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Interaction of triprolidine hydrochloride with serum albumins: Thermodynamic and binding characteristics, and influence of site probes☆

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ABSTRACT

The interaction between triprolidine hydrochloride (TRP) to serum albumins *viz.* bovine serum albumin (BSA) and human serum albumin (HSA) has been studied by spectroscopic methods. The experimental results revealed the static quenching mechanism in the interaction of TRP with protein. The number of binding sites close to unity for both TRP–BSA and TRP–HSA indicated the presence of single class of binding site for the drug in protein. The binding constant values of TRP–BSA and TRP–HSA were observed to be $4.75 \pm 0.018 \times 10^3$ and $2.42 \pm 0.024 \times 10^4\ M^{-1}$ at 294 K, respectively. Thermodynamic parameters indicated that the hydrogen bond and van der Waals forces played the major role in the binding of TRP to proteins. The distance of separation between the serum albumin and TRP was obtained from the Förster's theory of non-radioactive energy transfer. The metal ions *viz.*, K^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} were found to influence the binding of the drug to protein. Displacement experiments indicated the binding of TRP to Sudlow's site I on both BSA and HSA. The CD, 3D fluorescence spectra and FT-IR spectral results revealed the changes in the secondary structure of protein upon interaction with TRP.

1. Introduction

Serum albumins are the most abundant proteins in blood plasma. They aid in the transport, distribution and metabolism of many endogenous and exogenous ligands [1]. So, serum albumin is employed as a model for studying drug–protein interaction *in vitro*. Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components that then function as carriers [2]. Among albumins, BSA and HSA have been studied extensively. BSA shows 76% similarity with that of HSA [3]. BSA has a single-chain 582 amino acid residues, two of which are tryptophans located within the hydrophobic pocket of domain II A at positions 134 and 214 while HSA consists of a single polypeptide chain of 585 amino acid residues in which the single tryptophan 214 residue measures the drug-binding affinity [2]. The affinities of drugs to protein would influence the concentration of drugs at the binding sites and duration of the effectual drugs and consequently contribute to their magnitude of biological actions *in vivo*. Generally, the weak binding leads to a shorter lifetime or poor distribution, while strong binding decreases the concentration of free drug in plasma. So, pharmaceutical firms need standardized screens for protein binding in new drug design and for fixing dose limits. Some research groups have reported the interactions of different drugs with serum albumins [4–7].

Triprolidine hydrochloride [TRP] (Fig. 1) is a pyridine derivative with antihistamine properties. It is a potent histamine H_1-receptor antagonist (H_1-blocker), with a rapid onset and long duration action, almost up to 12 h. It is probably effective for the symptomatic treatment of seasonal and perennial allergic rhinitis, vasomotor rhinitis, allergic conjunctivitis due to allergens, foods and prevention of allergic reactions to blood or plasma [8]. The most common side effects include sedation, dizziness, incoordination, gastrointestinal disturbances, nausea, vomiting and diarrhea. It may also produce blurred vision, dryness of mouth, tightness of chest, blood disorders including agranulocytosis and haemolyticanaemia [9]. Because of its pharmacological importance, the study of binding phenomena will provide the basic information on the pharmacological actions, bio-information, bio-distribution, etc. Therefore, it was thought worthwhile to investigate the interactions of protein with TRP. The present paper reports the results of interactions between TRP and serum albumins by spectroscopic (fluorescence, synchronous fluorescence, UV–vis absorption, FT-IR, 3D fluorescence and CD) techniques. Further, this study investigates whether there is any significant difference between BSA and HSA with regard to binding as they have different number of intrinsic fluorophores (tryptophan residues).

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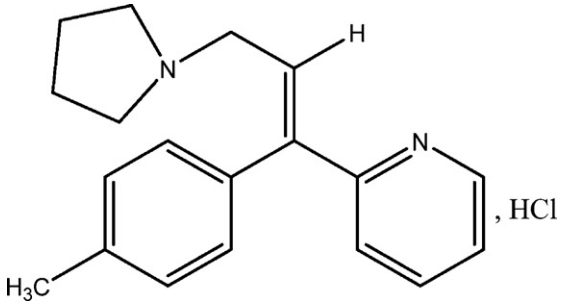

Fig. 1. The structure of triprolidine hydrochloride (TRP).

