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Research Article

CONFORMATIONAL CHANGES OF SERUM ALBUMIN UPON COMPLEXATION WITH AMPHIPHILIC DRUG IMIPRAMINE HYDROCHLORIDE

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Abstract: Imipramine hydrochloride (IMP) is a cationic amphiphilic molecule belonging to the antidepressant category of drugs. Here we report the interaction of IMP with serum albumins (i.e., human (HSA) and bovine (BSA)) using various biophysical methods. Absorption spectroscopy provides qualitative information about the interaction and complex formation between IMP and serum albumins. The binding parameters and the corresponding thermodynamic parameters have been estimated by fluorescence quenching method. The results revealed that hydrophobic forces were the predominant intermolecular forces between serum albumins and IMP. The quenching rate constant (k_q) values decrease with increase in temperature which indicates a static quenching procedure. At very low concentrations of drug, the conformational changes in HSA/BSA due to interaction were investigated by circular dichroism (CD). For different molar ratios of protein and drug, the far-UV CD spectra showed an increase in α-helicity of serum albumins with the increment in α-helical structural content being slightly higher for IMP-BSA complex.

Keywords: Human serum albumin; Bovine serum albumin; IMP; fluorescence quenching; circular dichroism.

Introduction

Molecular biology is an emerging field of research which employs small molecules to modulate complex biological processes. In order to treat mental illness or depression, which is common in all diseases groups, various small molecules have been identified as antidepressants. Imipramine hydrochloride (IMP), 5-[3-(dimethylamino)propyl]-10,11-dihydro-5H-dibenz [b,f]-azepinemonohydrochloride, is such a small cationic amphiphilc drug molecule (Scheme 1) that blocks the reuptake of certain neurotransmitters such as norepinephrine and serotonin, making it a good candidate for mood stabilization (Jain *et al.*, 2002). Most of the amphiphilic drugs show their activity by interacting with biological membranes (Schreier *et al.*, 2000). In this regard, these drugs have to be carried to their binding sites of action by means of protein carriers, serum albumins, with which they bind with different affinities. The fates of drug molecules are thus correlated with their binding abilities with serum albumins. Strong binding can decrease the concentration of free drug in plasma, whereas weak binding can lead to a low circulation time or poor distribution (Cheema *et al.*, 2007). Towards this endeavor, it is quite interesting to study the binding capacity and structure of the complexes between serum albumins and drugs in order to find out the means by which clinical efficacy of drugs can be tuned.

Among the family of globular proteins, human serum albumin (HSA) and bovine serum

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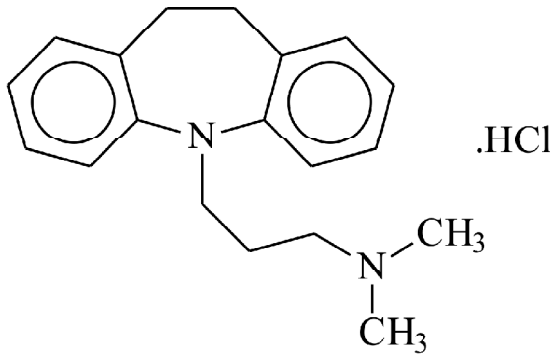

Scheme 1

albumin (BSA) are most studied proteins for many years due to their architecture, functional role and abundance in blood plasma. From the functionality point of view, serum albumins play vital role in maintaining the colloidal osmotic pressure of blood and distributing exogenous and endogenous molecules and metabolites such as nutrients, hormones, fatty acids and many diverse drugs (such as anticoagulants, tranquillizers, and general anesthetics), which make them suitable as a model for studying drug-protein interaction in *in-vitro* (Chakrabarty *et al*., 2007). The three-dimensional structure of human serum albumin (HSA), as determined by X-ray crystallography, comprises of three domains I, II and III (Carter and Ho, 1994). Each domain contains two subdomains, named IAB, IC, IIAB, IIC, IIIAB, IIIC, respectively. Bovine serum albumin (BSA) presents 76% sequence similarity with human serum albumin (HSA). One of the main differences within the two proteins is that BSA has two tryptophan residues (Trp134 and Trp214) while HSA has only one (Trp214). The extra tryptophan residue in BSA is buried in a hydrophobic sack and has been suggested to lie near the surface of the albumin molecule in the second α-helix of the first domain (He and Carter, 1992). The conformation of a protein in solution depends on the delicate balance of electrostatic, hydrogen bonding, van der Waals, and hydrophobic interactions between the amino acid residues of polypeptide chains, resulting in a folded structure of protein (Cheema *et al*., 2007). The three dimensional folded structure of protein is responsible for most of the biological functions that occur in living organisms. This fragile structure of protein gets affected (or unfolded) by the binding of small molecules (through preferential interactions of both the polar and nonpolar amino acids with the chemicals rather than with water) or change in the physiological conditions like pH (ionization of nonpolar residues), temperature (complex interplay between enthalpic and entropic effects) and by variation in ionic strength. The correct three dimensional structures are essential to function, although some parts of functional proteins may remain unfolded. Several neurodegenerative and other diseases are believed to result from the accumulation of amyloid fibrils formed by misfolded proteins (Selkoe, 2003). In this context, a perfect knowledge of the mechanism underlying protein folding-unfolding is necessary.

