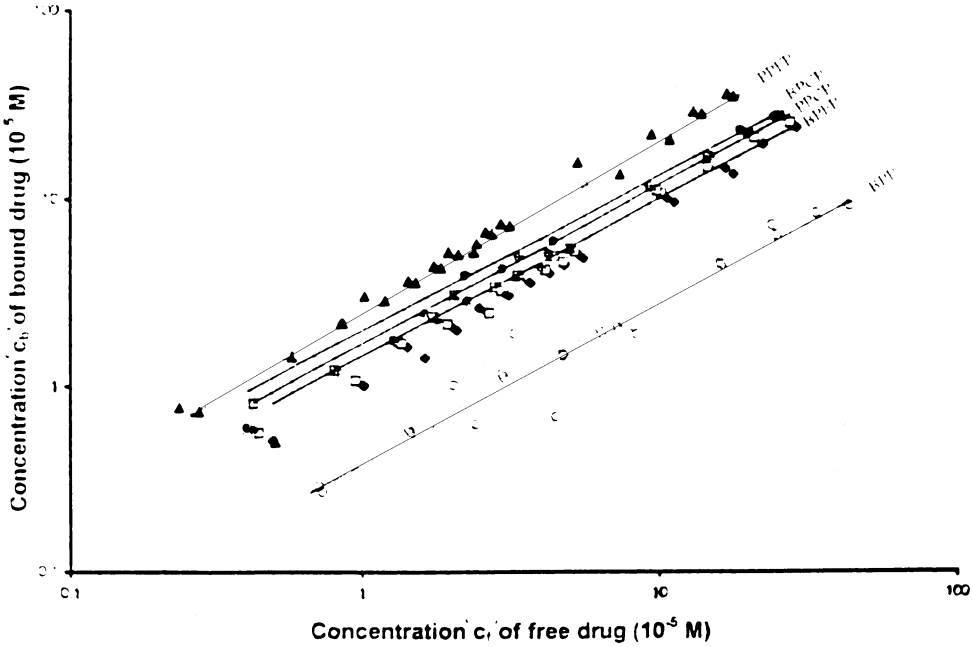


Fig. 2. Binding capacity of a 1% albumin solution for phenoxazine derivatives. Ordinate: concentration 'c_b' of bound phenoxazine derivative (0.1×10^{-5} to 100×10^{-5} M). Abscissa: concentration of free phenoxazine derivative (0.1×10^{-5} to 100×10^{-5} M). Binding measurements were carried out in 1% BSA solution (pH 6.8, 22°C, incubated at 37°C for 6 h). Each point represents a single experiment. This plot is performed in order to obtain the binding constants 'm' and 'k', see also Table 3. For statistical data see Table 4.

