RESEARCH PAPER

Accepted 5 March 1999
Received 21 September 1998
Indian J. Pharm. Sci. 1999, 51 (3) 143-148

Mode of Interaction of Metronidazole with Bovine Serum Albumin

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Mechanism of interaction of metronidazole with bovine serum albumin 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. The reaction was found to be first order with rate constant 1.832 s^{-1} and activation energy 13.194 KJ mol^{-1}. The drug does not compete with hydrophobic probe, 8-anilino-1-naphthalene sulphonic acid sodium salt (ANS) for hydrophobic sites on the surface. The decrease in critical micellar concentration (CMC) of cationic surfactant, cetyltrimethylammonium bromide (CTAB) in the presence of metronidazole showed that the drug does not have predominantly hydrophobic character and is not solubilized inside the micelle. Reduction in surface tension of the solvent suggested that the drug has a weakly hydrophobic character. Stern-Volmer analysis of fluorescence quenching data showed that both tryptophan residues of BSA are involved in the drug-protein interaction. The high magnitude of the rate constant for quenching, k_q ($10^{13} M^{-1} s^{-1}$) could be attributed to increase in the encounter radii of tryptophan-metronidazole due to hydrogen bonding interaction between BSA and drug. Thermodynamic parameters, obtained from data at different temperatures, showed that the binding of metronidazole to BSA involves the formation of both hydrogen and hydrophobic bonds. However, much smaller magnitude of ΔS° value showed that the hydrogen bond formation interaction predominates.

Drug-protein binding has important pharmacokinetic and pharmacodynamic implications[1-3]. The extent of binding and the nature of binding forces affect the availability of drug at the site of action. Serum albumin, being the major binding protein for drugs and other physiological substances, is considered as a model for studying drug-protein interaction *in vitro*[4].

Although metronidazole has been very widely used in the treatment of intestinal amoebiasis, trichomoniasis, giardiasis[5-7] and as radiosensitizer in radiation therapy[8], there appears to be not much information about the mechanism of protein binding of metronidazole. Some preliminary studies have been reported by Bangbose and Bababunmi[9] and other[10] using equilibrium dialysis and proton nmr techniques. Sulkowska *et al.*,[11] while studying the effect of urea and ionic strength on the association of metronidazole with bovine serum albumin, have shown that the drug is released on unfolding the protein. Interaction of metronidazole with nucleic acids has been reported by La Russo *et al*[12]. However, detailed investigations on the nature of interaction involved have not been reported. Present paper reports various aspects of the mechanism of interaction of metronidazole with bovine serum albumin using fluorescence spectroscopic technique.

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EXPERIMENTAL

Bovine serum albumin (BSA) and 8-anilino-1-naphthalene sulphonic acid sodium salt (ANS) have been purchased from Sigma Chemical Co., USA. A pure sample of metronidazole was kindly provided by Unique Pharmaceutical Laboratories Ltd., Ankaleshwar, Gujarat. 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.

Perkin Elmer fluorescence spectrophotometer (MPF 44B) equipped with a 150 W xenon lamp source was used.

Metronidazole-BSA interaction: 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 0.5 at the excitation wavelength in order to avoid inner filter effect. Thermodynamic parameters for drug-protein interaction were determined from experiments conducted at three different temperatures, 2,20 and 30°. Fluorescence spectroscopic data was analysed using the ward method[13] to obtain association constant and the number of available binding sites.

Kinetic Studies : Metronidazole was taken in excess (160 μM) and increasing amounts of BSA (2-10 μM) were added. Rate of the reaction was followed by setting the fluorescence spectrophotometer in time drive mode and measuring intensity of fluorescence w.r.t. time at an excitation wavelength of 296 nm and emission wavelength of 344 nm (the emission maximum of BSA).

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 concentration was kept fixed at 20 μM and ANS/drug concentration was varied from 4 to 25 μM. Fluorescence spectra were 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 5,10,15 and 20 μM drug. BSA concentration was again kept fixed at 20 μM and ANS concentration was varied from 4 to 25 μM. Fluorescence spectra were recorded in the range 350 to 550 nm after excitation at 370 nm.

