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Spectroscopic Studies on the Interaction of N-alkyl Phenothiazines with Bovine Serum Albumin

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Binding of N-Alkyl phenothiazines (NAP) to bovine serum albumin (BSA) was studied by spectroscopic methods. It was found that the 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. Stern-Volmer plots indicated the presence of static component in the quenching mechanism. The high magnitude of rate constant of quenching indicated that the process of energy transfer occurs by intermolecular interaction and thus the drug-binding site is in close proximity to tryptophan residues of BSA. Binding studies in presence of hydrophobic probe, 8-anilino-1-naphthalein-sulphonic acid showed that there is hydrophobic interaction between drug and the probe and they do not share common sites in BSA. Thermodynamic parameters obtained from data at different temperatures showed that the binding of NAP to BSA predominantly involve hydrophobic forces. The effects of some cations and anions common ions were investigated on NAP-BSA interactions. The CD spectrum of BSA in presence of drug showed that binding of drug leads to change in the helicity of the protein.

key words: Interaction studies, N-alkyl phenothiazines, bovine serum albumin

INTRODUCTION

Bovine serum albumin (BSA), one of the major components in the plasma protein, is a single-chain 582 amino acid globular nonglycoprotein cross-linked with 17 cystine residues (8 disulfide bonds and 1 free thiol). It is divided into three linearly arranged, structurally distinct, and evolutionarily related domains (I-III); each domain is composed of two subdomains (A and B). BSA has two tryptophans embedded in two different domains: Trp-134 located in proximity of the protein surface, but buried in a hydrophobic pocket of domain IIA, and Trp-214 located in an internal part of domain IIIA. Like other serum albumins, BSA has a wide range of physiological functions involving the binding, transport and delivery of fatty acids, porphyrins, bilirubin, tryptophan, thyroxine and steroids. It is home to specific binding sites for metals, pharmaceuticals and dyes. It is well known that many of drugs are bound to serum proteins, especially serum albumin. The effectiveness of drugs depends on their binding ability. Hence, this kind of work has stimulated many researchers to carry out the studies on drug-protein interactions [1-6]. These results may provide salient information of the structural features that determine the therapeutic effectiveness of drugs, and hence become a important research field in chemistry, life sciences and clinical medicine.

N-alkyl phenothiazines (NAP) are widely used as anticholinergic, antihistaminic, tranquillisers, anticancer, psychotherapeutic, antiemetic and antipsychotic drugs. Reports on binding of drugs to plasma proteins using gel filtration and micro calorimetric techniques [2,7] are available in the literature. But, these techniques are laborious and time consuming and the results, at times, are not reproducible. Moreover, the nature of binding forces is not clear. In addition, these conventional methods are often inapplicable to the analyses of strongly bound drugs because of technical problems such as drug adsorption on the membrane and the leakage of bound drug through membrane.

In vitro binding of phenothiazine neuroleptics to human erythrocytes and alpha acid glyco protein [7] has been reported. However, there is no general agreement about the relative contribution of hydrophobic and ionic interaction 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. Literature survey indicated that the attempts have not been made so far to investigate the interaction between the selected NAP and BSA. In view of this, we planned to carry out the detailed investigations on the mode of interaction of phenothiazine derivatives with bovine serum albumin (BSA) using fluorescence spectroscopy, spectrophotometry and circular dichroism methods.

MATERIALS AND METHODS

Fluorescence measurements were performed on a Hitachi spectrofluorimeter Model F-2000 equipped with a 150W Xenon lamp and slit width of 10 nm. A 1.00 cm quartz cell was used

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for measurements. CD measurements were made on a JASCO-810 spectropolarimeter using a 1.00 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 200-300 nm.

Bovine Serum Albumin (BSA, Fraction V, approximately 99%; protease free and essentially γ-globulin free) and 1-anilino-8-naphthalein-sulphonic acid (ANS) were obtained from Sigma Chemical Company, St Louis, USA. N-alkyl phenothiazines were obtained as gift samples from different manufacturers. All other materials were of analytical reagent grade. The solutions of drugs and BSA were prepared in 0.1M phosphate buffer of pH 7.4 containing 0.15 M NaCl. BSA solution was prepared based on its molecular weight of 65,000.