To understand the pharmacological actions of a particular drug, the mode of binding between the drug and protein should be studied. In this study, the main focus of work was to find the binding of drug with serum albumins leading to conformational changes and structural characterization of the complexes formed between HSA/BSA with imipramine hydrochloride by the techniques of UV-visible absorption and steady state fluorescence spectroscopy. Moreover, far UV-CD (circular dichroism) was employed to confirm the conformational changes of the proteins upon binding with drug.

Materials and Methods

Materials - Imipramine hydrochloride, human serum albumin (free from fatty acid) (HSA) and bovine serum albumin (free from fatty acid) were purchased from Sigma and were used without further purification. All other reagents were of analytical grade. All the experiments were performed in tris-hydrochloride buffer of pH 7.4 (20 mM). The concentration of protein was determined spectrophotometrically using $\varepsilon_{1\%}^{1cm}$ of 5.3 $M^{-1}cm^{-1}$ (HSA) and 6.5 $M^{-1}cm^{-1}$ (BSA) at 280 nm by using Perkin Elmer Lambda 25 spectrophotometer.

UV-Visible spectroscopic measurements - The UV measurements were carried out in such a way that the HSA and BSA concentrations were kept constant at 10 μM, while the concentration of drug was varied from 10 μM to 150 μM. The blank used

was that of appropriate drug solution and the Lamda 25 spectrophotometer was used for recording the absorbance.

Fluorescence measurements - Fluorescence measurements were carried out with Shimadzu-RFPC5301 spectrofluorimeter equipped with a PC. The fluorescence spectra were measured at three different temperatures (25°C, 37°C and 45°C) with a 1 cm path length cell and a thermostatically controlled cell holder attached to Neslab's RTE – 110 water bath (accuracy 0.1°C). The excitation and emission slits were set at 10 nm and 3 nm, respectively, for HSA while the excitation and emission slits were set at 5 nm and 3 nm, respectively, for BSA. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra were recorded in the range of 300–400 nm.

Circular dichroism measurements - Circular dichroism measurements were carried out with a JASCO spectropolarimeter (model J–815) equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulfonic acid. All the CD measurements were carried out at 30 °C. The spectra were collected with scan speed of 20 nm/min and response time of 1 sec. The observed ellipticities were converted into the mean residue ellipticities (MRE), deg. $cm^2.dmol^{-1}$, given by

$$MRE = \text{observed CD (mdeg)} / (10\, C_p n l) \quad (1)$$

where C_p is the molar concentration of protein, n the number of amino acid residues and l the path length.