Critical Micellar concentration : CMC of cetyltrimethylammonium bromide (CTAB) was determined in the presence and absence of 40 μM metronidazole using hydrophobic probe, ANS[14]. ANS concentration was kept fixed (120 μM) and CTAB concentration was varied from 2.50×10^{-4} to 1.75×10^{-3} M. Fluorescence spectra were recorded in the range 350 to 550 nm after excitation at 370 nm.

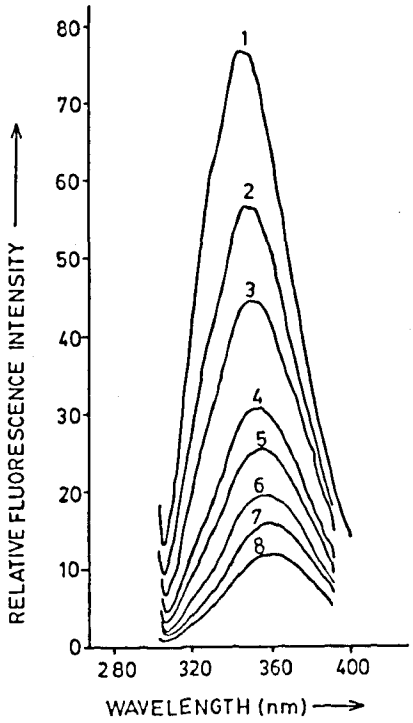

Fig. 1 : Fluorescence spectra of bovine serum albumin in the presence of metronidazole

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

Surface Tension measurements : Surface activity of the drug was determined by surface tension measurements. Surface tension of 0.8% drug solution was measured by drop weight and drop number methods using a stalagmometer. Surface tension data was expressed as surface pressure, π which is the difference between the surface tension of the solvent and that of the drug solution.

RESULTS AND DISCUSSION

Metronidazole-BSA interaction

Fluorescence spectra of BSA in the presence of increasing amounts of metronidazole drug are shown in Fig. 1. It is observed that on interaction with the drug, λ_{max} of tryptophan fluorescence in BSA shifts to longer wavelength (344 to 360 nm on the addition of 0 to 140 μM drug to 24 μM BSA). Drug is also observed to quench the fluorescence of BSA. Shift in λ_{max} towards longer wavelength and decrease in intensity of fluorescence is

