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Binding of trazodone hydrochloride with human serum albumin: A spectroscopic study

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Abstract

The binding of trazodone hydrochloride (TZH) to human serum albumin (HSA) was investigated by spectroscopic techniques. Various binding parameters have been evaluated. Negative enthalpy and positive entropy values indicated that both hydrogen bond and hydrophobic forces played a major role in the binding of TZH to HSA. The distance, r between donor (HSA) and acceptor (TZH) was found to be 2.16 nm based on the Förster's theory of non-radiation energy transfer. The circular dichroism data indicated that the α-helicity of HSA decreased upon interaction with TZH. The binding constant of HSA–TZH was found to decrease in presence of common ions and hence, shortened the stored time of drug in blood plasma.

Keywords: Human serum albumin; Trazodone hydrochloride; Fluorescence quenching; Fluorescence resonance energy transfer; Thermodynamic parameters

1. Introduction

Human serum albumin (HSA) is a principal extracellular protein with a high concentration in blood plasma. The globular protein consists of a single polypeptide chain of 585 amino acid residues and has many important physiological functions [1]. It is composed of three structurally similar domains (I, II and III), each containing two subdomains (A and B), and is stabilized by 17 disulphide bridges. Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in subdomains IIA and IIIA which are site I and site II [2]. The sole tryptophan residue (Trp-214) of HSA is in subdomain IIA [3].

HSA considerably contributes to colloid osmotic blood pressure and realize transport and distribution of many molecules and metabolites, such as fatty acids, amino acids, hormones, cations and anions, and many diverse drugs. It has been shown that the distribution, free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to HSA [4]. Drug interactions at protein binding level will in most cases significantly affect the apparent distribution volume of the drugs and also affect the elimination rate of drugs. Therefore, the studies on this aspect can provide information of the structural features that determine the therapeutic effectiveness of drugs, and have been an interesting research field in chemistry, life sciences and clinical medicine.

Trazodone, known as 8-[3-[4-(3-chlorophenyl)piperazin-1-yl]propyl]-6,8,9-triazabicyclo[4.3.0]nona-2,4,9-trien-7-one (Fig. 1) is used as an antidepressive agent [5]. It has been shown to be effective in patients with major depressive disorders and other subsets of depressive disorders. It is generally more useful in depressive disorders associated with insomnia and anxiety. This drug does not aggravate psychotic symptoms in patients with schizophrenia or schizoaffective disorders.

The interactions of drugs with proteins are often investigated by spectroscopic techniques as these are sensitive and relatively easy to use. They have several advantages over conventional approaches such as affinity and size exclusion chromatography, equilibrium dialysis, ultra filtration and ultracentrifugation, which suffer from lack of sensitivity or long analysis time or both and use of protein concentrations far in excess of the dissociation constant for the drug–protein complex [6,7] and for drug–protein interaction studies. In the present paper, we report the mechanism of interaction of trazodone hydrochloride (TZH) with HSA by three spectral methods for the first time.

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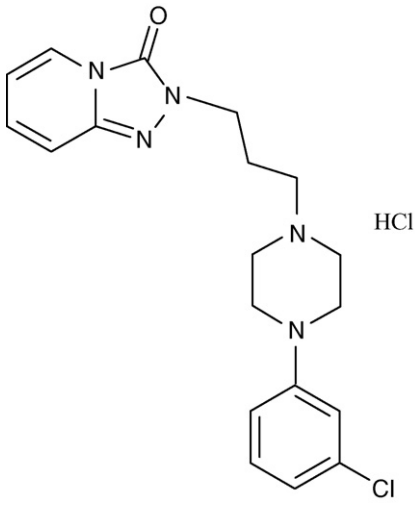

Fig. 1. Molecular structure of trazodone hydrochloride.

2. Experimental

2.1. Materials

Human serum albumin was obtained from Sigma Chemical Company, St. Louis, USA. Trazodone hydrochloride was obtained as a gift sample from Protec, India. The solutions of TZH and HSA were prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. HSA solution was prepared based on its molecular weight of 66,000. All other materials were of analytical reagent grade and double distilled water was used throughout.