2. Experimental

2.1. Instrumental

All fluorescence measurements were carried out on a spectrofluorimeter (Hitachi F-2500, Tokyo, Japan) equipped with a 150 W Xenon lamp source and 1 cm path-length quartz cell. The required temperature was maintained by a circulating water bath (CYBeRLAB CB 2000, MA, USA). For excitation and emission, the slit widths were set at 5 nm. CARY 100 UV–vis spectrophotometer (Varian, North America) equipped with 1.0 cm quartz cells was used. Circular dichroism measurements were made on a Jasco J-715 spectropolarimeter (Tokyo, Japan) in a cell of 1 mm path length at room temperature. FI-IR measurements were carried out at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (Waltham, MA, USA) equipped with a Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were recorded via the ATR method with a resolution of 4 cm^{-1} using 60 scans.

2.2. Reagents

BSA (Fraction V, Protease-free, essentially γ-globulin free), and HSA (fatty acid free) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and used without further purification. Warfarin (analytical standard), ibuprofen (analytical standard) and digitoxin (≥92.0%) were purchased from Sigma–Aldrich (Bangalore, India). Triprolidine hydrochloride was obtained as a gift sample from Pioneer laboratories India Pvt. Ltd. (Indore, India). Sodium dihydrogen orthophosphate dihydrate (Analar grade), disodium hydrogen orthophosphate dihydrate (Analar grade) and sodium chloride (Analar grade) were received from s.d.fiNE-CHEM Ltd. (Mumbai, India). The molecular weights of BSA and HSA are assumed to be approximately 65,000 and 66,000, respectively. Stock solutions of BSA and HSA (each of 250 μM) were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. A stock solution (1000 μM) of TRP was prepared in water and then stored at 4 °C. All chemicals used were of analytical reagent grade and millipore water (ELIX-10 & MILLI-Q gradient, Bangalore, India) was used throughout the study.

2.3. Procedures

2.3.1. Fluorescence measurements

On the basis of preliminary investigations, intrinsic fluorescence was measured ($n = 5$ replicates) at 289, 294 and 299 K in the range of 300–400 nm (for BSA) and 285–405 nm (for HSA) upon excitation at 296 and 280 nm for BSA and HSA, respectively. The concentration of protein was fixed at 2.5 μM while that of drug was varied in the range of 0–22.5 μM.

2.3.2. Binding studies in the presence of site probes

The displacement experiments were performed using different site probes *viz*., warfarin, ibuprofen and digitoxin for site I, II and III, respectively [10] by keeping the concentration of protein and probe constant (2.5 μM each) and varying the concentration of drug. The fluorescence emission spectra ($n = 5$ replicates) were recorded and the binding constant values of drug–protein–probe were evaluated.

2.3.3. Effects of common ions

The fluorescence spectra of TRP–BSA and TRP–HSA ($n = 5$ replicates) were recorded in the presence of some cations *viz*. K^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} at 340 nm upon excitation at 296 and 280 nm, for BSA and HSA, respectively. The overall concentrations of BSA, HSA and common ion were maintained at 2.5 μM.

2.3.4. UV absorption studies

The UV absorption measurements of BSA and HSA in the presence and absence of TRP ($n = 4$ replicates) were recorded in the range of 235–335 nm. Concentrations of BSA and HSA were fixed at 2.5 μM while that of the drug was changed from 0 to 20 μM for both BSA and HSA. The absorbance values of drug–protein mixtures in the concentration range employed in the study did not exceed 0.05 at the excitation wavelength.

2.3.5. Circular dichroism (CD) measurements

CD spectra of protein (5 μM) were recorded in the range of 200–250 nm in the presence and absence of drug ($n = 3$ replicates). The protein to drug concentration was varied (1:9 and 1:16) and CD spectra were recorded.

2.3.6. FT-IR measurements

The FT-IR spectra of protein (2.5 μM) in the presence and absence of TRP were recorded in the range of 1500–1700 cm^{-1} with three scans averaged for each spectrum. The corresponding absorbance contributions of buffer and free TRP solutions were recorded and digitally subtracted with the same instrumental parameters.

2.3.7. Synchronous fluorescence measurements

The synchronous fluorescence characteristics of TRP–BSA and TRP–HSA were measured ($n = 5$ replicates) at different scanning intervals of $\Delta\lambda$ ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$). When $\Delta\lambda = 15$ nm, the spectrum characteristic of protein tyrosine residues was observed and when $\Delta\lambda = 60$ nm, the spectrum characteristic of protein tryptophan residues was observed.