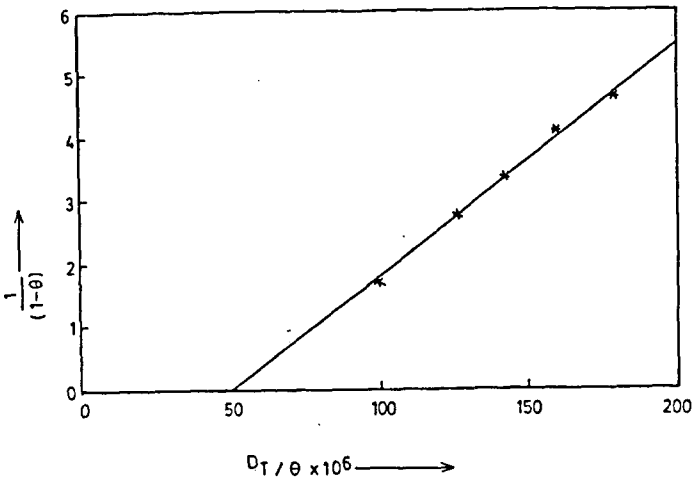

Fig. 2 : 1/(1-0) versus D_T/0 plots for the binding of metronidazole to BSA

θ is the fraction of drug bound and D_T is the total drug concentration

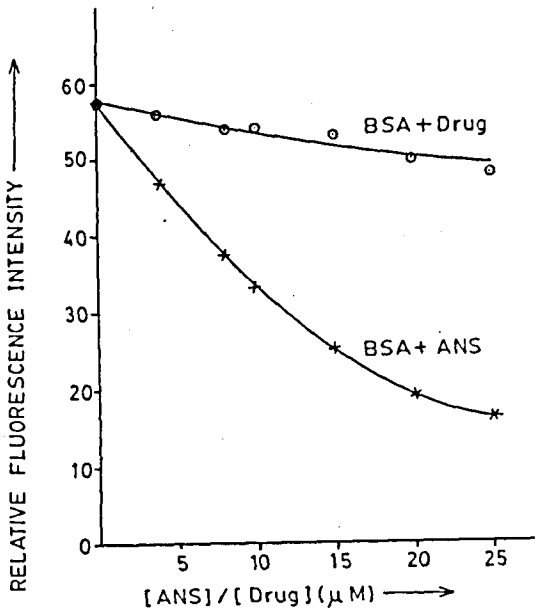

Fig. 3 : The effect of quenchers, metronidazole and ANS on the tryptophan fluorescence of BSA

BSA concentration was kept fixed (20 μM). Excitation wavelength was 296 nm and Emission wavelength was 344 nm

usually caused by increase in polarity of the solvent. The fraction of drug bound, 0, was determined according to Weber and Young[15,16] using the following equation;

$$0 = \frac{F_0 - F_p}{F_0}$$

where F_p and F_0 denote the fluorescence intensities of the protein in a solution with a given concentration of drug and without drug, respectively. 0 represents the fraction of sites on the protein occupied by molecules. Fluorescence data was analysed using the method described by Ward[13]. It has been shown that for equivalent and independent binding sites,

$$\frac{1}{(1-0)K} = \frac{[D_T]}{0} - n[P_T] \qquad (2)$$

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/(1-0) versus $[D_T]$/0 plot for BSA-metronidazole 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 3.61×10^4 and 2.08, 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.

Kinetic Studies

Fluorescence emission of drug (160 μM) in the presence of increasing amounts of BSA (2-10 μM) was monitored with respect to time. The reaction was complete in 2.5 to 3 seconds. Rate of reaction, r, expressed as ΔF/t was determined at time 1.5 sec for different concentrations of BSA. Since $r=K[C]^n$, log r was plotted against log C and from the slope and intercept of the linear plot, rate constant, k and the order of reaction, r were obtained. The reaction was found to be first order and the rate constant was found to be 1.832 s^{-1}. Kinetic studies performed at four different temperatures, 16, 23,30 and 40°, enabled determination of energy of activation for the reaction. From the slope of log k vs. 1/T plot, activation energy of the reaction was found to be 13.194 KJ mol^{-1}

Binding Studies in the presence of hydrophobic probe, ANS

Fluorescence spectra of 20 μM BSA in the presence of increasing amounts (4 to 25 μM) of metronidazole 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 large for ANS as compared to that for drug (cf. Fig.3). ANS bound to BSA, calculated from the fraction of occupied sites (0), was 72% whereas, the drug bound to BSA was only 19%, 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 the molecules of the interacting species[17]. 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 partial hydrophilic character. Thus drug and ANS do not share common binding sites in BSA.

In another set of experiments, BSA-ANS interaction was studied in the presence and absence of 5,10,15 and 20 μM metronidazole. It was found that the presence of drug had no effect on the fluorescence spectra. This again shows that the metronidazole does not compete with ANS for hydrophobic sites on the surface.

CMC OF CTAB

To understand further the nature of interaction involved, critical micellar concentration (CMC)of a cationic surfactant, cetyltrimethylammonium bromide (CTAB) was determined in the presence and absence of metronidazole. ANS is virtually non-fluorescent in aqueous solution and becomes highly fluorescent in non-polar solvents. Large increase in the intensity of ANS fluorescence on association of surfactant monomers to form micelles was employed as the basis of CMC determination[14]. Fluorescence intensity of 120 μM ANS was determined in the presence of increasing concentrations (2.5×10^{-4} to 1.75×10^{-3}M) of CTAB after excitation at 370 nm. Relative fluorescence intensity at λ_{max} (482-486nm) was plotted against the concentration of CTAB (Fig. 4). It was found that the presence of drug decreased the CMC of CTAB from 9.875×10^{-4} M to 7.500×10^{-4} M.