2.2. Apparatus

The absorption spectra were recorded on a double beam CARY 50-BIO UV–vis spectrophotometer (Varian, Australia) equipped with a 150 W Xenon lamp and a slit width of 5 nm. A quartz cell of 1.00 cm was used for measurements. Fluorescence measurements were performed on a spectrofluorometer Model F-2000 (Hitachi, Japan) equipped with a 150 W Xenon lamp and a slit width of 5 nm. A 1.00 cm quartz cell was used for measurements. The CD measurements were made on a JASCO-J-715 spectropolarimeter (Tokyo, Japan) using a 0.1 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectrum in the range of 200–250 nm.

2.3. Procedures

2.3.1. TZH–HSA interactions

Based on preliminary investigations, HSA concentration was kept fixed at 12 μM while the drug concentration was varied from 1.25 to 7.5 μM. Fluorescence spectra of HSA in absence and presence of different amounts of drug were recorded at three temperatures (288, 302 and 309 K) in the range of 300–500 nm upon excitation at 280 nm in each case in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl.

2.3.2. Circular dichroism (CD) measurements

The CD measurements of HSA in the presence and absence of TZH were made in the range of 200–250 nm using a 0.1 cm cell at 0.2 nm intervals, with 3 scans averaged for each CD spectra. A stock solution of 150 μM HSA was prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. The HSA to drug concentration was varied (1:6, 1:12 and 1:18) and the CD spectrum was recorded.

2.3.3. Effects of some common ions

The solutions of SO_4^{2-}, F^- and NO_3^- were prepared from sodium salts while those of cations were made from the respective metal chloride except in case of V^{5+} solution where ammonium metavanadate was used. The fluorescence spectra of TZH–HSA were recorded in presence and absence of various common ions *viz*., SO_4^{2-}, F^-, NO_3^-, Mg^{2+}, Cu^{2+}, K^+, Ca^{2+} and V^{5+}, separately, in the range of 300–500 nm upon excitation at 280 nm in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. The concentration of HSA was fixed at 12 μM and that of common ion was fixed at an overall concentration of 6.25 μM.

3. Results and discussion

3.1. Interactions between TZH and HSA

It is reported in the literature that the binding of small molecules to HSA could induce the conformational change of HSA, because the intramolecular forces involved to maintain the secondary structure could be altered [8]. For macromolecules, the fluorescence measurements can give some information of the binding of small molecule substances to protein, such as the binding mechanism, binding mode, binding constants, binding studies, intermolecular distances, etc. HSA has only three intrinsic fluorophores *viz*., tryptophan, tyrosine and phenylalanine. In fact, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized, or near an amino group, a carboxyl group, or a tryptophan. This viewpoint was well supported by the experimental observations made by Sulkowska [9]. That is, the change of intrinsic fluorescence intensity of HSA is that of fluorescence intensity of tryptophan residue when small molecule substances are bound to HSA. The fluorescence quenching spectra of HSA in presence of different concentrations of TZH are shown in Fig. 2. As can be seen from Fig. 2, HSA has strong fluorescence emission with a peak at 336 nm upon excitation at 280 nm. The fluorescence intensity of HSA decreased regularly in presence of TZH. For TZH, its maximum emission wavelength is 438 nm and fluorescence intensity increases in the presence of HSA. This shows that the binding of TZH to HSA quenched the intrinsic fluorescence of HSA, whereas the fluorescence of TZH was enhanced. These results indicated that there were strong interactions and non-radiative energy transfer between TZH and HSA [10]. Moreover, the occurrence of an isoactinic point at 384 nm might also indicate the existence of bound and free TZH in equilibrium [10].

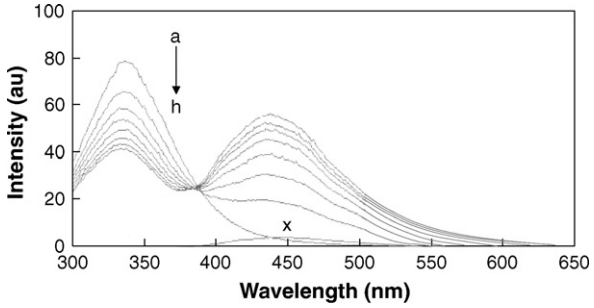

Fig. 2. Fluorescence spectra of TZH (x) and HSA in the presence of TZH (b–h). HSA concentration was 12 μM (a) while that of TZH was at 1.25 (b), 2.5 (c), 3.75 (d), 5 (e), 6.25 (f), 7.5 (g) and 8.75 μM (h). The concentration of TZH alone was 1.25 μM (x).