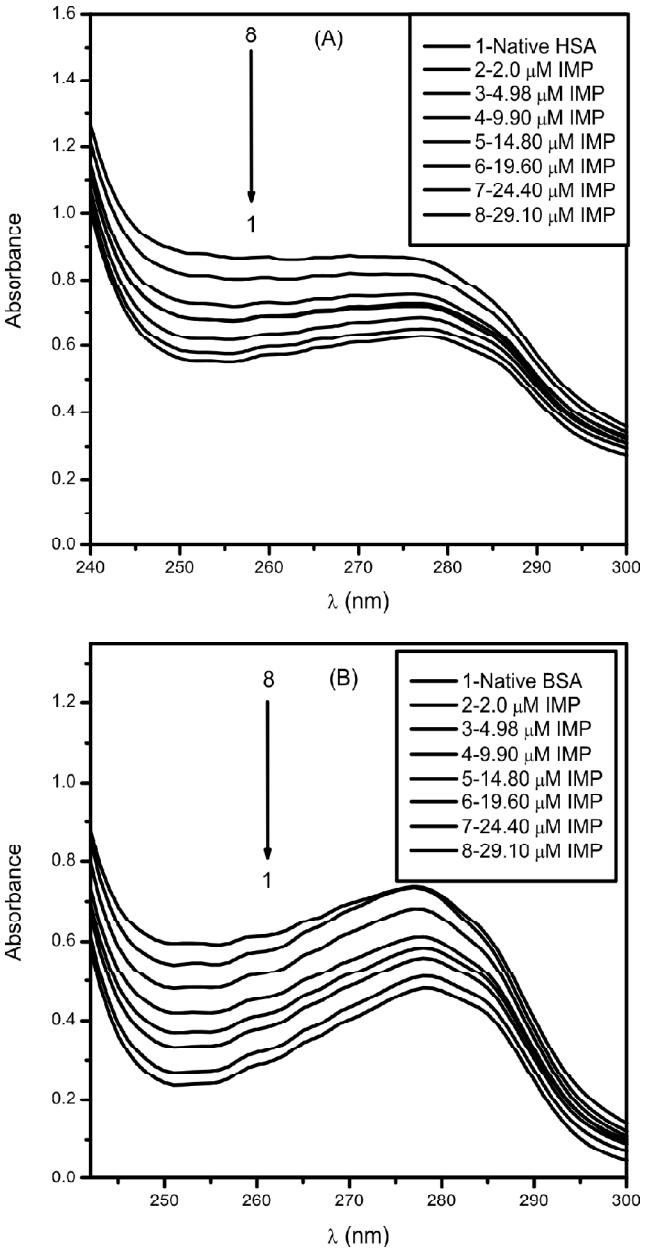

Figure 1: **Ultraviolet absorbance spectra of HAS, BSA and HSA-IMP (A) BSA-IMP (B) complexes**

Results and Discussion

UV Absorption Spectra of HSA/BSA in the presence of IMP

UV-visible absorption measurement is a very important method for the determination of structural changes and complex formation (Gore *et al.*, 2012). The absorption spectra of HSA/BSA and their complexes with IMP were studied at physiological pH and are shown in Figure 1. We are mainly concerned with the changes of the UV-visible absorption spectrum near 280 nm, which is the maximal absorption wavelength of HSA/BSA. As shown in Figure 1A and 1B, the absorption peak intensity increased with the addition of IMP. Furthermore, the formation of HSA/BSA-IMP complex resulted in a slight shift of spectrum toward shorter wavelength indicating the interaction between IMP-HSA/BSA. This also suggests that the chromophore of protein is placed in a more hydrophobic environment after addition of IMP. The obvious enhancement of absorbance intensity and the change in the absorption spectrum verify the formation of a new complex between IMP and HSA/BSA.

Fluorescence

The tryptophan, tyrosine and phenylalanine amino acid residues act as fluorophore probes in serum albumin (Carter and Ho, 1994). However, the intrinsic fluorescence of serum albumin is almost led by tryptophan alone, as phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost quenched if it is ionized or is near an amino group, a carboxyl group, or a tryptophan (Li *et al.*, 2012). Quenching in fluorescence is a decrease in the quantam yield of fluorescence from a fluorophore. It may be dynamic quenching, resulting from the collisional encounter between the drug and protein, or static quenching, resulting from the formation of a ground-state complex between the drug and protein. Dynamic and static quenching can be distinguished by their differing dependence on temperature. Increase in temperature results in larger duffusion coefficients, therefore, dynamic quenching constants are expected to increase. In contrast, increased temperature is likely to result in decreasing stability of complexes and thus lower the values of the static quenching constants.

The binding of antidepressant drug imipramine hydrochloride (IMP) with serum albumins (i.e., HSA and BSA), by maintaining the concentration of serum albumins constant (i.e., 2 μM) and varying the concentration of drug from 0.2 μM to 6 μM, was studied by fluorescence spectroscopy. The spectra were recorded after excitation of proteins at 280 (excites both tryptophan and tyrosine) and 295 nm (excites only tryptophan groups) for all the drug-serum albumin complexes at three temperatures (25 °C, 37 °C, and 45 °C). On critical analysis of the spectra of HSA and BSA in presence of IMP, no significant changes were observed between the two proteins; this leads to the conclusion that the target site for IMP in both cases is same. As we know, HSA contains only one tryptophan residue (Trp214), which resides in domain II. While in BSA, the tryptophan residues could be either Trp134 or Trp214. Hence it could be safely concluded that the Trp134 is not the target in the BSA. The fluorescence intensity of HSA/BSA decreased regularly with the increasing concentration of IMP (Figure 2).