It is known that the repulsion between head groups of surfactant increases the free energy of micellization and thus hinders micelle formation. The presence of oppositely charged ions at the boundary of micelle reduces the repulsion between head groups of surfactant thereby facilitating micelle formation. The CMC, therefore,

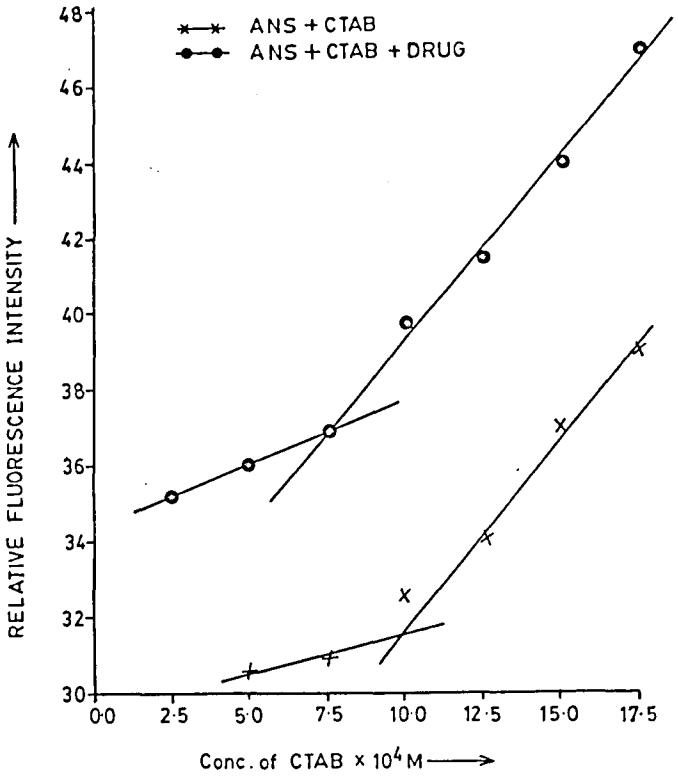

Fig. 4 : Relative fluorescence intensity of ANS (482-486 nm) corresponding to λmax as a function of CTAB concentration

ANS concentration was kept fixed (120 μM) and CTAB concentration was varied from 2.50×10^{-4} to 1.75×10^{-3}M. Excitation wavelength was 370 nm.

decreases. At pH 7.4 drug is predominantly in the ionized form (pK_a = 2.5) and thus carries a negative charge on the alcoholic-CH_2-CH_2-OH group. The decrease in CMC shows that the negatively charged drug molecules interact with positively charged-NH^+_4 groups at the boundary of CTAB micelle thereby decreasing the CMC. A drug with hydrophobic character will be solubilized in the micelle with negligible change in CMC. The decrease in CMC shows that the drug does not have predominantly hydrophobic character and is not solubilized inside the micelle.

Surface Activity

Surface tension (γ_{soln}) of 0.8% solution of metronidazole in phosphate buffer(pH 7.4)at 34°, determined by drop weight and drop number methods, was found to be 7.003×10^{-2} and 7.015×10^{-2}N m-1, respectively. The

average value of γ_{soln} being 7.009×10^{-2} N m^{-1}. Thus surface activity, expressed as surface pressure, $\pi = \gamma_{solvent} - \gamma_{soln}$, was found to be 4.5×10^{-4} N m^{-1}.

Reduction in surface tension of solvent or increase in surface pressure, π, is due to hydrophobicity of the drug molecule. However, the order of π value suggests that the drug has only a weakly hydrophobic character.