2.3.8. 3D fluorescence measurements

20 μl (2.5 μM) protein was transferred in to a quartz cell, diluted to 2.0 ml with phosphate buffer and mixed well. To this, 180 μl of 22.5 μM TRP was added and the three-dimensional fluorescence spectra ($n = 3$ replicates) were recorded with the setting of the excitation wavelength range of 200–350 nm and emission wavelength range of 200–550 nm.

3. Results and discussion

3.1. Fluorescence quenching studies

Fluorescence spectroscopy is the most suitable technique to investigate the interaction of a drug with a biomolecule as it has high sensitivity and easy to carryout. Tryptophan residue makes major contribution to the intrinsic fluorescence of protein. The fluorescence of tryptophan residues is extremely sensitive to its environment and can be monitored to understand the binding of small molecules to protein such as the binding mechanism, binding mode, binding constant, binding sites and intermolecular distance.

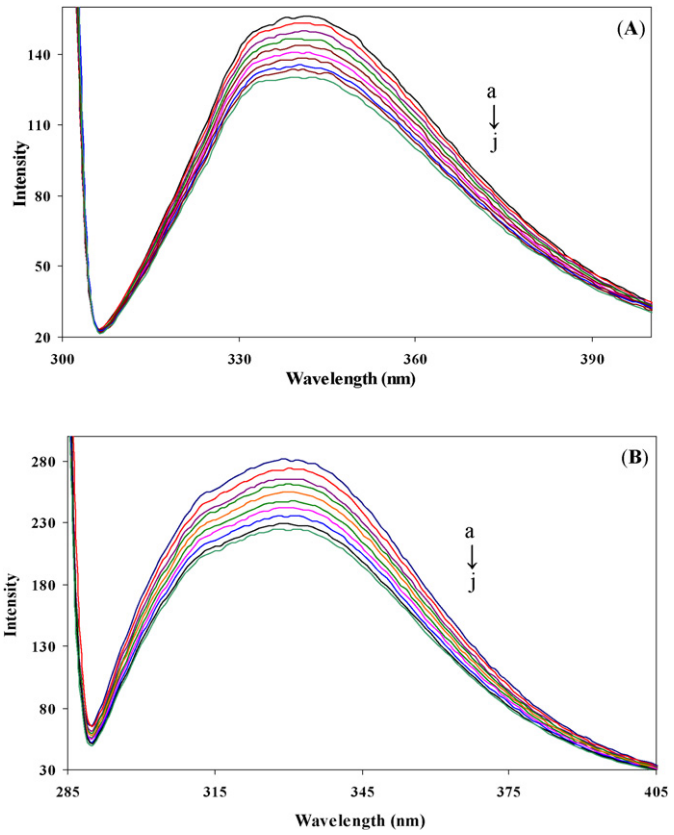

Fig. 2. Fluorescence quenching spectra of (A) BSA (2.5 μM) and (B) HSA (2.5 μM) in the presence of increasing amounts of TRP following the excitation at 296 nm for BSA and 280 nm for HSA. (a–j) Indicate the emission spectra of protein in presence of 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 22.5 μM TRP. This figure indicates the concentration dependent quenching of protein fluorescence by drug.

The decrease in fluorescence intensity of a compound by molecular reactions is called quenching. As can be seen from Fig. 2(A) and (B), the addition of increasing concentrations of TRP quenched the fluorescence intensity of protein. The possible quenching mechanism may be either dynamic or static or both. The dynamic and static quenching can be distinguished by their difference on temperature dependence. The quenching rate constants decrease with increase in temperature for static quenching while reverse is true for dynamic quenching [11].

The fluorescence results obtained at 289, 294 and 299 K were subjected to the Stern–Volmer equation [12] and the values of Stern–Volmer quenching constant, K_{sv} were calculated (not shown). These values were found to decrease with increase in temperature. Further, the magnitude of quenching rate constant $k_q(= K_{sv}/\tau_o)$ was noticed to be higher than that of the maximum value reported for the diffusion controlled process (2×10^{10} M^{-1} s^{-1}) [12] indicating thereby that the quenching mechanism was initiated by complex formation between TRP and protein rather than by dynamic collision. Therefore, the data were analyzed employing the modified Stern–Volmer equation shown below [12]:

$$\frac{F_o}{F_o - F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \quad (1)$$

where F and F_o are the fluorescence intensities of protein in the presence and absence of TRP, respectively; K_a is the modified Stern–Volmer association constant, [Q] is the concentration of quencher and f_a is the fraction of the initial fluorescence that is accessible to quencher. The plot of $F_o/(F_o - F)$ *versus* 1/[Q] (figure not shown) displayed the linear trend and yielded the values of ${f_a}^{-1}$ (from the intercept) and $(f_a K)^{-1}$ from the slope. The value of f_a was found to be 0.72 for drug–BSA and 0.75 for drug–HSA indicating that only 72% and 75% of the initial fluorescence of respective protein was accessible for quenching. The corresponding K_a values are shown in Table 1. The decreasing trend of K_a with increase in temperature revealed the presence of static quenching mechanism in the binding of drug to protein.

