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In vitro Study of the Mechanism of Interaction of Trifluoperazine Dihydrochloride with Bovine Serum Albumin

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Mechanism of interaction of antipsychotic drug trifluoperazine dihydrochloride with bovine serum albumin (BSA) has been reported. Association constant for drug-protein binding showed that the interactions are non-covalent in nature and there are two independent binding sites. Binding studies in the presence of hydrophobic probe, 8-anilino-1-naphthalene sulphonic acid, sodium salt (ANS) showed that there is hydrophobic interaction between drug and ANS and they do not share common sites in BSA. Small decrease in critical micellar concentration (CMC) of anionic surfactant, sodium dodecyl sulphate (SDS) in the presence of drug showed that the ionic character of drug also contributes to binding. Stern-Volmer analysis of fluorescence quenching data showed that the fraction of fluorophore (protein) accessible to the quencher (drug), was close to unity indicating thereby that both tryptophan residues of BSA are involved in drug-protein interaction. The rate constant for quenching, greater than 10^{10} $M^{-1}s^{-1}$, indicated that the TFP binding site is in close proximity to tryptophan residues of BSA.

The binding of phenothiazine derivatives to plasma proteins is a determinant factor in their pharmacokinetic and hence in their therapeutic properties[1-3], since most of the drugs are more than 90% bound to serum albumin. Various aspects of the binding of some of the tricyclic psychoactive drugs to plasma proteins have been reported in recent years using equilibrium dialysis and microcalorimetric techniques[4-7]. However, the nature of binding forces, that is, the relative contribution of hydrophobic and ionic interactions, is not clear. Moreover, very few studies involving fluorescence spectrophotometric technique are available[8]. Fluorescent probes, such as 1-anilino- 8-naphthalenesulfonic acid, can be used as valuable tools to understand the nature of interaction involved[9,10].

Trifluoperazine, 10-[3-(4-methylpiperazin-yl) propyl]-2-trifluoromethyl phenothiazine (TFP), is a widely used antipsychotic drug. Its interaction with erythrocyte cells, human plasma, α_1-acid glycoprotein and human serum albumin has been reported in recent years[4-6]. It has been suggested that hydrophobic interaction is primarily involved. However, contribution of electrostatic interaction has also been found by Miyoshi *et al*[6].

In the present work, Mechanism of interaction of trifluoperazine dihydrochloride with bovine serum albumin has been studied using fluorescence spectrophotometric technique.

EXPERIMENTAL

Bovine serum albumin (BSA) and 8-anilino-1-naphthalene sulphonic acid, sodium salt (ANS) have been purchased from Sigma Chemical Co., USA. Other reagents used were of analytical grade. BSA solutions were prepared based on molecular weight of 65,000. All experiments were carried out in 0.05 M phosphate buffer of pH 7.4 using fluorescence spectroscopic technique. Due to limited solubility of drug in aqueous buffer, TFP solutions were prepared in a buffer containing 5% alcohol by volume. Perkin Elmer fluorescence spectrophotometer (MPF 44B) equipped with a 150 W xenon lamp source was used.

Trifluoperazine - BSA interaction :

Some preliminary experiments were carried out to select optimum protein and drug concentrations for drug-protein interaction. On the basis of the preliminary experiments, BSA concentration was kept fixed at 24 μM and drug concentration was varied from 20 to 140 μM. Fluorescence spectra were recorded at 20° 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 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[11] to obtain association constant and the number of available binding sites.

Binding Studies in the presence of hydrophobic probe, ANS :

Experiments were also carried out in the presence of hydrophobic probe, ANS. In the first set of experiments, interaction of drug and ANS with BSA was studied under identical conditions. BSA, ANS and drug concentrations were selected on the basis of preliminary experiments. BSA concentration was kept constant at 20 μM and ANS/drug concentration was varied from 4 to 25 μM. Protein fluorescence was recorded in the range 280-400 nm after excitation at 296 nm. In the second set of experiments, BSA-ANS interaction was studied in the presence and absence of 20 μM drug. BSA concentration was again kept fixed at 20 μM and ANS concentration was varied from 4 to 25 μM. ANS fluorescence was recorded in the range 350-550 nm after excitation at 370 nm.

Critical Micellar Concentration :

Critical Micellar Concentration (CMC) of anionic surfactant, sodium dodecyl sulphate (SDS) was determined in the presence and absence of 50 μM drug using conductance measurements. Since conductance methods are less sensitive than spectroscopic methods, a higher concentration of drug had to be used. SDS concentration was varied from 1 mM to 15 mM. CMC values were obtained from the conductivity versus concentration of surfactant plots.