In order to invoke possibility of quenching type (static or dynamic), data were analyzed using the Stern-Volmer equation (Lakowica, 1999).

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \qquad (2)$$

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} the Stern–Volmer quenching constant, k_q stands for bimolecular quenching constant, τ_0 for the life time of flurophore in the absence of quencher and [Q] the concentration of quencher (i.e., drug). On the basis of linear fit plots of Eq. (2), the K_{sv} values were obtained (Table 1). The value of τ_0 for biopolymers is 10^{-8} s (Togashi and Ryder, 2008). Accordingly, the values of k_q are 1.80×10^{12}, 1.14×10^{12}, and 0.66×10^{12} l/mol.s, at 293.15, 310.15 and 318.15 K, respectively.

In general, the maximum collision quenching constant (k_q) of various kinds of biomolecules is 2.0×10^{10} l/mol.s. In the present study, the K_{sv} values decreased with increase in temperature and values of k_q are far larger than 2.0×10^{10}l/mol.s (the maximum value reported for diffusion quenching rate constant of various quenchers with the biopolymer) (Cheng and Zhang, 2008), which indicates that the probable mechanism for IMP-HSA/BSA interaction is static quenching, i.e., the formation of a complex. Hence, fluorescence measurement is in total agreement with absorption studies, confirming that there is complex formation between HSA/BSA and IMP.

For HSA/BSA–IMP complexes, K_{SV} values were slightly more at 280 nm than at 295 nm but the values are more with BSA than HSA at both wavelengths. These results suggest that for HSA/BSA–drug complexes the quenching of fluorescence intensity by tyrosine residues is more effective in comparison to tryptophan residues. A noticed blue shift at maximum emission wavelength of serum albumin is likely because of the loss of compact structure of hydrophobic sub-domains where tryptophan was identified (Sułkowska, 2002). Nevertheless, the micro-environment about the tyrosine residues did not undergo evident changes during the binding process. Moreover, BSA-drug complex shows more shift as compared to HSA-drug complex.

