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Spectroscopic Investigations on the Interaction of Bovine Serum Albumin with Amoxicillin and Cloxacillin

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The mechanism of interaction of two drugs *viz.*, amoxicillin and cloxacillin with bovine serum albumin has been investigated using fluorescence absorption and circular dichroism spectroscopy. The quenching mechanism of fluorescence of bovine serum albumin by amoxicillin and cloxacillin was discussed. The binding sites number n and apparent binding constant K were measured by fluorescence quenching method. The thermodynamic parameters obtained from data at different temperatures were calculated. The distance r between donor (bovine serum albumin) and acceptor (amoxicillin and cloxacillin) was obtained according to Forster theory of non-radiative energy transfer. The effect of common ions on binding constant was also investigated. The results of synchronous fluorescence spectra, UV-vis absorption spectra and circular dichroism of BSA in presence of amoxicillin and cloxacillin show that the conformation of bovine serum albumin changed

keywords: antibacterial drugs, bovine serum albumin, fluorescence quenching, UV-vis spectroscopy, thermodynamic parameters, energy transfer

INTRODUCTION

Serum albumins are the most abundant proteins in the circulatory system of a wide variety of organisms. Being the major macromolecule contributing to the osmotic blood pressure they can play a dominant role in drug disposition and efficacy. Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components, which then function as carriers. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cells in vivo and in vitro. Consequently, it is important to study the interaction of drugs with this protein. The effectiveness of drugs depends on their binding ability[1-4]. The selected drugs, amoxicillin (AMX) and cloxacillin (CLX) are broad-spectrum penicillin, active against certain Gram-positive and Gram-negative organisms, and widely used as antibacterial agents.

Fluorescence and UV-vis absorption spectroscopies are powerful tools for the study of the reactivity of chemical and biological system. The aim of this work was to determine the affinity of AMX and CLX to bovine serum albumin (BSA), and to investigate the thermodynamics of their interaction. To resolve this problem the UV, circular dichroism and fluorescence properties of AMX and CLX as well as BSA were investigated[5].

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MATERIALS AND METHODS

BSA, Fraction V 99 % protease-free essentially γ-globulin free prepared from pasteurized serum. Purified by heat treatment and organic solvent precipitation was obtained from Sigma Chemical Company, St Louis, USA. AMX and CLX drugs were obtained as gift samples from CIPLA LTD, India. The solutions of AMX and CLX and BSA were prepared in 0.1M phosphate buffer of pH 7.4 containing 0.15 M NaCl. All other materials were of analytical reagent grade and doubly distilled water was used throughout.

Fluorescence measurements were performed on a Hitachi spectrofluorometer Model F- 2000 equipped with a 150W Xenon lamp and slit width of 10 nm, and using a 1.00 cm quartz cell was used. Peltier Accesory (temperature control) attached Varian CARY 50 BIO UV-vis spectrophotometer was used for scanning UV-vis spectra. CD measurements were made on a JASCO-810 spectropolarometer using a 1.00cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 195-260 nm.

Spectroscopic measurements

Drug-BSA interaction

Some preliminary studies were carried out to select optimum protein and AMX and CLX concentrations for interaction. On the basis of preliminary experiments, BSA concentration was kept fixed at 10 mM and AMX and CLX concentration was varied from 10 to 140 μM. Fluorescence spectra were recorded at room temperature 29°C in the range 300-500 nm after excitation at 296 nm in each case. The fluorescence measurements were performed at different

temperatures (298, 302, 306, and 310 K). Excitation wavelength was 296 nm. Appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence. The absorption spectra of BSA, AMX and CLX and their mixture were performed at room temperature. A stock solution of 0.1 μM BSA was prepared in 0.01M phosphate buffer containing 0.15M NaCl. The BSA to AMX/CLX concentration was varied (1:1,1:3 and 1:5) and the CD spectrum was recorded.