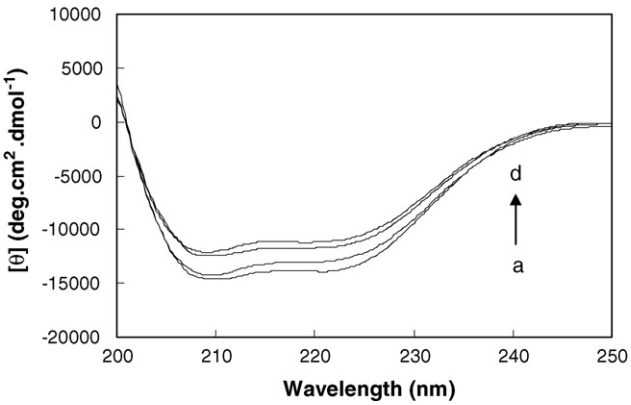

Fig. 4. CD Spectra HSA, and HSA–TZH system. HSA concentration was kept fixed at 5 μM (a). In HSA–TZH system, the TZH concentration was 30 (b), 60 (c) and 90 μM (d).

UV–vis absorption measurement is a very simple method and applicable to explore the structural change [11] and to know the complex formation [12]. In the present study, we have recorded the UV absorption spectra of TZH, HSA and TZH–HSA system (Fig. 3). The UV absorption intensity of HSA increased with the variation of TZH concentration. Further, a slight blue shift of maximum peak position was noticed possibly due to complex formation between TZH and HSA [13]. It also indicated that the peptide strands of protein molecules extended more upon the addition of TZH to HSA and the hydrophobicity was decreased [11].

CD was employed in the study to monitor the conformational change in the protein. The CD spectra of HSA in presence of lower concentrations of TZH did not exhibit appreciable changes in conformation of HSA in terms of α-helicity. However, appreciable changes in α-helicity values were noticed in presence of higher concentration of TZH (1:6, 1:12 and 1:18 molar ratios of HSA to drug). The CD spectra of HSA in the absence (line a) and presence of TZH (lines b–d) are shown in Fig. 4. The CD spectra of HSA exhibited two negative bands in the UV region at 208 and 218 nm, characteristic of an α-helical structure of protein [14]. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 dmol^{-1} according to the following equation:

$$\text{MRE} = \frac{\text{Observed CD (mdeg)}}{C_P n l \times 10} \quad (1)$$

where C_P is the molar concentration of the protein, n the number of amino acid residues and l the path length. The α-helical contents of free and combined HSA were calculated from MRE values at 208 nm using the equation [14]:

$$\alpha\text{-Helix}\,(\%) = \frac{[-\text{MRE}_{208} - 4000]}{[33,000 - 4000]} \times 100 \quad (2)$$

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 a pure α-helix at 208 nm. From the above equation, the α-helicity in the secondary structure of HSA was determined. They differed from that of 35.7% in free HSA to 27.4% in HSA–TZH complex, which was indicative of the loss of α-helicity upon interaction. The CD spectra of HSA in presence and absence of TZH are observed to be similar in shape, indicating that the structure of HSA is also predominantly α-helical [15].

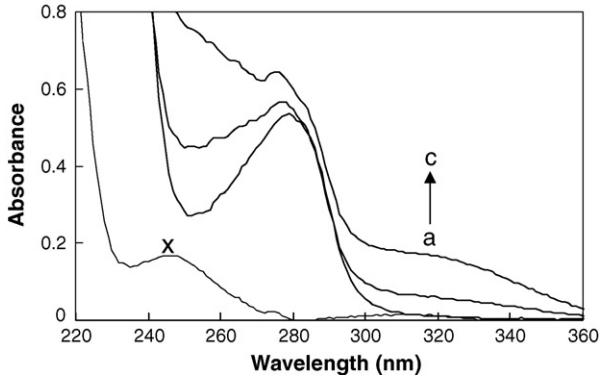

Fig. 3. Absorbance spectra of HSA, TZH and HSA–TZH system. HSA concentration was at 12 μM (a). TZH concentration for TZH–HSA system was at 12 (b) and 36 μM (c). A concentration of 12 μM TZH (x) was used for TZH only.