EXPERIMENTAL SECTION

NAP-BSA interactions

On the basis of preliminary experiments, BSA concentration was kept fixed at 10 μM and drug concentration was varied from 10 to 140 μM. Fluorescence spectra were recorded at room temperature (29°C) in the range 300-500 nm upon excitation at 296 nm in each case. The absorbances of drug-protein mixtures in the concentration range employed for the experiment did not exceed 0.05 at the excitation wavelength to avoid inner filter effect.

Effects of common ions

The fluorescence spectra of NAP-BSA were recorded in presence and absence of various additives *viz.*, dextrose, starch, magnesium stearate, urea and lauric acid (each of 0.2 M), common ions *viz.*, SO_4^{2-}, F^-, NO_3^-, I^-, CH_3COO^-, Mg^{2+}, Co^{2+}, K^+, Ni^{2+} and V^{5+}, and different drugs at 344 nm upon excitation at 296 nm. The concentration of BSA was fixed at 20 μM while that of different drug/common ion was varied from 10-100 μM and that of additive was maintained at 20 μM for the study.

Circular dichroism (CD) measurements

CD measurements of BSA in the presence and absence of selected drugs were made in the range of 200-300 nm using a 1.00 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectra. A stock solution of 0.1 μM BSA was prepared in 0.01 M phosphate buffer containing 0.15 M NaCl. The BSA to drug concentration was varied (1:1, 1:3 and 1:5) and the CD spectrum was recorded.

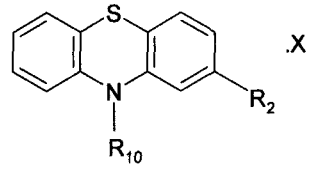
RESULTS AND DISCUSSION

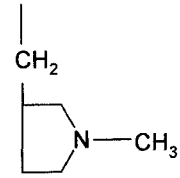
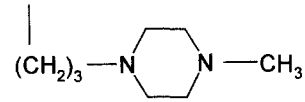
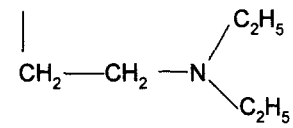
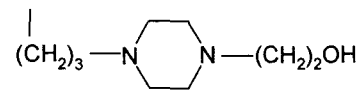
The structures of NAP employed in the present study are shown in Table 1.

Fluorescence studies

In order to examine whether NAP bind to BSA or not, fluorescence measurements were carried out. The conformational changes of BSA were evaluated by the measurement of intrinsic fluorescence intensity of protein before and after addition of drug. Fluorescence measurements give information about molecular environment in a vicinity of the chromophore molecules. Fluorescence spectra of BSA were recorded in the presence of increasing amounts of various drugs. The spectra

Table 1. Structures of N-alkyl phenothiazines



Name of drug	R_2	R_{10}	X
Methdilazine hydrochloride (MDH)	-		HCl
Prochlorperazine dimaleate (PCPD)	-Cl		$2C_4H_4O_4$
Diethazine hydrochloride (DH)	-		HCl
Perphenazine dimaleate (PPD)	-Cl		$2C_4H_4O_4$