RESULTS AND DISCUSSION

The structures of AMX and CLX used in the present study are shown in Table 1.

Fluorescence studies

Fluorescence spectra of BSA in the presence of increasing amounts of various CLX are shown (Fig. 1). It could be interpreted that the complex formed between AMX/CLX and BSA quenched the fluorescence of tryptophan moiety of BSA. The fraction of drug bound, θ, was determined[1,6] using the equation, $\theta = (F_o - F) / F_o$ ----------(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. Theè represents the fraction of site on the protein occupied by drug molecule. Fluorescence data was analyzed using the method described by Ward[7]. It has been shown that for equivalent and independent binding 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 is the number of binding sites, [Dt] is the total drug concentration and $[P_T]$ total protein concentration. A plot of 1/(1- θ) *versus* [Dt]/θ was shown in Fig. 2. The values of K and n, obtained from the slope and intercept of such plots were found to be 1.52 X10^4 and 6.27 X10^4 M^{-1} and, 2.11 and 3.63 for AMX and CLX, respectively. The higher association constant (K) values of 10^4 and the number of binding sites concluded that under the conditions of the experiment, the binding sites are equivalent and independent. The order of K value is consistent with non-covalent interactions. Association constant for the AMX/CLX-BSA system showed that the interaction is non-covalent in nature and that there occurs only a partial occupation of a binding site.

Table 1. Structure of AMX and CLX

Drug	Structure
AMX	
CLX	

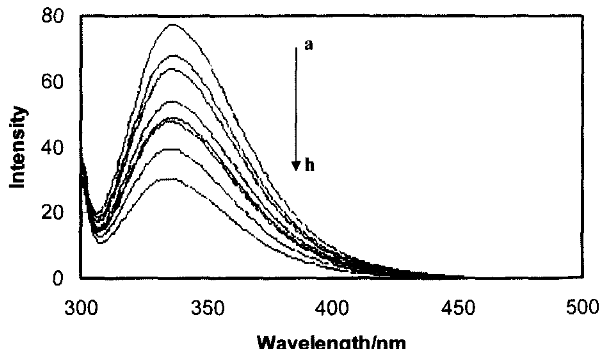

Figure 1. Fluorescence spectra of BSA (10 μM) in the presence of CLX (a-0, b-20, c-40, d-80, e-100, f-140, g-160 and h-180 μM).

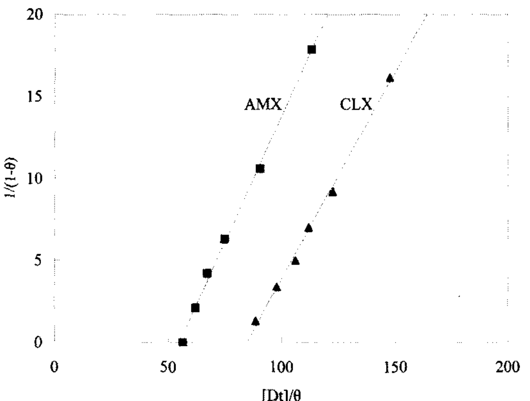

Figure 2. 1/(1-θ) versus [Dt]/θ plot for the binding of AMX and CLX to BSA.

Stern-Volmer analysis

The fluorescence quenching data are usually analysed by the Stern-Volmer equation:

$$F_o/F = 1 + K_{SV}[Q] \qquad (3)$$

Where F_o and F are the steady-state fluorescence intensities in the absence and presence of quencher (AMX and CLX), respectively. K_{SV} is the Stern-Volmer quenching constant and [Q] the concentration of quencher. The Stern-Volmer quenching constant K_{SV} of BSA and tryptophan residues fluorescence by AMX and CLX at different temperatures are shown in table 2. These results indicate that the probable quenching mechanism of fluorescence of BSA by AMX and CLX is a static quenching procedure because the K_{SV} decreased with rising temperature.

