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Mechanism of Interaction of Phenothiazine Derivatives with Serum Albumin

NEELAM SEEDHER* AND MAMTA KANOJIA
Department of Chemistry, Panjab University, Chandigarh-160014

The mechanism of interaction of five phenothiazine derivatives with serum albumin was investigated using fluorescence spectroscopy. It was found that phenothiazine ring common to all drugs makes major contribution to interaction. However, the nature of alkylamino group at position 10 influences the protein binding significantly. Binding affinities could be related to parachor values of drugs. Fluorescence intensity data in the presence of additives showed that hydrophobic interactions play a significant role. Stern-Volmer plots indicated the presence of a static component in the quenching mechanism. Results also showed that both tryptophan residues of protein are accessible to drug molecules.The high magnitude of the rate constant of quenching indicated that the process of energy transfer occurs by intermolecular interaction forces and thus drug binding site is in close proximity to tryptophan residues of BSA.

Drug-protein interactions are important since most of the administered drugs are extensively and reversibly bound to serum albumin and drug is transported mainly as a complex with protein. The nature and magnitude of drug-protein interaction significantly influences the biological activity of a drug[1-3]. Binding parameters are useful in studying the pharmacological response of a drug and design of dosage forms[4,5].

In vitro binding of some phenothiazine derivatives to human erythrocytes[6], serum albumin[7-9] and α_1-acid glycoprotein[10,11] have been reported in recent years. However, there is no general agreement about the relative contribution of hydrophobic and ionic interactions and also which part of the molecule, that is, phenothiazine ring or substituents at positions 2 or 10 on the phenothiazine ring, are involved in binding. In the present work interaction of five phenothiazine derivatives with serum albumin has been studied using fluorescence spectroscopic technique.

EXPERIMENTAL

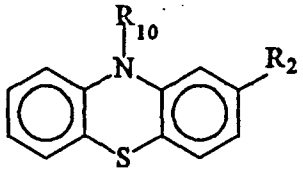
Serum albumin, bovine (BSA, Fraction V) was obtained from Sigma Chemical Company, St. Louis, USA. Phenothiazine derivatives were obtained as gifts from various manufacturers. All other materials were of analytical grade and all solutions were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solutions were prepared based on molecular weight of 65000. Perkin Elmer fluorescence spectrophotometer (MPF-44B) equipped with a 150W Xenon lamp source was used.

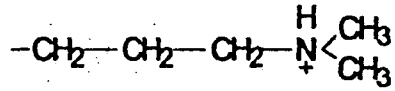
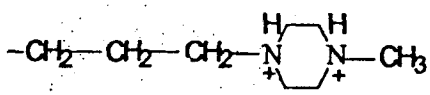
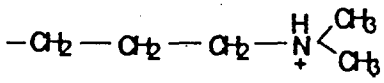
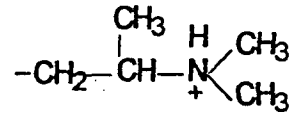
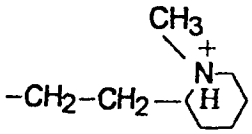
Phenothiazine-albumin interaction:

Structures of phenothiazine derivatives used in the present study are shown in Table 1. Interaction of five phenothiazine derivatives, chlorpromazine hydrochloride, trifluoperazine dihydrochloride, triflupromazine hydrochloride, promethazine hydrochloride and thioridazine hydrochloride with serum albumin was studied using fluorescence spectrophotometric technique. On the basis of the preliminary experiments, albumin concentration was kept fixed at 24 μM and drug concentration was varied from 20 to 140 μM. Fluorescence spectra were recorded at 37° in the range 280-400 nm keeping excitation wavelength 296 nm in each case. The absorbances of drug-protein mixtures in the concentration range employed for

*For correspondence

TABLE 1: STRUCTURES OF PHENOTHIAZINE DERIVATIVES INVESTIGATED IN THE PRESENT STUDY



Name of Drug	R_2	R_{10}
Chlopromazine hydrochloride	-Cl	
Trifluoperazine dihydrochloride	$-CF_3$	
Triflupromazine hydrochloride	$-CF_3$	
Promethazine hydrochloride	-H	
Thioridazine hydrochloride	$-SCH_3$	

the experiments did not exceed 0.05 at the excitation wavelength in order to avoid inner filter effect. Fluorescence spectroscopic data was analysed using the Ward method[14] to obtain association constants for drug-protein binding. Fluorescence spectra of BSA-promethazine hydrochloride system was also recorded in the presence of additives, urea and lauric acid.