3.2. Binding mechanism and binding parameters

A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity [16]. The quenching rate constants decrease with increasing temperature for static quenching, but the reverse effect is observed for dynamic quenching [17].

A possible quenching mechanism is evident from the Stern–Volmer plots (Fig. 5) of HSA–TZH system at different temperatures (288, 302 and 309 K). The Stern–Volmer plots are linear with the slopes decreasing with increasing temperature. The values of K_{SV} and R^2 at different temperatures are shown in Table 1 and these indicate the occurrence of a static quenching

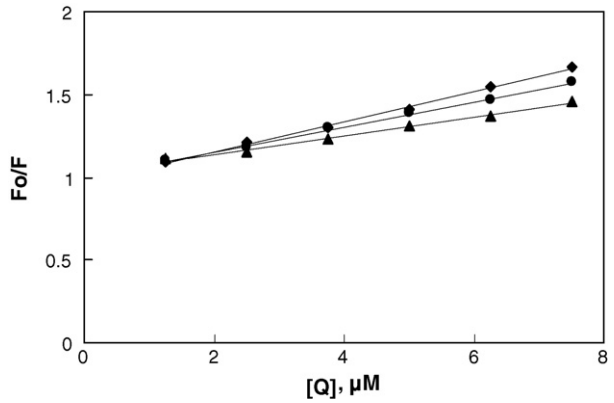

Fig. 5. The Stern–Volmer curves for the binding of TZH with HSA at 288 (♦), 302 (●) and 309 K (▲). Excitation wavelength was 280 nm, [HSA] = 12 μM.

interaction between TZH and HSA. In order to invoke this possibility, the mechanism is assumed to involve dynamic quenching. The quenching equation [17] is represented by:

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (3)$$

where F and F_0 are the fluorescence intensities with and without quencher, respectively. The K_q, K_{SV}, τ_0 and $[Q]$ are the quenching rate constant of the biomolecule, the dynamic quenching constant, the average lifetime of the biomolecule without quencher and the concentration of quencher, respectively. Obviously,

$$K_{SV} = K_q\tau_0 \quad (4)$$

Because the fluorescence lifetime of the biopolymer is 10^{-8} s [18] and K_{SV} is the slope of linear regressions (Fig. 5), the quenching rate constant, K_q can be calculated. The K_q values decrease from 7.74×10^{12} L to 6.34×10^{12} L mol^{-1} s^{-1}. The order of magnitude of the quenching rate constant, K_q is 10^{12} in the present work. However, the maximum scatter collision quenching constant, K_q of various quenchers with the biopolymer is 2×10^{10} L mol^{-1} s^{-1} [13]. Clearly, the rate constant of the protein quenching procedure initiated by TZH is greater than the K_q for the scatter mechanism. This implies that the quenching is not initiated by dynamic collision but originates from the formation of a complex. The static quenching equation [14] can be expressed as follows:

$$\log\frac{(F_0 - F)}{F} = \log K + n\log[Q] \quad (5)$$

From the plot of $\log(F_0 - F)/F$ versus $\log[Q]$, the binding constant, K of TZH–HSA and the binding sites, n can be obtained from the intercept and slope, respectively. The values of K were found to be $3.36 \pm 0.004 \times 10^4$, $2.4 \pm 0.002 \times 10^4$ and $1.95 \pm 0.003 \times 10^4$ M^{-1} and those of n were noticed to be 0.96 ± 0.001, 0.93 ± 0.003 and 0.91 ± 0.002, respectively at 288, 302 and 309 K. It was noticed that the binding constant decreased with increasing of temperature, resulting in a reduction of the stability of the TZH–HSA complex. The values of binding sites for TZH on HSA were all almost equal to unity indicating that there was one independent class of binding sites on HSA for TZH. Hence, TZH most likely binds to the hydrophobic pocket located in subdomain IIA; that is to say, Trp-214 is near or within the binding site [14]. The binding constants between them are remarkable and the effect of temperature is small. This suggests that there is a strong interaction and the formation of a complex between TZH and HSA. This clearly implies that TZH can be stored and removed by the proteins in the body.