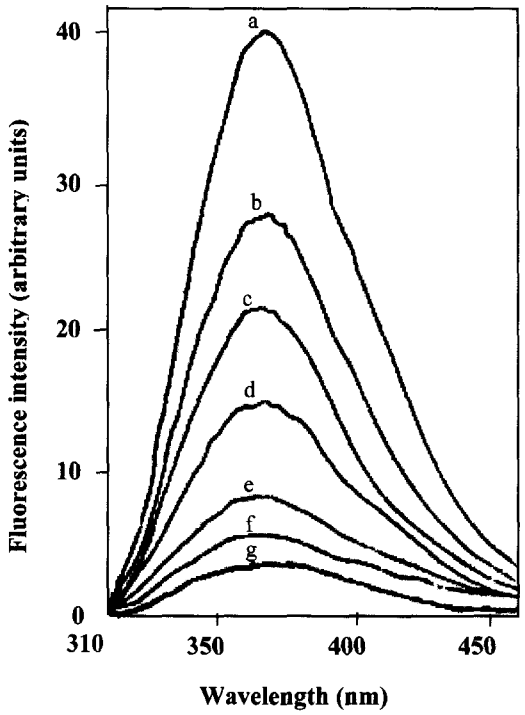


Figure 1. Fluorescence spectra of BSA in the presence of MDH. BSA concentration was kept fixed (10 μM). IPH concentration was a-0, b-20, c-30, d-40, e-60, f-80 and g-100 μM.

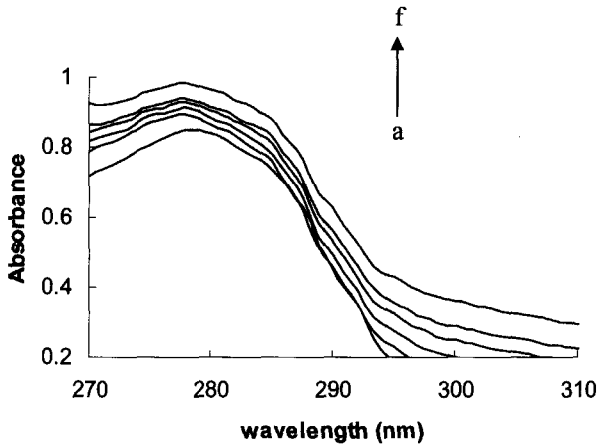


Figure 2. Absorbance spectra of BSA in the presence of MDH. BSA concentration was kept fixed (20 μM). MDH concentration was a-0, b-13.2, c-26.4, d-40, e-53.3 and f-66.6 μM.

of one of the representative drugs, MDH are shown in Fig. 1. It was noticed that NAP exhibited a concentration-dependent quenching of the intrinsic fluorescence of BSA, without altering the emission maximum, indicating that NAP bound to BSA and possibly formed complexes. In order to confirm the formation of the complexes the absorption spectra of BSA in presence of NAP were recorded. The absorption spectra of BSA in presence of a representative drug, MDH are shown in Fig. 2. From this it is evident that the protein undergoes conformational change upon interaction with NAP. The fraction of drug bound, θ, was determined according to Weber and

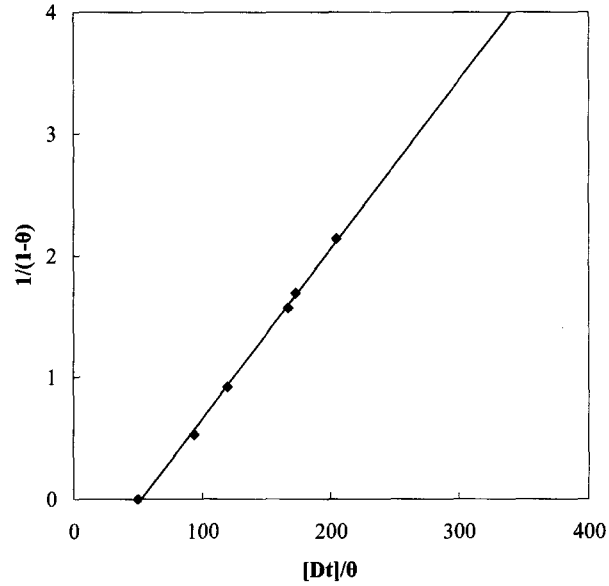


Figure 3. 1/(1-θ) *versus* $[D_t]/\theta$ plot for the binding of MDH to BSA.