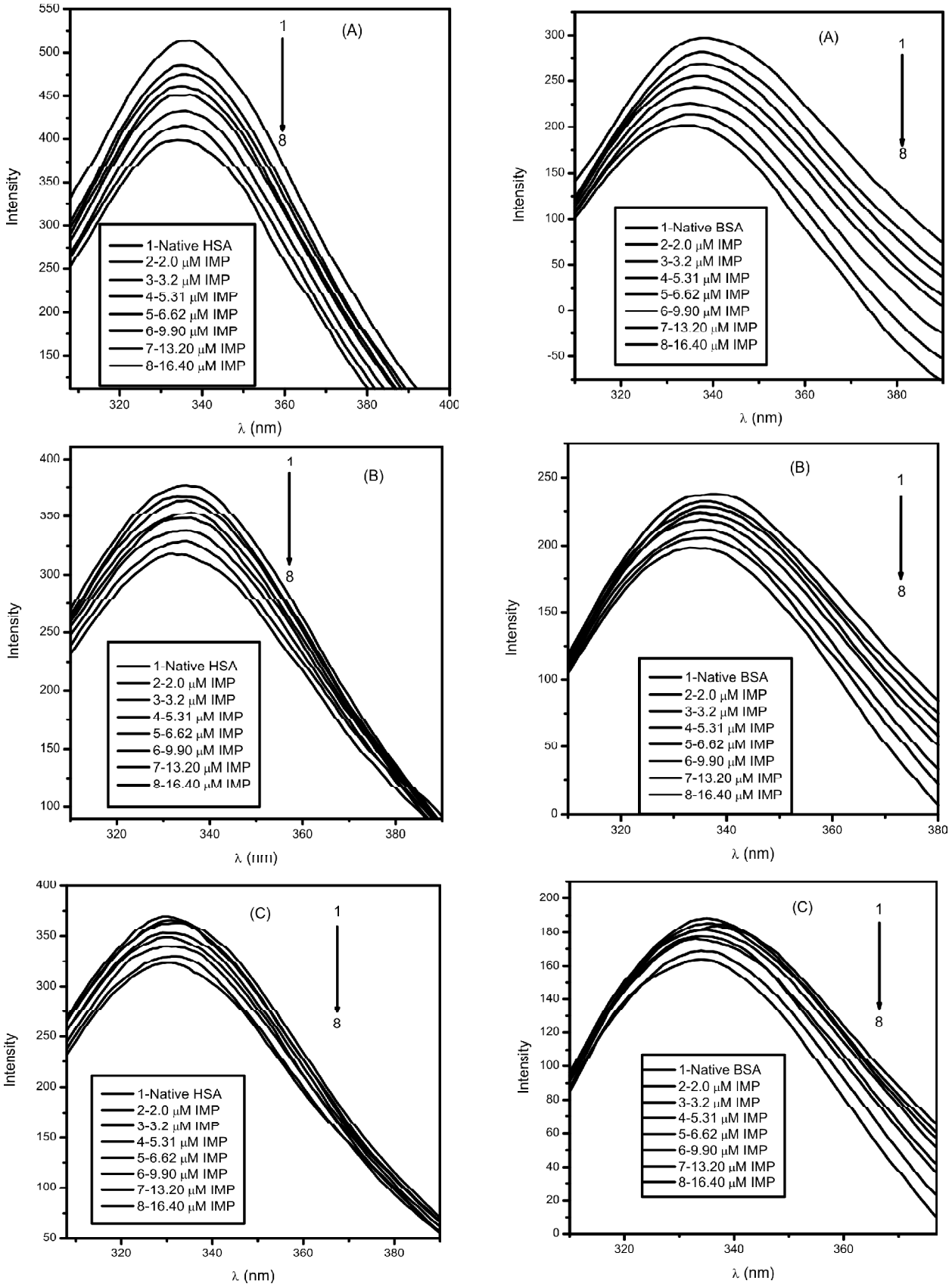

Figure 2: **Fluorescence emission spectra of native HSA/BSA and HSA/BSA-IMP complexes, excited at 280 nm at different temperatures (298.15 K (A), 311.15 K (B), 311.15 K (C))**

Analysis of binding equilibria

For static quenching, the relationship between fluorescence intensity and concentration of a quencher can be described by the equation shown below (Min *et al.*, 2004):

$$\log \frac{F_0 - F}{F} = \log K + n \log[Q] \quad (3)$$

where K and n are the binding constant and the number of binding sites, respectively. From a plot of $\log \frac{F_0 - F}{F}$ vs. log [Q], the *K* of IMP with HSA/BSA and the binding sites n can be obtained from the intercept and the slope (Table 1). The value of K is significant to understand the distribution of the drug in plasma since a weak binding can lead to a short lifetime or poor distribution, as strong binding can decrease the concentration of free drug in plasma. The larger values of K observed in the present study suggest the presence of strong binding between IMP and HSA/BSA. Further, the binding constant values decrease with increase in temperature indicating the reduction in stability of drug–HSA/BSA complexs. The complex could have been partly decomposed when temperature increases. The binding capability is more in case of BSA and IMP as compared to HSA, also more at 280 nm with respect to 295 nm. As seen in Table 1, the values of *n* are were approximately equal to 1 at different temperatures, which indicate the existence of just one main binding site in HSA/BSA for IMP.

Determination of binding force

Considering the dependence of binding constant on temperature, a thermodynamic process was believed to be responsible for the formation of a complex. Therefore, the thermodynamic parameters dependent on temperature were examined in order to further characterize the acting forces between IMP and HSA or BSA. The working forces between a small molecule and macromolecule mainly led in hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interaction forces. The

Table 1
Binding parameters, i.e, Stern-Volmer quenching constant, K_{sv}, number of binding sites, n, binding constant, K, and thermodynamic parameters for HSA/BSA with IMP at 280 and 295 nm excitation wavelengths at different temperatures