Stern-Volmer Analysis

Fluorescence quenching data was also analysed by the modified Stern-Volmer plot[18]

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{[Q] f_a K_q} \qquad (3)$$

where F_0 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 $F_0/(F_0-F)$ 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 (1.11) 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 1.91×10^4 M^{-1}. For a bimolecular quenching process, $K_q = K_q t_0$ where t_0 is the lifetime in the absence of quencher and K_q is the rate constant for quenching. As t_0 value for tryptophan fluoresence in proteins is known to be $\approx 10^{-9}$ s, 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 the 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 tryptophanmetronidazole

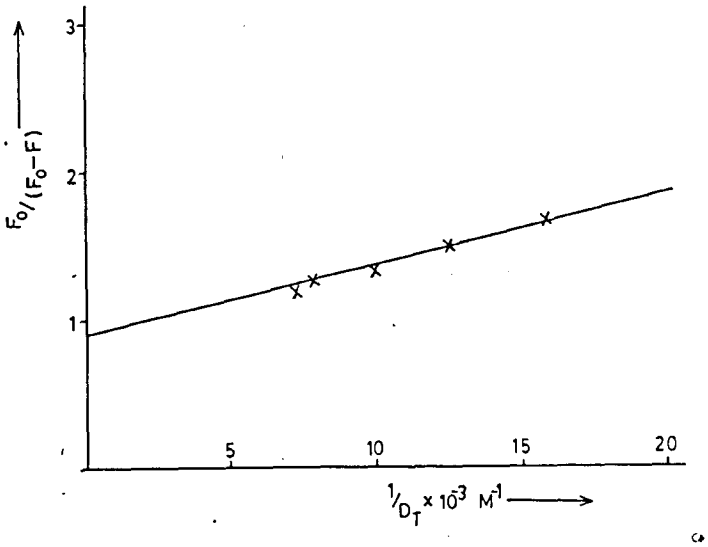

Fig. 5 : Stern-Volmer plot $\{F_0/(F_0-F)$ versus $1/D_T\}$ for the binding of metronidazole to BSA

F_0 and F are the fluorescence intensities in the absence and presence of metronidazole. D_T is the total drug concentration.

($R_{tryp\text{-}metro}$) due to hydrogen bonding interaction between BSA and drug. Thus drug is bound to BSA by hydrogen bond at a site which is close to the tryptophan residues.

Thermodynamics of drug-protein interaction

Thermodynamic parameters for the binding of metronidazole to BSA were determined by carrying out the binding studies at three different temperatures, 2,20 and 30°. Other conditions being same as described previously in experimental section. Association constant, K was found to decrease with increase in temperature Since,

$$\log K = \frac{-\Delta H^\circ}{2.303\ RT} + \frac{\Delta S^\circ}{2.303\ R} \qquad (5)$$

log K versus 1/T plot enabled determination of standard enthalpy change, ΔH° and standard entropy change, ΔS° for the binding process. ΔH°, ΔS° and ΔG° values were found to be -4.31 KJ mol^{-1}, +72.53 mol^{-1} and -25/56 KJ mol^{-1}, respectively. Negative ΔH° value indicates hydrogen bond formation between the substrates, whereas, positive ΔS° value indicates hydrophobic character of binding. These observations, based on thermodynamic considerations, therefore, suggest that the binding of metronidazole to serum albumin involves the formation

of both hydrogen and hydrophobic bonds. However, the magnitude of ΔS° is much less than ΔH° showing thereby that the hydrogen bond formation interaction predominates.

The nature of interaction between drug and protein may thus be summarised as under. The finding that drug does not compete with hydrophobic probe ANS for common sites on BSA and decrease in CMC of CTAB in the presence of drug suggest hydrophilic nature of binding whereas lowering of surface tension of the solvent shows that the drug has a weakly hydrophobic character. Stern-Volmer analysis predicts hydrogen bonding interaction between drug and protein in which both tryptophan residues of BSA are involved. Large negative ΔH° and small positive ΔS° again show that weak hydrophobic bonding and relatively strong hydrogen bonding interaction is involved.

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