3.1.1. Analysis of binding equilibria

For static quenching interaction, if it is assumed that there are similar and independent binding sites in the biomolecule, the binding constant, K and the number of binding sites, n can be calculated using the equation shown below [13]:

$$\log \frac{F_o - F}{F} = \log K + n \log [Q] \quad (2)$$

From the linear plot of $\log (F_o - F)/F$ *versus* $\log [Q]$, the values of K and n were obtained from the intercept and slope, respectively (Table 1). From the values of n, it may be inferred that there is one independent class of binding site on BSA and HSA for TRP. The values of K decreased with increase in temperature indicating the reduction of stability of the TRP–protein complex [14].

3.1.2. Binding mode and binding site

The active force between the drug and biomolecule may include hydrogen bond, van der Waals forces, electrostatic and hydrophobic interactions. The thermodynamic parameters, enthalpy change (ΔH°), entropy change (ΔS°) and free energy change (ΔG°) are important to propose the binding mode [7]. For this purpose, the binding studies were carried out at different temperatures (289, 294 and 299 K) and analyzed using the van't Hoff equation [15] given below:

$$\log K = \frac{-\Delta H^\circ}{2.303RT} + \frac{\Delta S^\circ}{2.303R} \quad (3)$$

The $\log K$ *versus* $1/T$ plot enabled the determination of ΔH° and ΔS° values for the binding process. The value of ΔG° was calculated using the equation given below [16]:

$$\Delta G^\circ = -2.303RT \log K \quad (4)$$

The ΔH° and ΔS° values were found to be −125.55 kJ mol^{-1} and −0.34 J mol^{-1} K^{-1} for TRP–BSA and, −48.22 kJ mol^{-1} and −0.095 J mol^{-1} K^{-1} for TRP–HSA, respectively; while the values of ΔG° were noticed to be −26.72, −25.01 and −23.30 kJ mol^{-1} for TRP–BSA and, −20.82, −20.82 and −19.87 kJ mol^{-1} for TRP–HSA at 289, 294 and 299 K, respectively. The negative values of ΔG° revealed that the interaction process is spontaneous. The $\Delta H^\circ < 0$

Table 1
The values of modified Stern–Volmer association constant, binding constant and the number of binding sites at different temperatures for TRP–BSA and TRP–HSA systems.

T (K)	TRP–BSA				TRP–HSA			
	$K_a \times 10^4$ (L mol^{-1})	$K \times 10^3$ (M^{-1})	n	[a]R^2	$K_a \times 10^4$ (L mol^{-1})	$K \times 10^3$ (M^{-1})	n	[a]R^2
289	1.40 ± 0.012	5.35	0.9621	0.9930	1.26 ± 0.025	7.17	1.1535	0.9968
294	1.06 ± 0.023	4.75	0.9479	0.9966	1.00 ± 0.014	2.42	1.0531	0.9969
299	0.73 ± 0.017	2.73	0.8903	0.9958	0.70 ± 0.021	1.25	0.9898	0.9986

[a] Correlation coefficient.

Table 2
The binding constants of TRP–BSA and TRP–HSA systems at 294 K in presence of some cations.

Metal ions	Binding constant (M^{-1})	
	TRP + BSA	TRP + HSA
–	$4.75 \pm 0.018 \times 10^3$	$2.42 \pm 0.024 \times 10^4$
K^+	$2.97 \pm 0.011 \times 10^3$	$0.41 \pm 0.021 \times 10^3$
Ca^{2+}	$3.32 \pm 0.022 \times 10^3$	$0.45 \pm 0.019 \times 10^3$
Co^{2+}	$2.86 \pm 0.024 \times 10^3$	$0.46 \pm 0.014 \times 10^3$
Cu^{2+}	$3.53 \pm 0.019 \times 10^3$	$0.92 \pm 0.022 \times 10^3$
Ni^{2+}	$2.71 \pm 0.016 \times 10^3$	$0.49 \pm 0.016 \times 10^3$
Mn^{2+}	$3.36 \pm 0.021 \times 10^3$	$0.48 \pm 0.018 \times 10^3$
Zn^{2+}	$3.41 \pm 0.023 \times 10^3$	$0.49 \pm 0.021 \times 10^3$

and $\Delta S^\circ < 0$ values indicated that the acting forces are mainly hydrogen bond and van der Waals forces [16].