RESULTS

Phenothiazine-Albumin interaction:

Fluorescence spectra of bovine serium albumin (BSA) was determined in the presence of increasing amounts of various drugs. Spectra for one of the drugs, chlorpromazine hydrochloride, are shown in fig. 1. It was observed that interaction of phenothiazine drugs with serum albumin did not result in any noticeable change in λ_{max} of tryptophan fluorescence in albumin. However, drugs were observed to quench the fluorescence of albumin. The fraction of drug bound, θ, was determined according

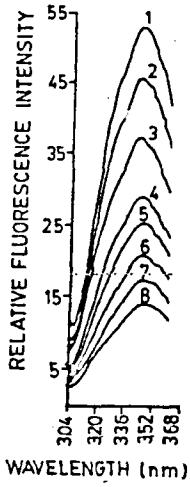


Fig. 1: Fluorescence spectra of bovine serum albumin in the presence of chlorpromazine hydrochloride.
BSA concentration was kept fixed (24 μM). CPZ concentration was 1-0 μM, 2-20 μM, 3-40μM, 4-60μM, 5-80μM, 6-100μM, 7-120μM, 8-140μM

TABLE 2: BINDING PARAMETERS FOR THE INTERACTION OF VARIOUS PHENOTHIAZINE DERIVATIVES WITH BOVINE SERUM ALBUMIN

Drug Sample	Association Constant $K \times 10^{-4}$	Number of Binding Sites n	Standard Free Energy Change ΔG^0(KJ mole^{-1})	Parachor $(Nm^{-1})^{1/4}m^3$
Chlorpromazine hydrochloride	4.155	3.45	-27.422	774.5
Trifluoperazine dihydrochloride	5.167	2.70	-27.985	1000.1
Triflupromazine hydrochloride	3.908	3.16	-27.264	806.6
Promethazine hydrochloride	3.153	3.16	-26.711	734.8
Thioridazine hydrochloride	4.441	1.64	-27.594	913.7

Association constant K and number of binding sites, n were obtained using the method described by Ward[14]. ΔG^0 was obtained from K using the relationship $\Delta G^0 = -RT \ln K$. Parachor values were calculated using data compiled by Quayle[15].

to Weber and Young[12] and Maruyama *et al.*[13] using the following equation

$$\theta = \frac{F_0 - F}{F_0} \qquad (1)$$

where F and F_0 denote the fluorescence intensities of protein in a solution with a given concentration of drug and without drug, respectively. θ represents the fraction of sites on the protein occupied by drug molecules. Fluorescence data was analysed using the method described by Ward.[14] It has been shown that for equivalent and independent bindings sites

$$\frac{1}{(1-\theta)K} = \frac{[D_T]}{\theta - n[P_T]} \qquad (2)$$

where K is the association constant for drug-protein interaction, n the number of binding sites, D_T the total drug concentration and $[P_T]$ the total protein concentration. $1/(1-\theta)$ versus $[D_T]/\theta$ plot for one representative system, serum albumin-chlorpromazine hydrochloride, is shown in fig. 2. K and n values obtained from the slope and intercept of such plots are given in Table 2. K values were of the order of 10^4 and the number of binding sites varied from 1.64 to 3.45.

Parachor, which is a measure of the molar volume of drug, was calculated for each drug from the atomic parachors and other structural features[15]. Values for different samples (Table 2) vary as trifluoperazine > thioridazine > triflupromazine > chlorpromazine > promethazine.

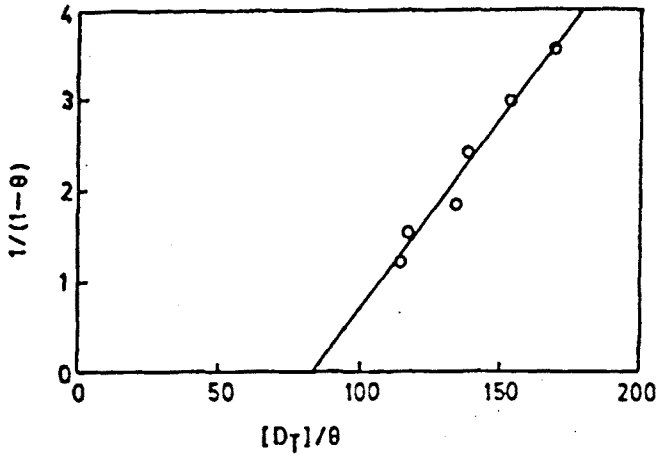


Fig. 2: $1/(1-\theta)$ versus D_T/θ plot.
Drug:chlorpromazine hydrochloride, $\theta = (F_0-F)/F_0$, is the fraction of drug bound and D_T is the total drug concentration.