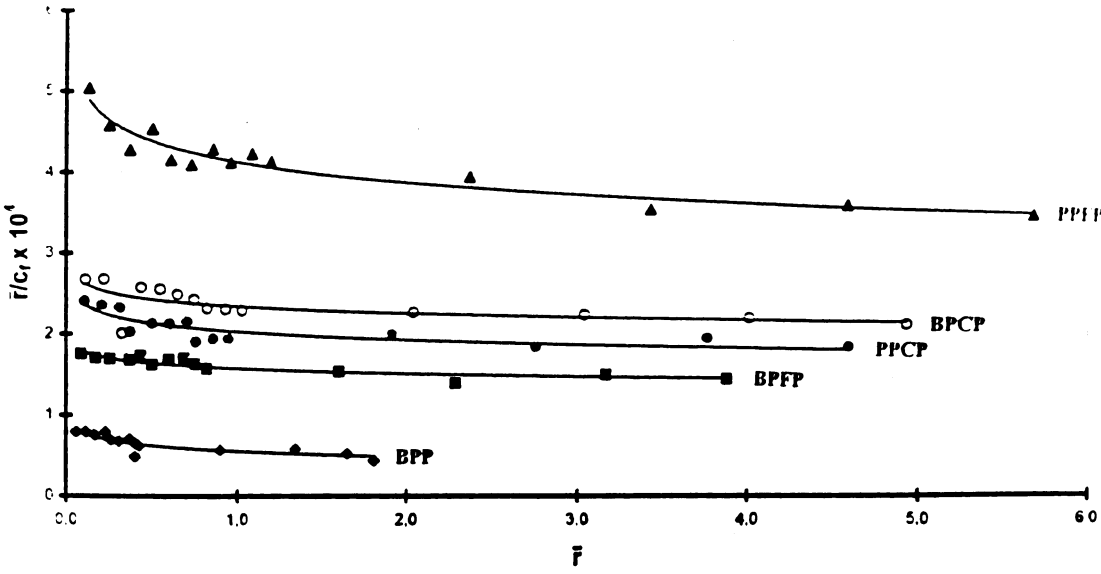


Fig. 3. Scatchard plot of the binding of the phenoxazine derivatives to bovine serum albumin. Ordinate: $\bar{r}/c_f$ in 10^{-4} M^{-1}. c_f, molar concentration of free phenoxazine derivative in the albumin solution. Abscissa: r, number of mol phenoxazine per mol albumin. All measurements were made in 1% albumin solution (pH 6.8, 22°C). Each point represents the mean value of two single experiments. For total binding constant K_1 see Table 3.

(β/α) function is preferred for this type of correlation because it is directly analogous to the organic solvent–buffer partition coefficient. The following equation was arrived at from the $\log(\beta/\alpha)$ and $\log_{10} P$ data for five phenoxazine derivatives substituted in position C-2 of the phenoxazine nucleus: $\log(\beta/\alpha) = -2.286 + 1.184 \log_{10} P$ (correlation coefficient $r = 0.9678$). For the five phenoxazine modulators, hydrophobicity decreased in the order: PPFP > PPCP > BPFP > BPCP > BPP. Hence, it is apparent from these results that the BSA binding of the phenoxazine derivatives with substituents in position C-2 of the phenoxazine nucleus increases with their hydrophobic character.

3.3. Displacement of phenoxazine modulators from their bovine serum albumin binding sites

Simple aromatic substances like benzoic acid or aniline are able to displace phenothiazines from their binding site on the albumin molecule [11,26]. In order to understand the BSA binding moieties of the phenoxazine modulators (BPP, BPCP,

Table 4
Statistical data for the binding of varying concentration of phenoxazine derivatives with bovine serum albumin

Name of phenoxazine derivative	Equation of the regression line in the double logarithmic system	No. of single experiments (n)	Correlation coefficient (r)	Significance of r
BPP	$y = 0.04588 + 0.226x$	28	0.9855	Significant
BPCP	$y = 0.0498 + 1.125x$	28	0.9960	Significant
BPFP	$y = 0.0393 + 0.8366x$	28	0.9979	Significant
PPCP	$y = 0.0402 + 0.996x$	28	0.9929	Significant
PPFP	$y = 0.0864 + 2.02x$	28	0.9929	Significant

Table 5
Statistical data for the displacement of phenoxazine derivatives from their albumin binding sites by hydroxyzine

Compound	Equation of the regression line	Parallel experiments (n)	Correlation coefficient (r)	Significance of 'r'
BPP + Hydroxyzine	$y = 76.20 + 0.234x$	26	0.7380	Significant
BPCP + Hydroxyzine	$y = 51.02 + 0.3685x$	26	0.7735	Significant
BPFP + Hydroxyzine	$y = 48.39 + 0.198x$	26	0.5017	Not significant
PPCP + Hydroxyzine	$y = 36.80 + 0.20x$	26	0.3568	Not significant
PPFP + Hydroxyzine	$y = 26.72 + 0.2992x$	26	0.9319	Significant

BPFP, PPCP and PPFP), the author has studied the displacement experiments by dialysis method using hydroxyzine and acetylsalicylic acid. In these experiments, the binding of the phenoxazine modulators to BSA was determined after the displacing agent, hydroxyzine or acetylsalicylic acid, was added to the incubation mixture.