Temperature (K)	$K_{SV}\times10^{-4}$ *(l/mol)*	R^2	*n*	$K\times10^{-4}$ *(l/mol)*	*ΔG (k]/mol)*	*ΔH (k]/mol)*	*ΔS (J/mol.K)*
				HSA			
				280 nm			
298.15	1.80	0.993	0.70	0.06	-21.56	37.08	196.71
311.15	1.14	0.994	0.97	0.80	-23.25	40.38	204.52
318.15	0.66	0.999	1.06	1.36	-25.18	42.22	211.86
				295 nm			
298.15	1.06	0.986	0.87	0.25	-19.39	63.46	277.91
311.15	0.31	0.998	0.99	0.28	-20.53	69.12	288.14
318.15	0.20	0.995	0.96	0.12	-18.75	72.26	286.10
				BSA			
				280 nm			
298.15	3.11	0.995	0.99	2.75	-25.34	41.56	224.41
311.15	1.27	0.990	1.02	1.74	-25.26	45.27	226.69
318.15	1.01	0.985	1.18	0.83	-23.87	47.33	223.80
				295 nm			
298.15	2.35	0.995	1.12	9.33	-22.66	52.35	251.61
311.15	0.77	0.999	1.05	1.32	-24.54	57.02	262.15
318.15	0.57	0.998	0.97	0.41	-22.01	59.61	256.55

thermodynamic parameters, free energy change (ΔG), enthalpy change (ΔH), and entropy change (ΔS) are the main evidences to determine the binding mode. The thermodynamic parameters were evaluated using the following equations:

$$\Delta G = -RT \ln K \quad (4)$$

$$\Delta H = -RT^2 \left[\frac{d \ln K}{dT} \right] \quad (5)$$

and

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (6)$$

where R and T are the gas constant and temperature in Kelvin scale, respectively. The results obtained are given in Table 1. The negative values of free energy (ΔG) show that the interaction process is spontaneous. The free energy (ΔG) is more in case of BSA and drug as compared to HSA and drug complexes, also more at 280 nm with respect to 295 nm (Table 1). It means that BSA-IMP binding is more spontaneous as compared to HSA-IMP binding. Ross and Subramanian (Ross and Subramanian, 1981) have quantified the sign and magnitude of the thermodynamic parameters related with various individual kinds of interaction that may take place in the protein association process, which can be easily resolved as: (i) $\Delta H > 0$ and $\Delta S > 0$, hydrophobic force; (ii) $\Delta H < 0$ and $\Delta S < 0$, van der Waals force and hydrogen bonding; (iii) $\Delta H < 0$ and $\Delta S > 0$, electrostatic interactions. On the basis of water structure, the positive value of ΔS can be considered as evidence in favor of hydrophobic interactions, since the water molecules arranged in an orderly fashion around the drug and protein acquire more random configuration. The positive values of enthalpy (ΔH) suggest unfeasible electrostatic interaction between the two components. The continuous adsorption of cationic drug on protein results in increase in net positive charge on protein and hence gives rise to repulsive type of interaction between the protein and drug. Hence, the binding of IMP and HSA/BSA is mainly entropy-driven, and the enthalpy is unfavorable for it. Thus, it is concluded that the hydrophobic force has a major role in the interaction, but the electrostatic interaction is also argued.

Change in secondary structure by far-UV CD

CD is one of the strong and sensitive spectroscopic tools in order to explore the secondary structure of proteins and also interaction of proteins with small molecules (Greenfield, 2006; Kelly and Price, 1997). The perturbation in the secondary structure of HSA and BSA have been studied with far-UV CD, in the range of 200-260nm. At physiological pH, the CD spectra of serum albumins exhibit two negative bands in the ultra violet region at 208 nm (n-π transition) and 222nm (n-π^* transition) which is the characteristic of α-helical protein. The acquired data suggest that the secondary structure of free HSA consists of ~64% of α-helix, ~6% of β-sheets and ~30 of random coils and free BSA consists of of ~66% of α-helix, ~6% of β-sheets and ~22 of random coils.

To study the influence of IMP on the secondary structure of HSA/BSA, the far –UV CD spectra were recorded using various molar ratios of proteins to drug (Figure 3). In all the drug-serum albumin complexes at 1:5, 1:10, and 1:20 molar ratios, α-helical structure is induced and β-sheets reduced (Table 2). The values of the negative peaks slightly increase at lower concentration of drug (lower ratio), which denotes that secondary structure is not greatly affected. However, noticeable increase of the ellipticity is noted in the presence of excess drug molecules, which arises from a folding process undergone by the protein. This process involves an important gain of α-helix content in the

Table 2
Secondary structural analysis for the native HSA/BSA and their complexes with amphiphilic drug IMP