Young [8], and Maruyama *et al.* [9] using the equation,

$$\theta=(F_o-F)/F_o \quad (1)$$

where F and F_o denote the fluorescence intensities of protein in a solution with a given concentration of drug and without drug, respectively. The fluorescence data was analysed as described by Ward [10]. It is known that for equivalent and independent binding sites the following equation holds good:

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

where K is the association constant for drug-protein interaction, n is the number of binding sites, $[P_T]$ is the total drug concentration and $[P_T]$ is the total protein concentration. A plot of $1/(1-\theta)$ *versus* $[D_t]/\theta$ for a representative member of NAP is shown in Fig. 3. The values of K and n, obtained from the slope and intercept of such plots are given in Table 2. Since the data fit equation 2 in all cases, it may be concluded that all the binding sites are equivalent and independent. Further, it was observed that the order of K value (10^4) was consistent with non-covalent interactions as reported [11]. These values are supported by standard free energy change (ΔG^0) values obtained from the relationship, $\Delta G^0=-2.303$ RT logK and are seen to be close to -28 kJ mol^{-1} (Table 2). This shows that the phenothiazine ring common to all drugs makes major contribution to interaction. Phenothiazine ring is the primary hydrophobic portion of the molecule but substituents at positions 2 and 10 of the ring may have an effect on surface activity of the molecule. The nature of the alkyl amino group at position 10 appears to influence the protein binding significantly. For different drug samples, the K values vary in the same manner as the hydrophobicity of 10-substituent: PPD>PCPD>DH>MDH. The PPD has the longest alkyl amino chain followed by PCPD.

Table 2. Binding parameters for the interaction of various NAP with BSA

Drug	Association Constant, $K \times 10^{-4}$, M^{-1}	Number of binding sites, (n)	Standard free energy change, ΔG_o (kJ $mole^{-1}$)	Parachor $(Nm^{-1})^{1/4}$ m^3
MDH	6.55	2.11	-27.87	665.6
DH	6.77	2.0	-27.92	840.0
PCPD	6.82	2.1	-27.94	940.0
PPD	8.50	2.13	-28.44	1255.2

However, MDH and DH have slightly different chain lengths. The hydrogen atom of the protonated amino group in PCPD and PPD forms hydrogen bond with an electron pair of chlorine atom at position 2 to develop a donor-acceptor 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 substituent at 2. Isaacson [12] 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.

Fluorescence intensity data was analyzed according to Stern-Volmer law [13],

$$F_o/F = 1 + K_q [Q] \quad (3)$$

By plotting F_o/F versus [Q], where F_o and F are the steady state fluorescence intensities at 344 nm in the absence and presence of quencher (drug) respectively, [Q] is the total drug concentration and K_q is the Stern-Volmer quenching constant. The Stern-Volmer plots (Fig. 4) exhibited positive deviation from straight line, thereby indicating the presence of a static component in the quenching mechanism [14]. According to Eftink and Ghiron [15] the upward curvature in the Stern-Volmer plots indicate that both tryptophan residues of BSA are exposed to quencher and the quenching constant of each tryptophan residue is nearly identical. A modified form of

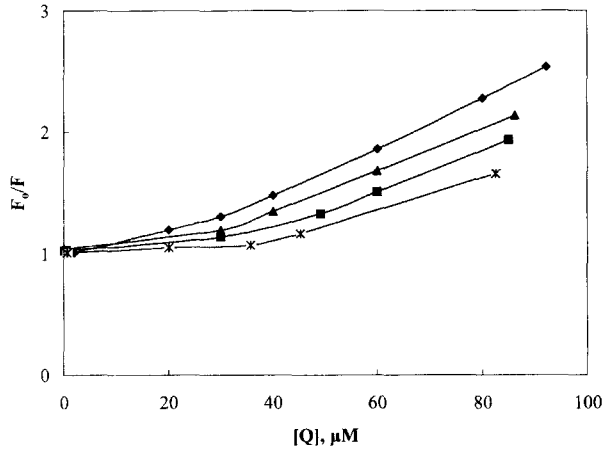


Figure 4. Stern-Volmer plot for the binding of MDH (■) PPD(◆), DH (▲) and PCPD (*) with BSA.