The participation of the side chain in the total binding of the phenoxazine derivatives is indicated in displacement experiments with hydroxyzine and the data are shown in Fig. 5. Examination of the data has revealed that hydroxyzine displaces BPP, BPFP and PPCP by about 2–3% and BPCP and PPFP by about 13–14% from their BSA binding sites. This might be explained by supposing that the aliphatic side chain between N^{10}- of phenoxazine nucleus and nitrogen atom of side chain tertiary amines is not in a position to contribute significantly to the binding of the phenoxazine modulators used [1].

Acetylsalicylic acid competes with the benzene rings of the phenothiazine ring system for binding

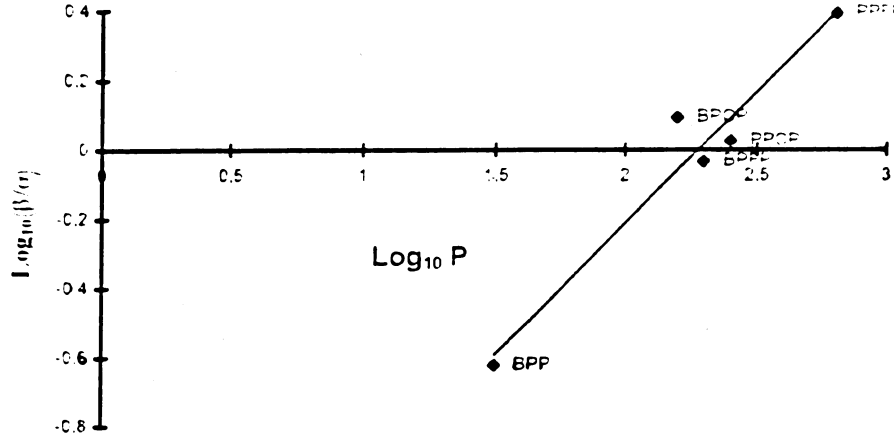


Fig. 4. Relation between albumin binding and partition coefficients of phenoxazine derivatives. Ordinate: $\log(\beta/\alpha)$, β is percent phenoxazine derivative bound, α is percent free. Abscissa: $\log_{10} P$, P, partition coefficient between n-octanol and buffer solution. The equation of the regression line: $\log(\beta/\alpha) = -2.286 + 1.184 \log_{10} P$.