3.3. Binding mode between HSA and TZH

The thermodynamic parameters, enthalpy (ΔH°) and entropy (ΔS°) of TZH–HSA interaction are important for confirming binding mode. For this purpose, the temperature dependence of binding constant was studied. Binding studies were carried out at 288, 302 and 309 K at which HSA does not under go any structural degradation. The molecular forces contributing to protein interactions with small molecular substrates may include van der Waals interactions, hydrogen bonds, ionic, electrostatic and hydrophobic interactions and so on. The thermodynamic parameters were evaluated using the equations:

$$\log K = \frac{-\Delta H^\circ}{2.303RT} + \frac{\Delta S^\circ}{2.303R} \quad (6)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (7)$$

where ΔH°, ΔG° and ΔS° are respectively enthalpy change, free energy change and entropy change. The plot of $\log K$ versus $1/T$ (Fig. 6) enabled the determination of ΔH°, ΔS° and ΔG° and these values are summarized in Table 1. Ross and Subramanian [19] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction. From the point of view of water structure, a positive ΔS° value is frequently taken as a typical evidence for hydrophobic interaction. Negative ΔH° value cannot be attributed to electrostatic interactions since for electrostatic interactions, ΔH° is very small, almost zero [19,20]. The negative ΔH° and positive ΔS° in case of TZH therefore showed that both hydrogen bond and hydrophobic interactions play a role in the binding of TZH to HSA [21,22].

Table 1
Thermodynamic parameters of HSA–TZH system

T (K)	$K_{SV} \times 10^{-4}$	R^2	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
288	7.74 ± 0.001	0.9978	−24.96 ± 0.003		
302	7.27 ± 0.002	0.9986	−25.35 ± 0.001	−18.53 ± 0.008	22.44 ± 0.011
309	6.34 ± 0.004	0.9984	−25.39 ± 0.002		

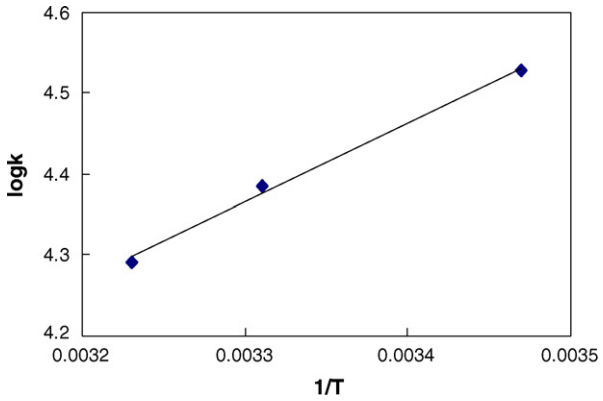

Fig. 6. Vant Hoff plot for the binding of TZH with HAS.

3.4. Energy transfer between TZH and HSA

The spectral studies revealed that the HSA could form a complex with TZH. HSA has a single tryptophan residue (Trp-214) and the fluorescence of HSA mainly comes from Trp-214. So, the distance between the Trp-214 and the bound TZH could be determined using fluorescence resonance energy transfer (FRET). The FRET occurs whenever the emission spectrum of fluorophore (donor) overlaps with the absorption spectrum of another molecule (acceptor). The overlap of the UV absorption spectrum of TZH with the fluorescence emission spectrum of HSA is shown in Fig. 7. The distance between the donor and acceptor and the extent of spectral overlap determines the extent of energy transfer. The distance between the donor and acceptor can be calculated according to Förster's theory [23].