3.1.3. Displacement studies

Sudlow et al. [10] have suggested two distinct binding sites on protein, sites I and II. Sites I and II of albumin show affinity for warfarin and ibuprofen, respectively. Digitoxin binding is known to be independent of sites I and II. In order to determine the specificity of TRP binding, competitive experiments were performed with warfarin, ibuprofen and digitoxin. The binding constant values were noticed to be $1.47 \pm 0.021 \times 10^3$, $4.61 \pm 0.015 \times 10^3$ and $4.68 \pm 0.023 \times 10^3\ M^{-1}$ for TRP–BSA and $1.36 \pm 0.041 \times 10^4$, $2.49 \pm 0.017 \times 10^4$ and $2.51 \pm 0.023 \times 10^4\ M^{-1}$ for TRP–HSA with warfarin, ibuprofen and digitoxin, respectively at 294 K. The binding constants of TRP–BSA and TRP–HSA in the absence of site probes were found to be $(4.75 \pm 0.018) \times 10^3$ and $(2.42 \pm 0.024) \times 10^4\ M^{-1}$ respectively at 294 K. As evident from the results, TRP was not significantly displaced by ibuprofen and digitoxin. However, warfarin (site I) exhibited a significant displacement of TRP suggesting that the TRP binding site on albumin was Sudlow's site I. Hence, the site I located in subdomain II A was proposed to be the main binding site for TRP in protein.

3.1.4. Effects of common ions on binding of TRP–protein

Some common ions are widely distributed in human and animals, which can affect the interactions of compounds with serum albumin. Therefore, we have examined the effects of some cations (K^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+}) on binding of TRP with protein at 294 K and the corresponding values of binding constant are incorporated in Table 2. The binding constant values of TRP–protein were noticed to be decreased in presence of above metal ions. This indicated that the drug would be quickly cleared from the blood [17]. In such an event, it necessitated to readjust the dose limits of the drug in presence of these metal ions.

3.1.5. UV–vis absorption studies

UV–vis absorption spectroscopy has been employed by researchers to study the interaction between the drug and protein. In the present study, we have recorded the absorption spectra of BSA/HSA in the presence of increasing amounts of drug (figures not shown). The absorbance of protein was found to be increased regularly upon the addition of TRP. This indicated that the peptide strands of protein molecules extended more upon the addition of TRP to BSA/HSA [18].

3.1.6. Fluorescence resonance energy transfer (FRET) between TRP and protein

Fluorescence resonance energy transfer (FRET) [12] is a good technique to determine the distance of separation (in nanometer scale) between the donor (fluorophore) and acceptor *in vitro* and *in vivo*. Basically, the FRET efficiency depends on three parameters *viz*: (i) the distance between donor and acceptor (which must be within the specified Förster distance of 2–8 nm); (ii) appreciable overlap between the donor fluorescence and acceptor absorption band and (iii) proper orientation of the transition dipole of the donor and acceptor. According to Förster, the efficiency (E) of FRET process depends on the inverse sixth-distance bound between donor and acceptor (r) and critical energy transfer distance or Förster radius (R_o) under the condition of 1:1 situation of donor to acceptor concentration and can be expressed by the following equation [19]:

$$E = \frac{R_o^6}{R_o + r^6} = \frac{F_o - F}{F_o} \quad (5)$$

where E is the efficiency of energy transfer and F and F_o are the fluorescence intensities of donor in the presence and absence of the acceptor, respectively. R_o can be expressed as

$$R_o^6 = 8.8 \times 10^{-25} k^2 n^{-4} \Phi J \quad (6)$$

where k^2 is the spatial orientation factor, n is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor and J is the overlap integral of emission spectrum of donor and absorption spectrum of acceptor (Fig. 4(A) and (B)). The term J is