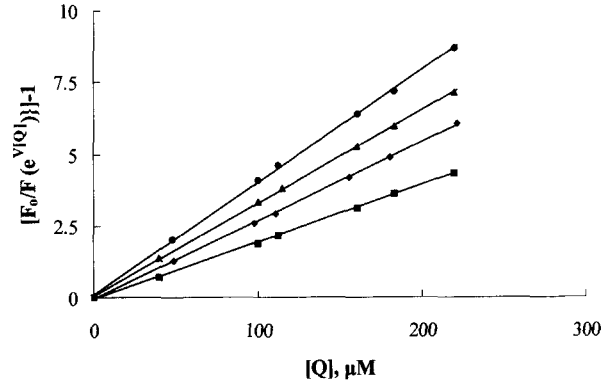


Figure 5. Plots of $[F_o/F (e^{V[Q]})]-1$ *versus* [Q] for MDH (■) PPD (◆), DH (▲) and PCPD (●).

Stern-Volmer equation [14] that describes quenching data when both dynamic and static quenching are operative is

$$F_o/F = 1 + K_q [Q] e^{V[Q]} \quad (4)$$

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

Fluorescence quenching data was also analyzed by modified Stern-Volmer plot,

$$F_o/(F_o-F) = 1/f_a + 1/[Q] f_a K_{SV} \quad (5)$$

where F_o and F are fluorescence intensities at 344 nm in the absence and presence of quencher, respectively, at $[Q]=D_t$, the total concentration of drug, K_{SV} is the Stern-Volmer quenching constant and f_a is the fraction of fluorophore (protein) accessible to the quencher (drug). From the plot of $F_o/(F_o-F)$ *versus* 1/[Q], (Fig. 6) the values of f_a and K_{SV} were determined. The value of f_a was found to be close to unity indicating thereby that both tryptophan residues of BSA are involved in the drug-protein interaction.

For a bimolecular quenching process, $K_q=k_q \tau_o$, where τ_o is the lifetime in the absence of quencher and k_q is the rate constant for quenching. As the reported τ_o value for tryptophan

Table 3. Static and collisional quenching constants for NAP-BSA systems

Phenothiazine derivative	Static quenching Constant, $V\times10^{-3}$ (M^{-1})	Collisional quenching constant, $K_q\times10^{-4}$ (M^{-1})	Collisional rate constant for quenching, $k_q\times10^{-12}$ ($M^{-1}s^{-1}$)
MDH	3.75	0.96	0.96
DH	4.95	0.79	0.79
PCPD	5.34	0.97	0.97
PPD	5.21	1.22	1.22

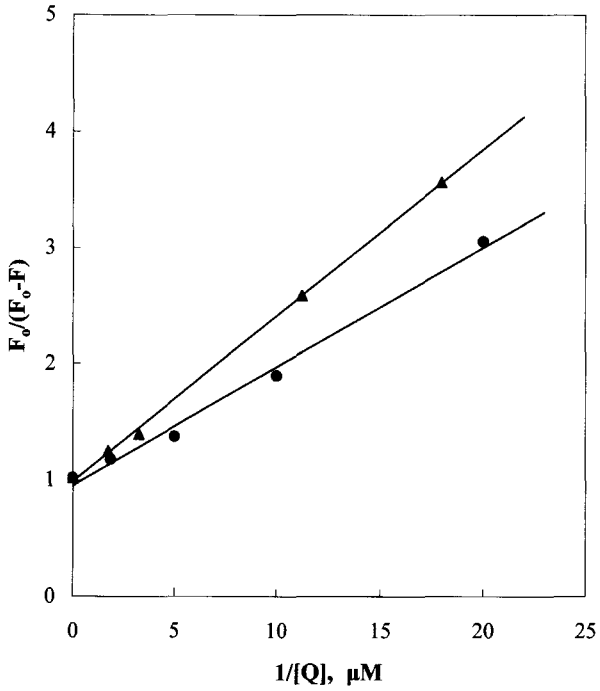


Figure 6. Plot of $F_o/(F_o-F)$ *versus* 1/[Q] for MDH (▲) and DH (●).