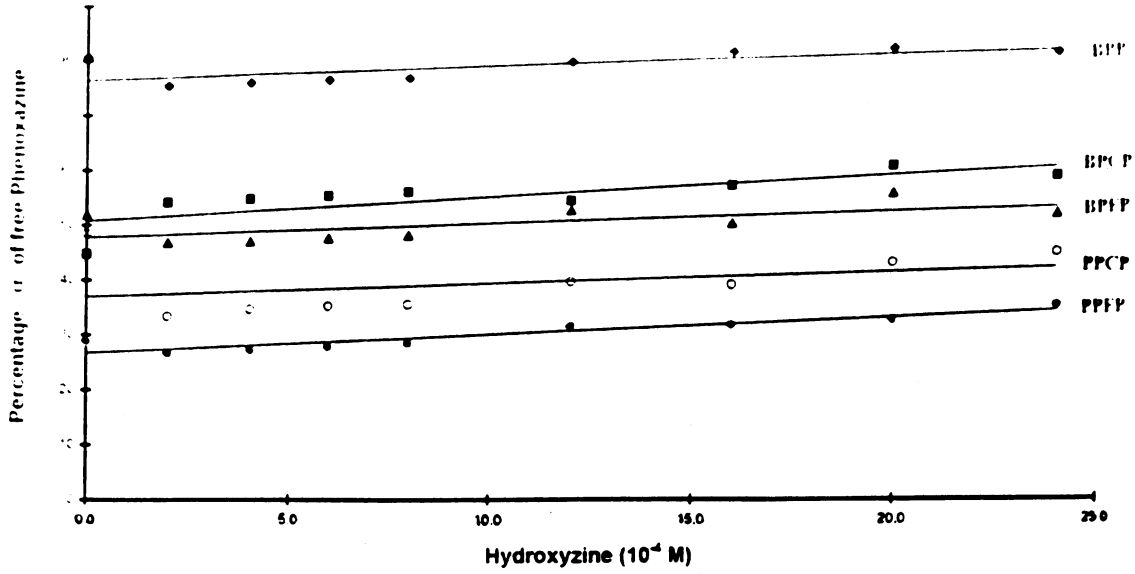


Fig. 5. Influence of hydroxyzine on the binding of phenoxazine derivatives to bovine serum albumin. Ordinate: percentage of free phenoxazine derivative in the albumin solution. Abscissa: total concentration of hydroxyzine (10^{-4} M). All measurements were made in 1% albumin solution containing 10^{-4} M phenoxazine derivative and varying concentrations of hydroxyzine. Each point represents the mean value of two single experiments. For statistical evaluation see Table 5.

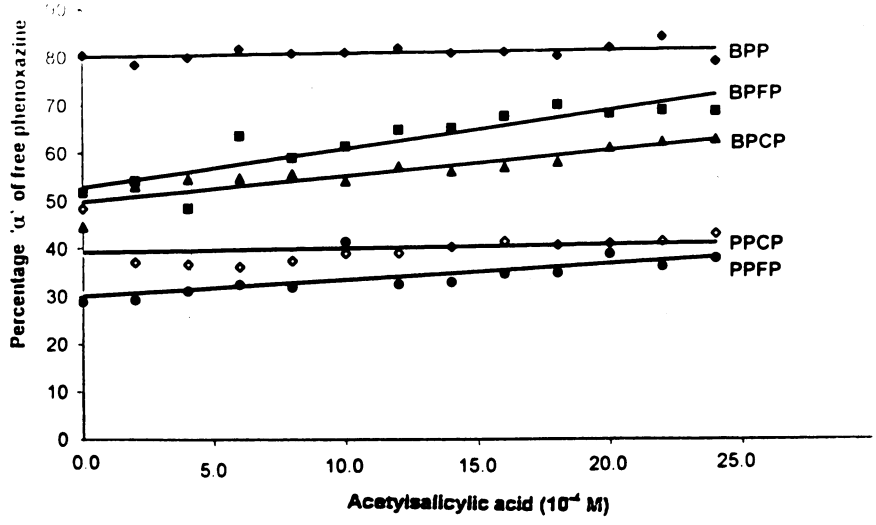


Fig. 6. Displacement of phenoxazine derivatives by acetylsalicylic acid from binding to bovine serum albumin. Ordinate: Percentage of free phenoxazine derivative. Abscissa: total concentration of acetylsalicylic acid (10^{-4} M). All measurements were made in a 1% albumin solution containing one of the phenoxazine derivatives 10^{-4} M and varying concentrations of acetylsalicylic acid. Each point represents the mean value of two single experiments.

to BSA [1]. Since acetylsalicylic acid competes for phenothiazine ring system for binding to BSA, the author has examined the effect of acetylsalicylic acid as a displacing agent to determine whether the benzene rings of phenoxazine modulator are involved in binding to BSA. The displacing experimental data are shown in Fig. 6. Examination of the data from Fig. 6 has highlighted that acetylsalicylic acid displaces BPP by about 2%, PPFP by about 9%, BPCP and BPFP by about 14–16% and PPCP by 4% from their BSA binding sites.

In summary, the results of the investigation suggest that possibly phenoxazines are bound to albumin by hydrophobic interactions of their benzene rings.

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