Effect of Additives:

To understand further the nature of interaction involved, fluorescence spectra of albumin-promethazine hydrochloride system was also determined in the presence of 0.02 M urea and saturated solution of lauric acid. Fluorescence intensity at 344 nm for various systems is given in Table 3. It is seen that the fluorescence intensity of albumin-drug system increases on the addition of urea and decreases on the addition of lauric acid.

TABLE 3: FLUORESCENCE INTENSITY OF BSA-DRUG SYSTEM IN THE PRESENCE OF ADDITIVES

S. No.	Sample	Fluorescence Intensity at 344 nm
1	only BSA	43.8
2	BSA + drug	19.7
3	BSA + drug + Urea	25.8
4	BSA + drug + Lauric acid	17.3

BSA and drug concentrations were 24 μM and 100 μM, respectively in each case. Urea concentration was 20 μM. Due to low solubility of lauric acid in water, saturated solution of lauric acid was used.

Stern - Volmer Analysis:

Fluorescence intensity data was also analyzed according to Stern-Volmer law[16,17] (Eq. 3) by plotting F_0/F versus [Q], where F_0 and F are the steady state fluorescence intensities at 344 nm in the absence and presence of quencher (drug), respectively and [Q] is the total drug concentration.

$$\frac{F_o}{F} = 1 + K_q[Q] \qquad (3)$$

The Stern-Volmer plots (fig. 3) showed positive deviation from straight line, suggesting the presence of a static component in the quenching mechanism[18]. A modified form of Stern-Volmer equation[18] that describes quenching data when both dynamic and static quenching are operative is

$$\frac{F_o}{F} = 1 + K_q[Q]\, eV[Q] \qquad (4)$$

where K_q is the collisional quenching constant or Stern-Volmer quenching constant and V is the static quenching constant. The static quenching constant, V was obtained from equation 4 by plotting $[F_o/\{Fexp(V[Q])\}]$-1 versus [Q] for varying V until a linear plot was obtained[18]. The collisional quenching constant, K_q was then obtained from the slope of $[F_o/\{Fexp(V[Q])\}]$-1 versus [Q] plots through origin (fig. 4). The values of V and K_q, so obtained are recorded in Table 4. The static quenching constant, V, is seen to be close to 5 x 10^3 M^{-1} in most cases.

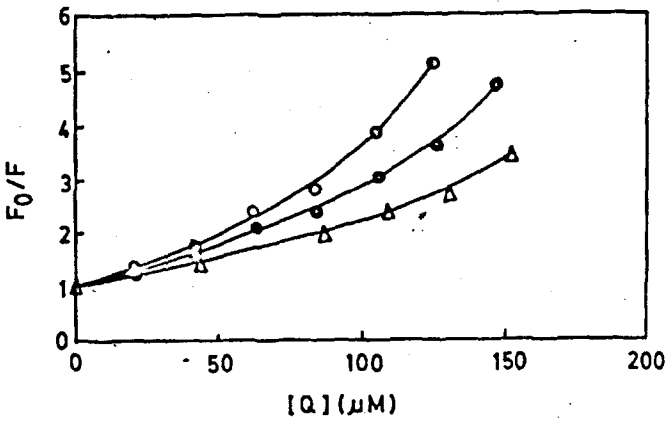


Fig. 3: Stern - Volmer plots for the binding of various phenothiazine derivatives to BSA.
F_0 and F are the fluorescence intensities in the absence and presence of drug. [Q] is the total drug concentration and V is the static quenching constant.
-O—O-Trifluoperazine, -●—●-Triflupromazine, -Δ—Δ- Promethazine