fluorescence in proteins is 10^{-8} s, the rate constant, k_q, would be of the order of $10^{12}M^{-1}s^{-1}$. Generally, the value of k_q depends on the probability of a collision between fluorophore and quencher. This probability depends on their rate of diffusion (D), their size and concentration. It can be shown that

$$k_q=4\pi\, aDN_a\times10^{-3} \quad (6)$$

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is sum of molecular radii and N_a is Avogadro's number. The maximum scatter collision quenching constant, k_q of various quenchers with the biopolymer is $2\times10^{10}M^{-1}s^{-1}$. The rate constant of protein quenching procedure initiated by NAP is greater than the k_q of the scatter procedure. This means that the quenching is not initiated by dynamic collision but from the formation of a complex [4].

Parachor, which is a measure of molar volume of drug, was calculated for each drug from the atomic parachors and other structural features [2]. Values for different samples varied in the order PPD>PCPD>DH>MDH. The order of parachor values is in close agreement with the order of K values. It thus appears that molecular size of drug also plays a significant role in the binding of phenothiazine derivatives to serum albumin. The large size drug molecule has larger hydrophobic area, which can interact with hydrophobic surface on the protein molecule.

Evaluation of thermodynamic parameters for BSA-NAP interactions

Small molecules bound to macromolecule by different binding modes including van der Waals, hydrogen bonding, electrostatic and hydrophobic interactions. The thermodynamic parameters, enthalpy change, ΔH° and entropy change, ÄS° are important for confirming binding mode. For this purpose, the temperature-dependence of the binding constant was studied at 286 K, 293 K, 302 K and 308 K. At these temperatures BSA doesn't undergo any structural degradation. The thermodynamic parameters were determined using van't Hoff equation,

$$\text{Log K} = -\Delta H^\circ / 2.303RT + \Delta S^\circ / 2.303\ R \quad (7)$$

The Log K *versus* 1/T plot (Fig. 7) enabled determination of ΔH° and ΔS° for the binding process. The ΔH°, ΔS° and ΔG° values were found to be in the range of +36.98 to +56.72 $kJmol^{-1}$, +215.12 to +282.38 $JK^{-1}mol^{-1}$ and -24.53 to -29.33 $kJmol^{-1}$, respectively (Table 4). The positive values found for the enthalpy and entropy changes seem to indicate that the hydrophobic contribution is the predominant intermolecular force stabilizing the BSA-NAP complexes [16]. These results together with spectral changes in the fluorescence emission spectra of BSA induced by phenothiazines suggest that the interaction may take place in subdomain IIA and IIIA since these subdomains have been proposed to bind drugs and other hydrophobic materials [17].

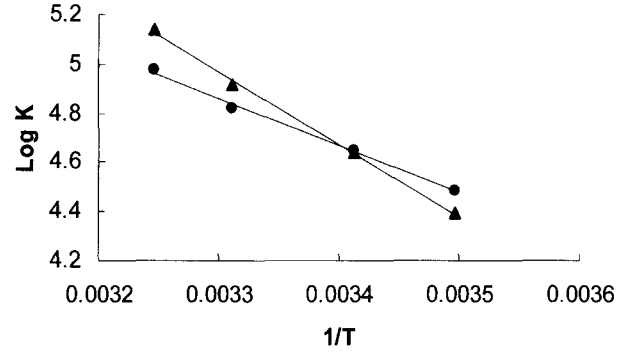


Figure 7. Plot of Log K *versus* 1/T for MDH (●) and PPD (▲).