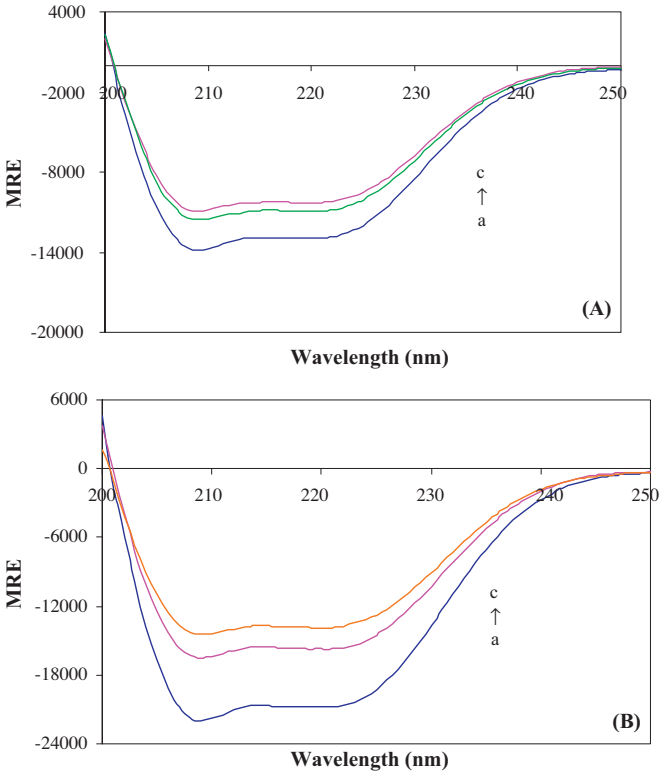

Fig. 3. CD spectra of: (A) BSA (5 μM) in the presence of TRP – (a) 0, (b) 40 and (c) 80 μM; and (B) HSA (5 μM) in the presence of TRP – (a) 0, (b) 40 and (c) 80 μM. The CD spectra indicate the changes in the secondary structure of protein upon interaction with TRP.

Table 3
Three-dimensional fluorescence spectral parameters of BSA and HSA in the absence and presence of TRP.

System	Peak 1 (nm)		Δλ (nm)	Intensity	Peak 2 (nm)		Δλ (nm)	Intensity
	λ_{ex}	λ_{em}			λ_{ex}	λ_{em}		
BSA	230	340	110	358.1	280	340	60	628
BSA–TRP	230	340	110	281.4	280	340	60	447.4
HSA	230	320	90	194	280	330	50	319
HSA–TRP	230	320	90	128.3	280	330	50	242.6