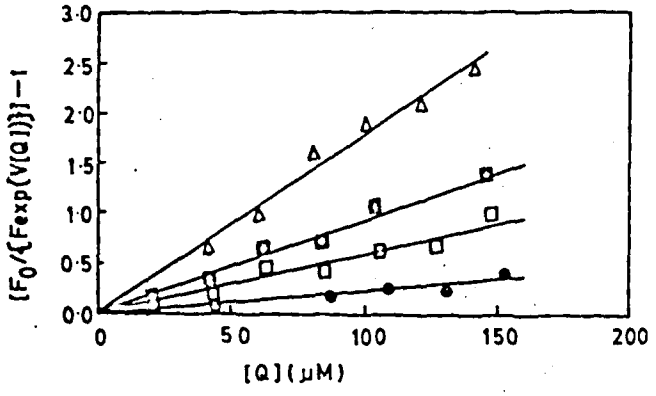


Fig. 4: Plots of $F_o/\{Fexp\,(V[Q])\}$- 1 versus [Q]
F_0 and F are the fluorescence intensities in the absence and presence of drug. [Q] is the total drug concentration and V is the static quenching constant
-●—●- Promethazine, -Δ—Δ-Thioridazine, -□—□-Triflupromazine, -□—□-Trifluoperazine

TABLE 4: STATIC AND COLLISIONAL QUENCHING CONSTANTS FOR VARIOUS DRUG-PROTEIN SYSTEMS

Drug Sample	Static quenching constant $V \times 10^{-3}$ (M-1)	Collisional quenching constant $K_q \times 10^{-4}(M^{-1})$	Collisional rate constant for quenching $K_q \times 10^{13}(M^{-1}s^{-1})$
Chlorpromazine hydrochloride	4.75	0.787	0.787
Trifluoperazine dihydrochloride	5.0	1.226	1.226
Triflupromazine hydrochloride	5.0	0.776	0.776
Promethazine hydrochloride	3.5	0.601	0.601
Thioridazine hydrochloride	2.5	2.181	2.181

K_q and V were obtained from Eqs. 3 and 4. $k_q = K_q/\pi_0$

DISCUSSION

Since the data fits equation 2 in all cases, it may be concluded that under the conditions of the experiment, all the binding sites are equivalent and independent. Association constants of the order of 10^4 have also been reported by other authors[7,9] for the interaction between phenothiazines and serum albumin. The order of K values is consistent with non-covalent interactions[9]. Standard free energy change, ΔG^0, calculated from association constant, K is also included in Table 2. ΔG^0 values are seen to be close to - 27 KJ mol^{-1} in all cases. This shows that the phenothiazine ring common to all drugs makes major contribution to interaction.

K values for different drug samples are seen to vary as trifluoperazine > thiordazine > chlorpromazine > triflupromazine > promethazine. Phenothiazine ring is the primary hydrophobic portion of the molecule but substituents on position 2 and 10 of the ring have an effect on surface activity of the molecule. Zografi and Munshi[19] have shown that holding other factors constant, substitution on position 2 of the phenothiazine ring enhances surface activity in the order CF_3 >> Cl > H. SCH_3 group of thioridazine should be least hydrophobic. The binding affinities do not decrease or increase in this order. Thus, the binding is not directly related to hydrophobicity or hydrophilicity of substituents at position 2 on the ring. However, it plays an indirect role by affecting the intramolecular interactions in the molecule, as discussed below.

The nature of alkylamino group at position 10 appears to influence the protein binding significantly. For different drug samples association constant, K varies in the same manner as the hydrophobicity of 10-substituent; trifluoperazine > thioridazine > chlorpromazine > triflupromazine > promethazine. Trifluoperazine has longest alkylamino chain followed by thioridazine. Chlorpromazine and triflupromazine have same chain length but differ in the substituent at 2-position of the phenothiazine ring. The hydrogen atom of the protonated amino group in chlorpromazine forms hydrogen bond with an electron pair of chlorine atom at position 2 to develop a donor-acceptor like arrangement thereby increasing the hydrophobicity of the molecule. A three atom chain length is necessary to bring the protonated amino nitrogen into proximity with the 2-substituent. The presence of a branched chain in promethazine reduces surface activity and thus hydrophobicity. Isaacson[20] has emphasized that for optimum biological activity, there should be a critical size of substituents on the nitrogen of amino group. This indicates the importance of this part of the molecule for receptor attachment. Once the size requirement is met the added chain length increases receptor-binding forces.