Table 4. Thermodynamic parameters of BSA-NAP systems

Drug	Temperature, (K)	Binding Constant $K \times 10^{-4}$, M^{-1}	$\Delta G°$ $kJmol^{-1}$	$\Delta H°$ $kJmol^{-1}$	$\Delta S°$ $Jmol^{-1} K^{-1}$
MDH	286	3.02	-24.53		
	293	4.47	-26.08	36.98	215.12
	302	6.60	-27.87		
	308	9.41	-29.33		
DH	286	3.01	-24.53		
	293	4.40	-26.05	37.45	216.70
	302	6.76	-27.92		
	308	9.41	-29.33		
PCPD	286	3.01	-24.53		
	293	4.27	-25.98	37.69	217.47
	302	6.80	-27.94		
	308	9.37	-29.32		
PPD	286	2.46	-24.04		
	293	4.36	-26.03	56.72	282.38
	302	8.31	-28.44		
	308	13.82	-30.31		

Binding Studies in the presence of ANS

In order to examine whether the hydrophobic probe, ANS and NAP share common binding sites in BSA or not, we have carried out the interaction studies of NAP with BSA in presence of ANS. Fluorescence spectra of 20 μM BSA in the presence of increasing amounts of drug (4-25 μM) and ANS were recorded after excitation at 296 nm. Both drug and ANS quench the fluorescence of BSA, but the magnitude of decrease in fluorescence intensity was much larger for ANS as compared to that for drug. ANS bound to BSA calculated from the fraction of occupied sites (θ) was found to be 75% where as the drug bound to BSA was found to be only 20% under identical conditions. It is known that excitation at 296 nm involves fluorescence due only to tryptophan residues of BSA. Further, under conditions of the experiment tryptophan residues of BSA are partially exposed and their accessibility depends upon the nature of molecules of the interacting species. It thus appears that whereas, tryptophan residues are fully accessible to the hydrophobic probe, ANS they are only partially accessible to the drug which has partially hydrophilic character. Thus, drug and ANS do not share common binding site in BSA.

In another set of experiments, BSA-ANS interaction was studied in the presence and absence of 5, 10, 15, and 20 μM of each drug by monitoring ANS fluorescence upon excitation at 370 nm. It was found that for a given concentration of ANS, fluorescence intensity increases when drug is added to BSA-ANS system. It is known [14] that ANS shows greatly increased fluorescence as a result of hydrophobic interactions with proteins and other macromolecules due to transfer of probe from an aqueous to non-polar environment. Increase in fluorescence intensity of BSA-ANS system on the addition of drug can be explained as follows: when drug is added to BSA-ANS system it can compete with ANS for the hydrophobic sites on the surface. In that case it would inhibit the binding of ANS, i.e. displaces ANS from its binding sites and the fluorescence intensity should decrease. But, the fluorescence intensity actually increases. This shows that ANS and drug do not share common sites in BSA. Increase in fluorescence intensity shows that the drug has the highly hydrophobic character, and thus, further shifts the fluorescence intensity of ANS to higher values.

Effects of some cations and anions

The fluorescence spectra of albumin and selected NAP were recorded in presence of various anions *viz.*, SO_4^{2-}, F^-, NO_3^-, I^-, PO_4^{3-} and CH_3COO^-, and cations *viz.*, Mg^{2+}, Co^{2+}, V^{5+}, K^+ and Ca^{2+} at 344 nm upon excitation at 296 nm. The fluorescence emission spectrum of NAP derivative in the presence of anions/cations shows that there is no interaction between anions/cations and NAP. But, there is a binding reaction between anions/cations and protein and the presence of anions/cations directly affects the binding between NAP and protein. Further, in order to study the effects of some ions and other drugs on the binding between NAP and BSA, binding constants were determined in presence of these ions and in presence of different drugs. The results of representative NAP are shown in Table 5. The competition between anions/cations/different drugs with selected NAP decreased the binding constants between protein and NAP, implying that the binding force between protein and NAP also decreased, thus, shortening the storage time of the NAP in blood plasma and enhancing the maximum effectiveness of the drug [16].