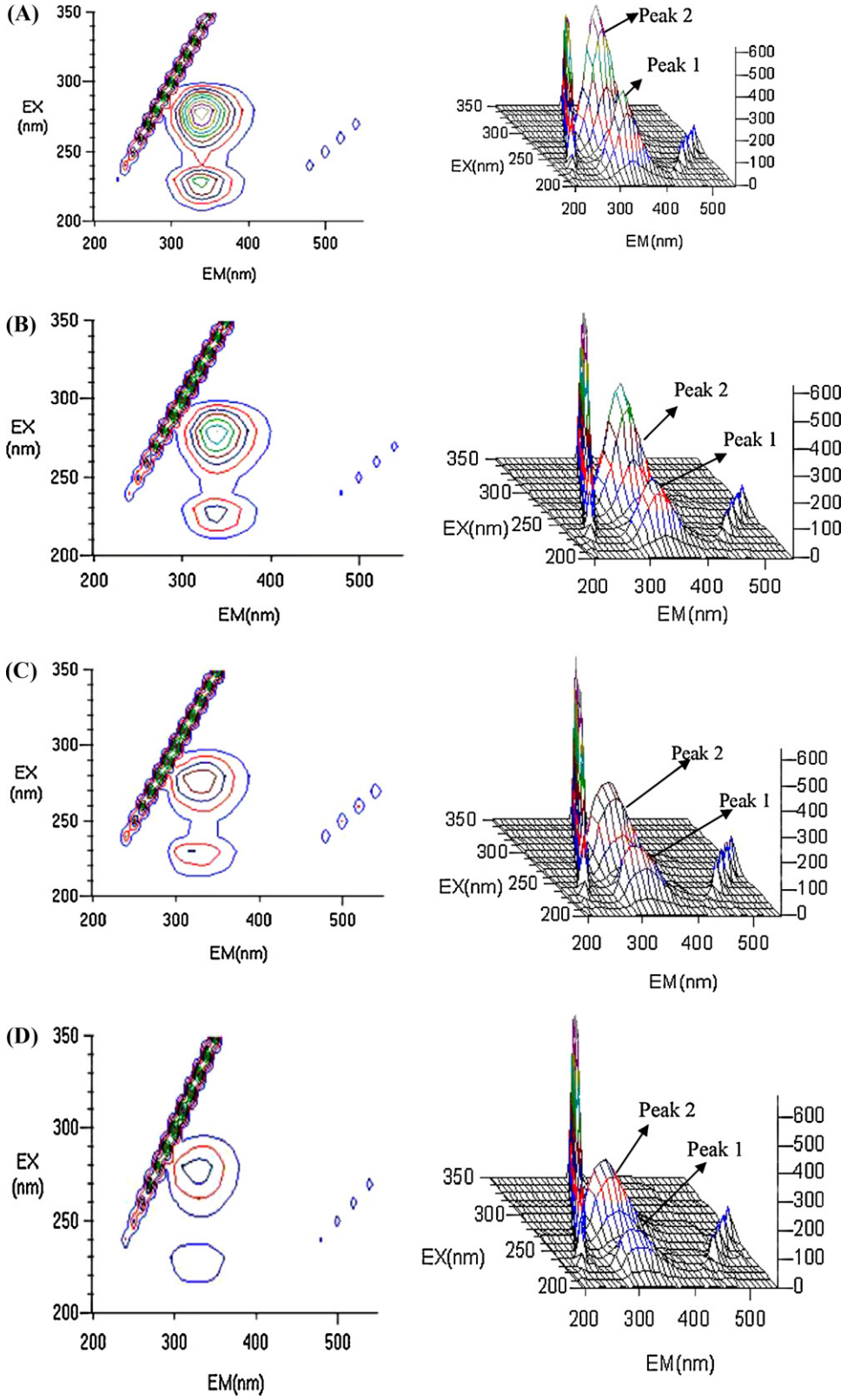

Fig. 4. The three-dimensional fluorescence spectra and corresponding contour diagrams of BSA (A), TRP–BSA (B), HSA (C) and TRP–HSA (D). $C_{Protein}$ = 2.5 μM and C_{TRP} = 22.5 μM. These diagrams reveal the conformational changes in BSA/HSA upon interaction with the drug.

expressed as

$$J = \frac{\int_o^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_o^\infty F(\lambda)d(\lambda)} \quad (7)$$

where $F(\lambda)$ is the fluorescence intensity of the donor of wavelength λ, $\varepsilon(\lambda)$ is the extinction coefficient of acceptor at λ. The overlap integral J was obtained by integrating the spectra (figures not shown). We observed good overlap between the emission spec-

trum of tryptophan with the absorption spectrum of the probe (drug). Using the values of $k^2 = 2/3$, $n = 1.36$ and $\Phi = 0.14$ for BSA and $k^2 = 2/3$, $n = 1.336$ and $\Phi = 0.118$ for HSA, the following data were obtained: $J = 3.45 \times 10^{-16}\,cm^3\,L\,mol^{-1}$, $R_o = 1.4\,nm$, $E = 0.266$ and $r = 1.70\,nm$ for TRP–BSA and $J = 3.26 \times 10^{-16}\,cm^3\,L\,mol^{-1}$, $R_o = 1.39\,nm$, $E = 0.207$ and $r = 1.73\,nm$ for TRP–HSA. The values of r indicate that the donor and acceptor are close to each other and hence have strong binding between them [20]. Further, the observed donor-to-acceptor distance, $r < 8\,nm$ revealed the presence of static quenching in the interaction [21].

3.1.7. FT-IR studies

FT-IR spectral studies are being employed for the investigation of secondary structure of protein and its dynamics. IR spectrum of the protein exhibits a number of amide bands, which represent different vibrations of the peptide moiety. The frequencies of amide I, II and III bands are sensitive to the secondary structure of proteins. The amide I and amide II peaks occur in the region of 1600–1700 cm^{-1} and 1500–1600 cm^{-1}, respectively. Amide I band (mainly C=O stretch) is more sensitive to the changes in protein secondary structure compared to amide II band [22]. The overlapped FT-IR spectra of free BSA and TRP–BSA (figure not shown), and free HSA and TRP–HSA (figure not shown) in phosphate buffer solution were recorded at 298 K. It was noticed that the peak position of amide I was shifted from 1648.8 to 1654.6 cm^{-1} and from 1623.7 to 1618.0 cm^{-1} while that of amide II was shifted from 1548.5 to 1537.0 cm^{-1} and from 1544.7 to 1554.3 cm^{-1}, respectively for TRP–BSA and TRP–HSA. This indicated that the secondary structure of protein was changed due to the interaction between TRP and BSA/HSA.