Molar ratio of drug: SA	*Conc. of drug(μM)*	*% α*	*% β*	*% RC*
Native HSA	0	62	5	33
HSA:IMP				
1:5	25	63	4	33
1:10	50	66	3	31
1:20	100	68	2	30
Native BSA	0	67	4	29
BSA:IMP				
1:5	25	69	4	27
1:10	50	72	3	25
1:20	100	77	2	23

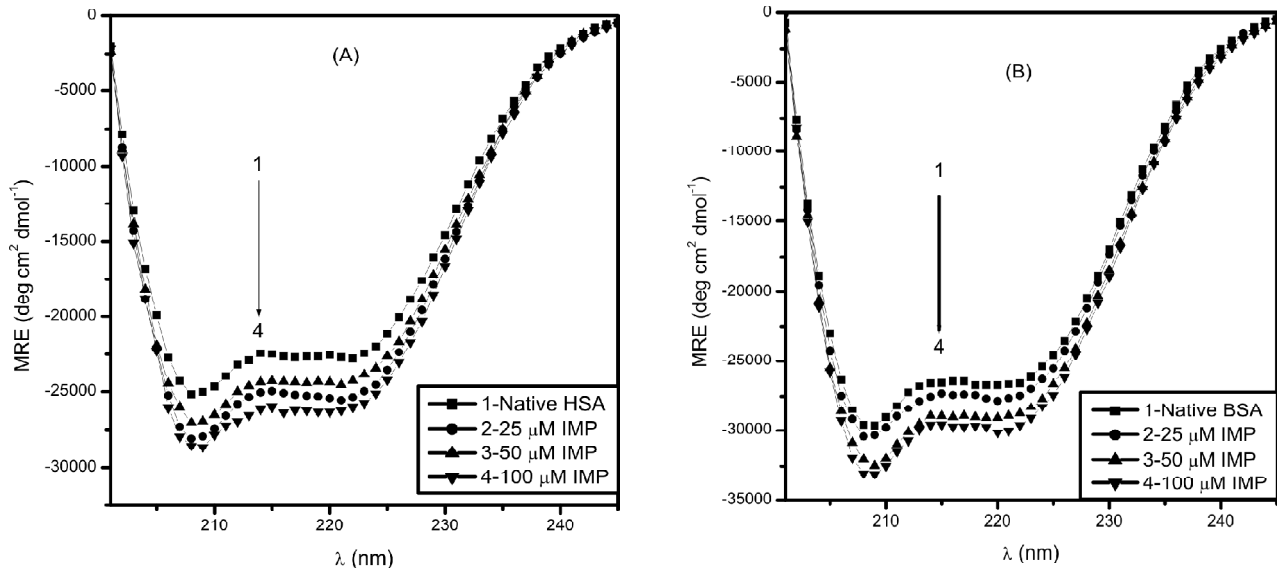

Figure 3: **CD spectra of native HSA/BSA and HSA-IMP (A) BSA-IMP (B) complexes**

secondary structure of the protein in contrast to a little loss in β-sheet and an extensive ordered conformation for drug, as seen in Table 2. This corroborates that, as the concentration of drug increases, the protein folding occurs, which can be facilitated by electrostatic interactions between drug molecules bound to the exposed binding sites. (Spaapen *et al.*, 1979).

Conclusion

The knowledge of the binding characteristic of biomolecules and drugs plays an important role in understanding the biological process. Under the steering of the information of the interactions between drug and serum albumins a wide variety of new drugs can be designed. The mechanism of IMP interacting with HSA/BSA was investigated by various spectroscopic methods under physiological conditions (i.e., pH 7.4). Experimental results indicated that IMP could bind with the serum albumins and quench the fluorescence. The binding constants of IMP interacting with HSA and BSA were obtained at various temperatures. Based on the Stern–Volmer equation, the quenching rate constants were evaluated and their values suggested that the fluorescence quenching was static process. The binding affinity is more in case of BSA and IMP as compared to HSA and IMP complexes. The results also suggest that the binding of drug molecules to serum albumins bring a conformational change in the secondary and tertiary structures, which was supported by the UV–vis absorption and the quantitatively studied data of the CD spectra.

Abbreviations

IMP, imipramine hydrochloride; HSA and BSA, human and bovine serum albumins; $K_{SV,}$ Stern–Volmer quenching constant; CD, Circular dichroism;

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