The order of the parachor values is in close agreement with the order of K values. It thus appears that the molecular size of drug also plays a significant role in the binding of phenothiazine derivatives to serum albumin. The large size drug molecules probably have larger hydrophobic area which can interact with hydrophobic surface on the protein molecule.

Urea is known to be a three-dimensional structure breaker and thereby weakens the hydrophobic interaction whereas lauric acid induces hydrophobic interaction because of its lengthy non-polar aliphatic chain[21]. Thus

both urea and lauric acid alter the microenvironment of the binding sites by affecting the iceberg structure of water. Since drug quenches the fluorescence of serum albumin, increase in fluorescence intensity of albumin-drug mixture in the presence of urea shows that urea inhibits drug-protein binding whereas corresponding decrease in the case of lauric acid shows that lauric acid facilitates drug-protein binding. It may thus be concluded that hydrophobic interactions play a significant role in the interaction of phenothiazine derivatives to serum albumin.

According to Eftink and Ghiron[18] upward curvature in the Stern-Volmer plot indicates that both tryptophan residues of BSA are exposed to quencher and the quenching constants of each tryptophan residue are nearly identical, while downward curvature indicates buried tryptophan residues. At a concentrations of 140 µM drug about 80% of the fluorescence intensity was quenched. The maximum quenching was obtained by extrapolating a plot of $(F_0-F)/F_0$ versus 1/[Q] to 1/[Q] = 0, corresponding to infinite concentration of drug. Fig. 5 shows that at infinite concentration of drug fluorescence quenching was more than 95% in each case. This again shows that both the tryptophan residues of BSA are accessible to drug molecules.

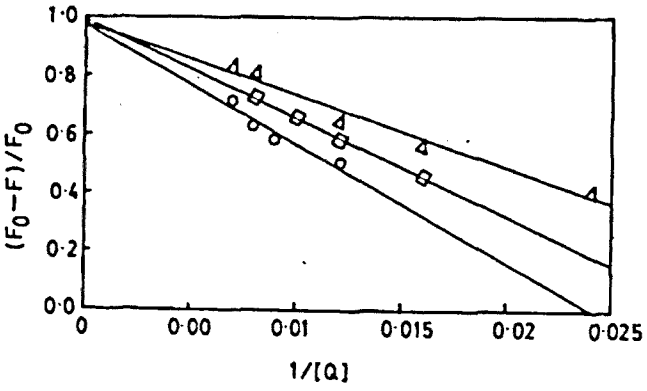


Fig. 5: Plots of $(F_0-F)/F_0$ versus 1/[Q] for various phenothiazine derivatives
F_0 and F are the fluorescence intensities in the absence and presence of drug. [Q] is the total drug concentration. -Δ—Δ-Trifluoperazine, -O—O- Promethazine, -□—□- Chlorpromazine

For a bimolecular quenching process, $K_q=k_q \pi_0$ where π_0 is the lifetime in the absence of quencher and k_q is the rate constant for quneching. π_0 value for tryptophan fluorescence in proteins is known to be $\approx 10^{-9}$ s[22]. Rate constant, k_q, calculated from K_q for different drugs is included in Table 4. The k_q values are seen to vary from 0.601 x 10^{13} to 2.181 x 10^{13} $M^{-1}s^{-1}$. The differences between different drugs may be due to steric or inductive effects altering the ability to form the excited collisional complex. Again the nature of substituent at position 10 of the phenothiazine ring seems important. The values being nearly same for trifluopromazine and chlorpromazine, slightly less for promethazine having a branched chain and significantly higher for trifluperazine and thioridazine having six membered piperazine and piperidine rings, respectively, in the side chain.

The probability of a collision between fluorophore and quencher depends on their rate of diffusion (D), their size and concentration[23]. It can be shown that

$$k_q = 4 \pi aDN_A \times 10^{-3} \qquad (5)$$

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is sum of molecular radii and N_A is the avogadro's number. The upper limit of k_q expected for a diffusion-controlled bimolecular process is 10^{10} $M^{-1}s^{-1}$ [24]. The high magnitude of k_q in the present study (~10^{13} $M^{-1}s^{-1}$) can probably be attributed to a specific long range interaction between drug molecules and tryptophan residues on protein. Thus the process of energy transfer occurs by intermolecular interaction forces between tryptophan and drug and this is possible only when the drug binding site is in close proximity to tryptophan residues of BSA.

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