3.1.8. Circular dichroism studies

Circular dichroism (CD) is a sensitive technique to monitor the conformational changes in the protein. The CD spectra of BSA/HSA in the absence (line a) and presence of TRP (lines b and c) are shown in Fig. 3(A) and (B), respectively. The CD spectra of HSA exhibited two negative bands in UV region at 208 and 220 nm, while that of BSA showed bands at 208 and 222 nm which are characteristic of α-helicity of protein [23]. The CD results are expressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ according to the following equation:

$$\text{MRE} = \frac{\text{observed CD (mdeg)}}{c_p n l \times 10} \quad (8)$$

where C_p is the molar concentration of protein, n is the number of amino acid residues and l is the path length. The α-helical contents of free and combined BSA and HSA are calculated from MRE values at 208 nm using the equation [24]:

$$\alpha\text{-helix}\,(\%) = \frac{(-\text{MRE}_{208} - 4000)}{33,000 - 4000} \times 100 \quad (9)$$

where MRE_{208} is the observed MRE value at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm and 33,000 is the MRE value of pure α-helix at 208 nm. Using the above equation, the α-helicity of BSA and HSA was calculated. It decreased from 60.98 ± 0.023% in free BSA to 51.02 ± 0.017% in TRP–BSA and from 61.08 ± 0.019% in free HSA to 42.53 ± 0.022% in TRP–HSA. Further, the CD spectra of protein in the presence and absence of TRP are observed to be similar in shape, indicating that the structure of BSA/HSA is also predominantly α-helical even after binding to drug [25].

3.1.9. Synchronous fluorescence studies

These studies are helpful to understand the environment of amino acid residues by measuring the possible shift in the wavelength emission maximum corresponding to the changes of polarity around the chromophore molecule [26]. When the D-value ($\Delta\lambda$) between excitation wavelength and emission wavelength is stabilized at 15 nm and 60 nm, the synchronous fluorescence give the characteristic information about the polarity of tyrosine (Tyr) and tryptophan (Trp) residue, respectively. We noticed that the addition of drug resulted in strong fluorescence quenching of BSA/HSA, but the maximum emission wavelength of both Tyr and Trp residues remained unchanged indicating the formation of complex between TRP and protein. With this, we could not obtain sufficient information on structural changes of the protein.

3.1.10. 3D fluorescence measurements

Application of three-dimensional fluorescence technique is seen in recent years for the investigation of conformational changes of proteins interacting with drugs [27]. The excitation wavelength, emission wavelength and fluorescence intensity can be used as axes in the fluorescence emission spectra. The maximum emission wavelength and fluorescence intensity of the residues showed a close relation to the polarity of their microenvironment. The three-dimensional fluorescence spectra and the corresponding contour diagrams for BSA, BSA–TRP, HSA and HSA–TRP are shown in Fig. 4(A)–(D), respectively. Two typical fluorescence peaks 1 and 2 were observed. Among these two, peak 2 mainly revealed the spectral characteristics of tryptophan and tyrosine residues. The reason is that, when protein is excited at 280 nm, it mainly reveals the intrinsic fluorescence of tryptophan and tyrosine residues. The excitation wavelength of peak 1 was noticed at 230 nm which was mainly attributed to the transition of $n \rightarrow \pi^*$ of protein's characteristic polypeptide backbone structure, C=O [28]. The results showed that the three-dimensional fluorescence contour map of protein and TRP–protein was clearly different. From Fig. 4, it is clear that the fluorescence intensities of peak 1 and 2 decreased significantly in the presence of TRP indicating the quenching of fluorescence of BSA/HSA by TRP. The results shown in Table 3 indicated the conformational changes of BSA/HSA upon interaction with the drug.

4. Conclusions

The present paper described the mechanism of interaction of TRP with proteins by spectroscopic techniques for the first time. TRP quenched the fluorescence of both BSA and HSA through static quenching mechanism. Displacement experiments indicated that the TRP interacted with protein at the site I of subdomain II A. Some metal ions were found to influence the binding of TRP with protein. This necessitated readjusting the dose limits of the drug in presence of the metal ions. This work also reported the distance between tryptophan and bound TRP for the first time based on the Förster's theory of energy transfer. The results of FT-IR, 3D fluorescence and CD studies revealed the changes in the secondary structure of protein upon interaction with TRP. This work assumes significance from the view point of pharmacokinetics and in turn to pharmaceutical industries.

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