Table 2. Stern-Volmer quenching constant K_{SV} at pH=7.4

T(K)	10^{-4} K_{SV} (L mol^{-1})		R^a	S.D.^{b}
	AMX	CLX		
298	2.241	6.882	0.9985	0.0274
302	1.524	6.274	0.9976	0.0312
306	1.321	5.214	0.9979	0.0277
310	1.227	3.339	0.9982	0.0339

R^a is the linear quotient
S.D.^{b} is the standard deviation

The rate constant for quenching, greater than 10^{10} $M^{-1}s^{-1}$, indicated that the drug-binding site is in close proximity to tryptophan residue of BSA. This again shows that both the tryptophan residues of BSA are accessible to drug molecule. For a bimolecular quenching process, $K_q = k_q \tau_o$ where, K_q is Stern-Volmer quenching constant, τo 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 equal to 10^{-9} s, the rate constant, k_q, would be of the order of 10^{13} M^{-1} s^{-1}. 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

$$k_q = 4\pi \text{ aDNa} \times 10^{-3} \quad (4)$$

Where, D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of molecular radii and Na 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 a specific long-range interaction between drug molecule and tryptophan residues on protein. Thus the process of energy transfer occurs by intermolecular interaction forces between tryptophan and AMX/CLX and this is possible only when the drug-binding site is in close proximity to tryptophan residues of BSA.

The determination of the force acting between AMX and CLX and BSA

The interaction forces between drug and biomoleule may include hydrophobic force, electrostatic interactions, van der Waals interactions or hydrogen bonds etc. The slope of a plot of the bimolecular quenching constant versus 1/T (T, absolute temperature) allows one to calculate the energy change for the quenching process since,

$$\text{Log } K = -\Delta H^o/2.303RT + \Delta S^o/2.303R \quad (5)$$

Log K *versus* 1/T plot enabled determination of standard enthalpy change, ΔH^o and standard entropy change, ΔS^o for the binding process. The ΔH^o, ΔS^o and ΔG^o values were found to be +1.92 and +1.23 $KJmol^{-1}$, +72.56 and +87.69 $JK^{-1}mol^{-1}$ and, −21.91 and −26.48 $KJmol^{-1}$ for AMX and CLX, respectively. The positive enthalpy (ΔH^o) and entropy (ΔS^o) values of the interaction of AMX and CLX and BSA indicate that the bonding is mainly entropy-driven and the enthalpy is unfavorable for it, the hydrophobic forces playing a major role in the reaction.[8] The low positive ΔH_o value indicates less-dominant hydrogen bond formation between the substrates while positive ΔS_o value indicates predominant hydrophobic character of binding (Large hydrophobic species such as proteins avoid the water molecules in aqueous solution as far as possible by associating into micelle-like structures with the non-polar portions in contact in the inner regions of the micelles, the polar ends facing the water molecules. This attraction of hydrophobic species, resulting from their unwelcome reception in water, is known as hydrophobic bonding or better, hydrophobic interaction). The positive ΔH_o and ΔS_o values observed in this case indicate less dominant hydrogen bond formation and predominant hydrophobic character of binding between BSA and AMX/CLX. These results together with spectral changes in the fluorescence emission spectra of BSA induced by AMX/CLX suggest that the interaction may take place in subdomain IA and IIA since these have been proposed to bind drugs and other hydrophobic materials.