Circular dichroism studies

The binding of phenothiazines was also confirmed by CD spectra. Typical CD spectra of a representative member of phenothiazines, MDH with BSA are shown in Fig. 8. As expected, the α-helices of protein show a strong double minimum at 220 nm and 209 nm [18]. The intensities of this

Table 5. Effects of anions and cations on binding constants of BSA-NAP interactions

Sample	Association Constant, M^{-1}	
	MDH	PCPD
BSA+drug	6.55×10^4	6.82×10^4
BSA+drug+SO_4^{2-}	3.6×10^3	1.23×10^3
BSA+drug+F^-	5.55×10^3	1.00×10^3
BSA+drug+NO_3^-	4.54×10^3	4.73×10^2
BSA+drug+I^-	2.97×10^3	2.07×10^3
BSA+drug+CH_3COO^-	7.78×10^3	1.07×10^3
BSA+drug+Mg^{2+}	1.07×10^2	0.07×10^2
BSA+drug+Co^{2+}	0.49×10^2	0.05×10^2
BSA+drug+K^+	2.54×10^2	0.11×10^2
BSA+drug+V^{5+}	0.12×10^2	0.08×10^2
BSA+drug+Ni^{2+}	0.17×10^2	0.09×10^2
BSA+drug+Ca^{2+}	1.49×10^2	0.16×10^2

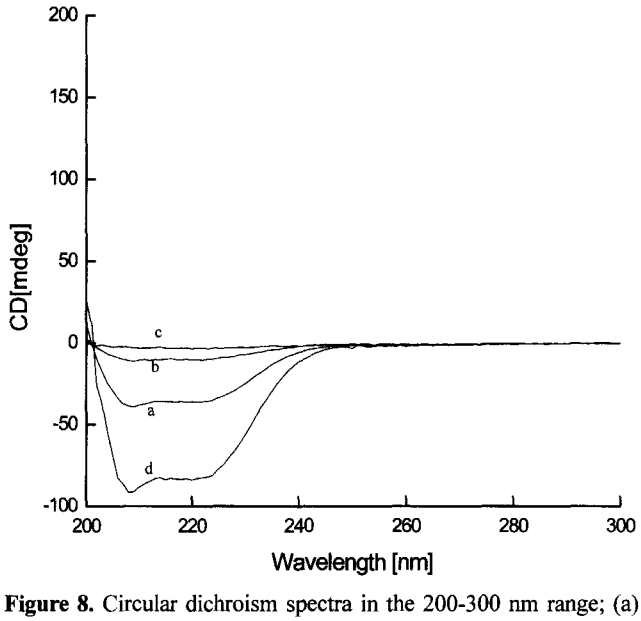


Figure 8. Circular dichroism spectra in the 200-300 nm range; (a) BSA, 0.1 μM; [BSA]: MDH=1:1 (b), 1:3 (c) and 1:5 (d).

double minimum reflect the amount of helicity of BSA and indicate that BSA contains more than 50% of α-helical structure. Upon addition of the drug to BSA (1:1) the extent of α-helicity of the protein decreases and hence the intensity of double minimum is reduced. On increased addition of drug to BSA (3:1), the double minimum start vanishing and later at 5:1, the intensity of the double minimum increased. This is indicative of increase in helicity when the drug is completely bound to BSA.

CONCLUSIONS

This paper provided an approach for studying the interactions of fluorescent protein with drugs using absorption, fluorescence and circular dichroism techniques. Comparison of the association constant values of drugs showed that the phenothiazine drugs containing -Cl in position C-2 have greater affinity to the protein than those containing -H in that position. The results also showed that BSA fluorescence quenched by NAP through both dynamic and static quenching for same quencher. All NAP interacted with BSA by hydrophobic association. The decreased binding constants of BSA-NAP complexes in presence of common ions indicated the decreased binding force between protein and NAP. This resulted in shortening the storage time of the NAP in blood plasma and enhancing the maximum effectiveness of the drug. The biological significance of this work is evident since albumin serves as a carrier molecule for multiple drugs. This report has a great significance in pharmacology and clinical medicine as well as methodology.

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