RESULTS AND DISCUSSION

Trifluoperazine-BSA interaction:

Fluorescence spectra of BSA in the presence of

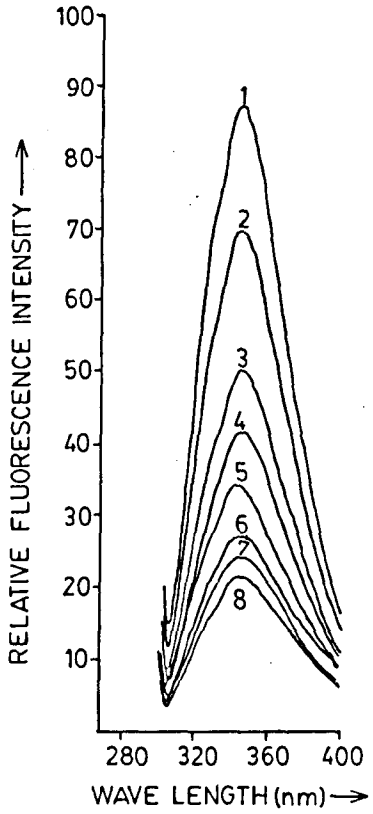

Fig. 1 : Fluorescence spectra of bovine serum albumin in the presence of trifluoperazine dihydrochloride

BSA concentration was kept fixed (24 μM). TFP concentration was 1-0 μM, 2-20μM, 3-40μM, 4-60μM, 5-80μM, 6-100μM, 7-120 μM, 8-140 μM

increasing amounts of drug are shown in Fig. 1. It was observed that the interaction of the drug with BSA did not result in any noticeable change in λ_{max} of tryptophan fluorescence in BSA. However, drug is observed to quench the fluorescence of BSA. The fraction of drug bound, θ, was determined according to Weber and Young[12,13] using the following equation.

$$\theta = \frac{F_o - F_p}{F_o} \qquad (1)$$

where F_p and F_o 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[11]. It has been shown that for equivalent and independent binding sites

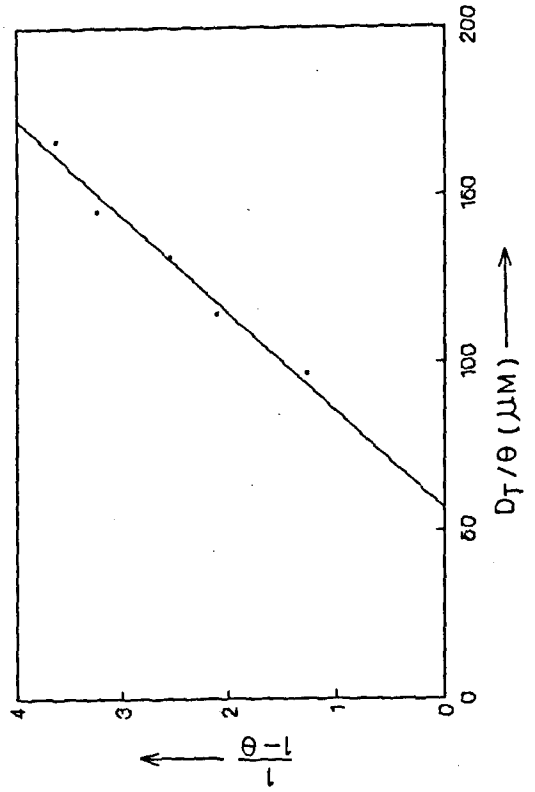

Fig. 2 : 1/(1-θ) versus D_T/θ plots for the binding of trifluoperazine dihydrochloride to BSA.
θ is the fraction of drug bound and D_T is the total drug concentration.

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

where K is the association constant for drug-protein interaction, n is the number of binding sites, D_T is the total drug concentration and $[P_T]$ is the total protein concentration. 1/(1-θ) versus $[D_T]/\theta$ plot for BSA-trifluoperazine system at 20° is shown in Fig. 2. K and n values obtained from the slope and intercept of Fig. 2 were found to be 3.44 x 10^4 M^{-1} and 2.35, respectively. Since the data fits equation 2, it may be concluded that there is only one set of independent binding sites. The order of K values shows that the interactions are non-covalent in nature[5].