Energy transfer between AMX and CLX and BSA

The overlap of the UV absorption spectra of AMX and CLX with the fluorescence emission spectra of BSA is shown in Fig. 3. According to Forster's non-radiative energy transfer theory, the energy transfer will happen under conditions: (a) the donor can produce fluorescence light; (b) fluorescence emission spectrum of the donor and UV absorption spectrum of the acceptor have more overlap; (c) the distance between the donor and the acceptor is close and lower than 7 nm. The energy transfer effect is related not only to the distance between the acceptor and the donor, but also to the critical energy transfer distance, that is

$$E = R_o^6/(R_o^6 + r^6) \quad (6)$$

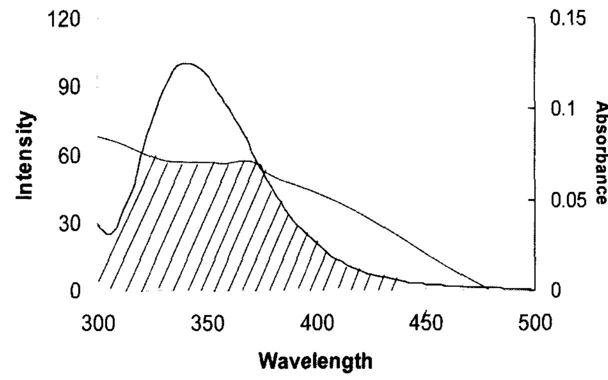

Figure 3. Spectral overlap of AMX absorption with BSA fluorescence c (BSA) = c (AMX) = 1.0X 10^{-5} mol L^{-1}.

Where r is the distance between the acceptor (A) and the donor (D), R_o is the critical distance when the transfer efficiency is 50 %, which can be calculated by $R_o^6 = 8.8 \times 10^{-25} k^2 \phi n^{-4} J$ (7)

Where k^2 is the spatial orientation factor of the dipole, n is the refractive index of the medium, ϕ is the fluorescence quantum yield of the donor, J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$J = \Sigma F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (8)$$

where F(λ) is the fluorescence intensity of the fluorescent donor at wavelength λ, e(λ) is the molar absorptivity of the acceptor at wavelength λ. The energy transfer efficiency is given by $E = (F_0-F) / F_0$ ---------(9)

It has been reported for BSA that, $k^2 = 2/3$, $\phi = 0.10$, n=1.46. J can be evaluated by integrating the spectra Fig.3. According to equations (6)-(9) we could calculate that J= 1.16 X 10^{-15} and 1.13 X 10^{-15} cm^3Lmol^{-1} for AMX and CLX, R_o= 2.25 and r = 2.38 and 2.34 nm for BSA. So the distance between CLX/ AMX and tryptophan residue in BSA is 2.38 nm for AMX and 2.34 nm for CLX. Obviously, they are lower than 7 nm after interaction between AMX/CLX and BSA. These accord with conditions of Forster's non-radiative energy transfer theory, indicating again the static quenching interaction between AMX/CLX and BSA.

The effect of other ions on the binding constant

The effect of common ions on the binding constants was investigated at 29°C. The results are summarized in table 3. It is shown that the binding constant between the protein and AMX and CLX increased in the presence of common ions, implying stronger binding between AMX and CLX and BSA. The higher binding constant obtained in the presence of metal ions might be resulted from the interaction of metal ion with AMX and CLX to form a complex, than the complex interacted with BSA thus, prolonging the storage time of the AMX and CLX in blood plasma and enhancing the maximum effectiveness of the drug. Therefore, in the presence of common ions AMX and CLX can be stored and removed better by BSA.

Table 3. The binding constants ($Lmol^{-1}$) between AMX, CLX and BSA at 29°C in the presence of common ions.

Ions	Association constant	
	AMX	CLX
Na^+	2.25 X 10^4	7.21 X 10^4
K+	2.69 X 10^4	6.42 X 10^4
Ca^+	2.06 X 10^5	7.65 X 10^4
Ba^{2+}	3.95 X 10^4	7.25 X 10^4
Mg^{+2}	3.23 X 10^4	6.65 X 10^5
Al^{3+}	2.21 X 10^4	3.21 X 10^5
Cl^-	2.23 X 10^4	7.19 X 10^4
Br^-	2.25 X 10^4	6.69 X 10^4
F^-	3.96 X 10^5	7.23 X 10^4
CO_3^{2-}	2.21 X 10^4	6.91 X 10^4
SO_4^{2-}	2.06 X 10^4	2.16 X 10^5
PO_4^{3-}	2.22 X 10^4	7.36 X 10^4

Conformation investigation

To explore the structural change of BSA by addition of AMX and CLX, we measured UV-vis spectra (Fig. 4) and circular dichroic spectra of BSA (Fig. 5) with various amounts of AMX and CLX. The UV-vis spectra of BSA at different contents of CLX show that in the visible region, the absorption peaks of these solutions showed moderate shifts towards shorter wavelength indicating that with the addition of AMX and CLX, the peptide strands of BSA molecules extended more and the hydrophobicity was decreased. The binding of AMX and CLX was also confirmed by circular

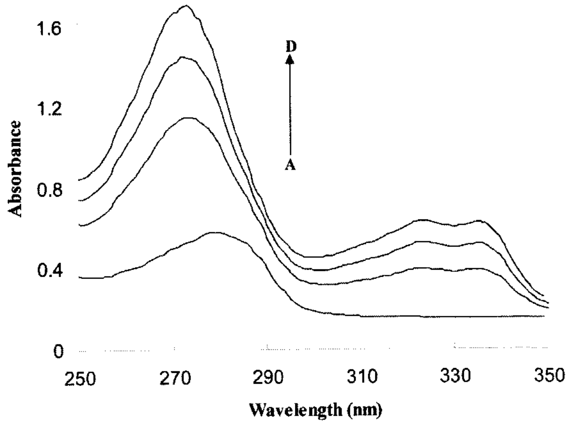

Figure 4. UV-vis spectra of BSA in the presence of various concentrations of AMX A-D, c(BSA) = 1.0 X 10^{-5} mol L^{-1}; c (CLX) (10^{-5} mol L^{-1}): 0; 2.6; 3.5; 6.

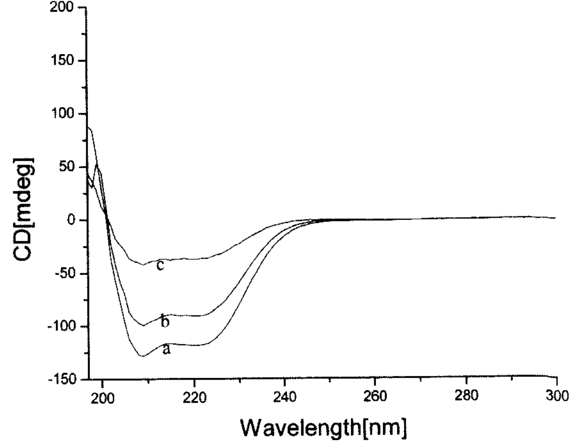

Figure 5. Circular Dichroism spectra in the 200-300 nm range: (a) BSA, 0.1 μM; [BSA]: CLX=1:1 (b); and 1:5 (c).

dichroism (CD) spectra. As expected the α-helices of protein show a strong double minimum at 220 nm and 209 nm[9]. The intensities of this double minimum reflect the amount of helicity of BSA and further these indicate that BSA contains more than 50% of α-helical structure. Upon the addition of the AMX to BSA (1:1, 3:1 and 5:1) the extent of á-helicity of the protein decreased and hence, the intensity of double minimum was reduced. This is indicative of change in helicity when the AMX is completely bound to BSA.

Conclusion

In this paper, the interaction of AMX and CLX with BSA was studied by spectroscopic methods including fluorescence spectroscopy, UV-vis absorption spectroscopy and circular dichroic spectroscopy. The results of synchronous fluorescence spectroscopy, circular dichroic and UV-vis spectra indicate that the secondary structure of BSA molecule is changed dramatically in the presence of AMX and CLX. The experimental results also indicate that the probable quenching mechanism of fluorescence of BSA by AMX and CLX is a static quenching procedure, the binding reaction is mainly entropy driven, and hydrophobic interactions played a major role in the reaction.

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