Fluorescence spectra of 20 μM BSA in the presence of increasing amounts (4 to 25 μM) of trifluoperazine and ANS were determined 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 (cf. Fig. 3). Percentage of ANS bound to BSA, calculated from the fraction of occupied sites (θ), was 81.2%

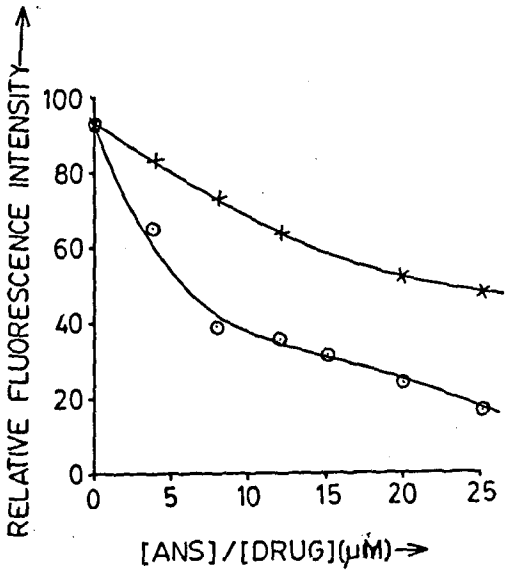

Fig. 3 : The effect of quenchers, trifluoperazine dihydrochloride (⟷) and ANS on (O-O) on the tryptophan fluorescence of BSA.
BSA concentration was kept fixed (20 μM). Excitation wavelength was 296 nm and Emission wavelength was 344 nm.

whereas the percentage of drug bound to BSA was only 51.6% 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, since tryptophan residues of BSA are not fully exposed, accessibility of interacting species varies with the nature and size of its molecules[14]. It, thus, appears that the drug and ANS do not share common sites in BSA.

In another set of experiments, BSA-ANS interaction was studied by monitoring ANS fluorescence (excitation wavelength 370 nm) in the presence and absence of 20 μM drug. Fluorescence spectra (350-500 nm) in the presence and absence of drug are shown in Fig. 4. It was found that for a given concentration of ANS, fluorescence intensity increases when drug is added to BSA-ANS system. It is known[9] that the hydrophobic probe, ANS shows greatly increased fluorescence as a result of hydrophobic interaction with proteins and other macromolecules due to the transfer of the probe from an aqueous to a non polar environment.

Increase in fluorescence intensity of BSA-ANS system on the addition of drug can be explained as follows.

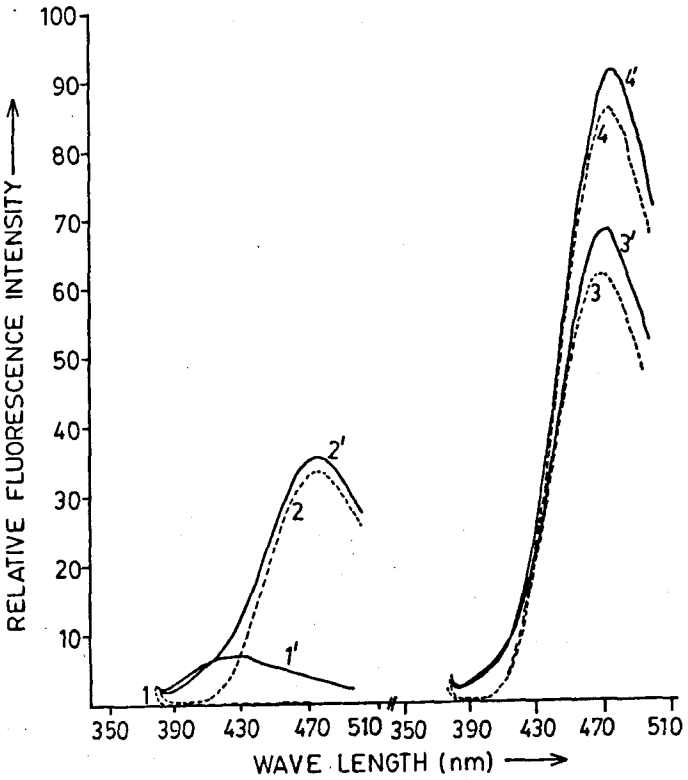

Fig. 4 : Fluorescence spectra of BSA-ANS in the presence (—) and absence (- - -) of drug.

BSA and drug concentrations were fixed at 20 μM. Excitation wavelength was 370 nm. 1,1'-BSA+0 μM ANS; 2,2'-BSA+4 μM ANS; 3,3'-BSA+8 μM ANS; 4,4'-BSA+12 μM ANS.

When drug is added to BSA-ANS system it can compete with ANS for hydrophobic sites on the surface. In that case it would inhibit the binding of ANS, i.e., displace ANS from its binding site 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 highly hydrophobic character and, thus, further shifts fluorescence of ANS to higher values. Hydrophobic interaction of phenothiazine neuroleptics with ANS has been reported by Jun and Ruenitz[9].

To understand further the nature of interaction involved, critical micellar concentration (CMC) of an anionic surfactant, sodium dodecyl sulphate (SDS) was determined in the presence and absence of 50 μM drug. CMC of SDS was found to be 8.20 mM which is in agreement with the literature value obtained by conductance method[15]. In the presence of 50μM drug, however, CMC value was reduced to 8.0 mM.

It is known that an additive solubilized inside the micelle increases the hydrophobicity of the medium and thus produces a small increase in CMC. On the other hand, attractive electrostatic interactions between anionic head groups of surfactant and drug cations reduce the CMC considerably. Only slight decrease in CMC value in the presence of drug shows that the drug also has some ionic character in addition to the predominantly hydrophobic character.

Fluorescence quenching data was also analysed by the modified Stern-Volmer plot[16]

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

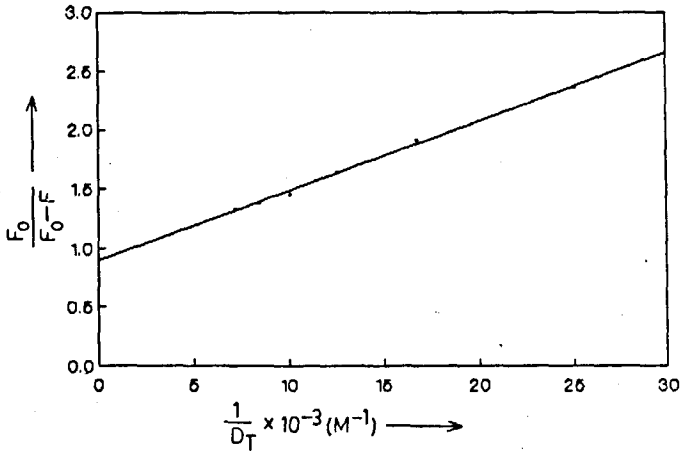

Fig. 5 : Stern - Volmer plot $\{F_o/F_o-F)$ versus $1/D_T\}$ for the binding of trifluoperazine dihydrochloride to BSA.

F_o and F are the fluorescence intensities in the absence and presence of trifluoperazine dihydrochloride. D_T is the total drug concentration

where F_o and F are the fluorescence intensities at 344 nm in the presence and absence of quencher, respectively, at concentration [Q] = D_T, the total concentration of drug. K_q is the Stern-Volmer quenching constant and f_a is the fraction of fluorophore (protein) accessible to the quencher (drug). From a plot of $F_o/(F_o-F)$ versus $1/D_T$, f_a and K_q were determined (cf. Fig. 5).

f_a, the fraction of fluorophore (protein) accessible to the quencher (drug), was found to be close to unity (1.12)

indicating thereby that both tryptophan residues of BSA are involved in the drug-protein interaction.

The Stern-Volmer quenching constant, K_q, was found to be 1.692 x 10^4 M^{-1}. 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 τ_o value for tryptophan fluorescence in proteins is known to be = 10^{-9} s[16], the rate constant, k_q, would be of the order of 10^{13} $M^{-1}s^{-1}$. 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

$$k_q = 4 \pi a D N_A \times 10^{-3} \qquad (4)$$

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} $M^{-1}s^{-1}$. The high magnitude of k_q in the present study (~10^{13} $M^{-1}s^{-1}$) can probably be attributed to increase in the encounter radii of tryptophantrifluoperazine dihydrochloride (R_{tryp}-TFP). This can happen only if the process of energy transfer and hence the quenching of tryptophan fluorescence occurs by intermolecular interaction forces such as ion-dipole and dipole-dipole between tryptophan and TFP. This is possible only when TFP binding site is in close proximity to tryptophan residues of BSA.

The nature of interaction between drug and protein may thus the summarised as under. The interaction is non-covalent in nature and there are two independent binding sites. The findings, that drug increases the fluorescence of ANS, show that the drug has a strong hydrophobic character. Only slight decrease in CMC of SDS in the presence of drug shows that ionic character of $>\overset{H}{\underset{+}{N}}-$ moiety also contributes slightly to binding. Stern-Volmer analysis predicts intermolecular interaction forces between drug and protein in which both tryptophan residues of BSA are involved.

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