The efficiency of energy transfer, E, is calculated using the equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{8}$$

where F and F_0 are the fluorescence intensities of HSA in the presence and absence of TZH, r the distance between acceptor and donor and R_0 the critical distance when the transfer efficiency is 50% [16]:

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^4 \Phi J \tag{9}$$

where k^2 is the spatial orientation factor of the dipole, N the refractive index of the medium, Φ the fluorescence quantum yield of the donor and J the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J [16] is given by:

$$\mathrm{J} = \frac{\sum F(\lambda)\varepsilon\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{10}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength, λ, $\varepsilon(\lambda)$ the molar absorption coefficient of the acceptor at wavelength, λ. In the present case, $K^2 = 2/3$, $N = 1.36$ and $\Phi = 0.118$ [13]. From Eqs. (8) and (10), we could able to calculate that $J = 1.22 \pm 0.005 \times 10^{-15}\ \mathrm{cm^3\,L\,mol^{-1}}$, $R_0 = 1.77 \pm 0.003$ nm, $E = 0.23 \pm 0.002$ and $r = 2.16 \pm 0.003$ nm. The donor-to-acceptor distance, $r < 7$ nm [13] indicated that the non-radiative energy transfer from HSA to TZH occurs with high possibility. This again indicates the static quenching interaction between HSA and TZH.

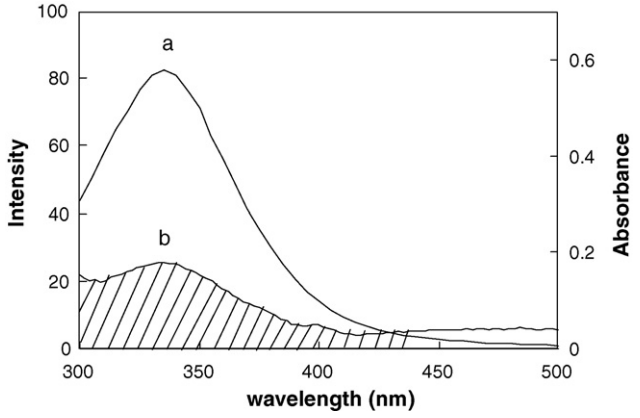

Fig. 7. The overlap of the fluorescence spectrum of HSA and the absorbance spectrum of TZH ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 336$ nm, [HSA]/[TZH] = 1:1. The fluorescence spectrum of HSA (a) and the absorption spectrum of TZH (b).

Table 2
Effects of common ions on binding constant of HSA–TZH

System	Association constant (M^{-1})
HAS + TZH	$2.08 \pm 0.002 \times 10^4$
HSA + TZH + V^{5+}	$7.94 \pm 0.004 \times 10^3$
HAS + TZH + K^+	$9.02 \pm 0.005 \times 10^3$
HAS + TZH + Cu^{2+}	$1.59 \pm 0.001 \times 10^3$
HSA + TZH + Ca^{2+}	$1.92 \pm 0.003 \times 10^3$
HSA + TZH + Mg^{2+}	$7.22 \pm 0.004 \times 10^3$
HSA + TZH + SO_4^{2-}	$2.15 \pm 0.003 \times 10^3$
HSA + TZH + NO_3^-	$3.18 \pm 0.002 \times 10^3$
HSA + TZH + F^-	$9.42 \pm 0.005 \times 10^3$

3.5. The effect of common ions on the binding constant

The effect of common ions *viz.*, SO_4^{2-}, F^-, NO_3^-, Mg^{2+}, Cu^{2+}, K^+, $Ca^{2+}$and V^{5+} on the binding of TZH to HSA was investigated at 302 K by recording the fluorescence intensity of TZH–HSA complex in the presence of each ion, separately in the range of 300–500 nm upon excitation at 280 nm. Under the experimental conditions, no cation gave precipitate in phosphate buffer. The effects of such cations on binding of a drug to HSA have also been reported in the literature [24]. The fluorescence emission spectrum of TZH in the presence of common ion shows that there is no interaction between the common ion and TZH. But, there is a binding reaction between the common ion and protein and thus the presence of common ion directly affects the binding between TZH and HSA. As evident from Table 2, the presence of common ions reduced the TZH–HSA binding constant. As a result, the binding force between protein and drug also decreased and shortened the stored time of drug in blood plasma. This may lead to the need for more doses of TZH to achieve the desired therapeutic effect [25].

4. Conclusions

This paper provided an approach for studying the interactions of fluorescent protein with TZH using absorption, fluorescence and CD techniques. The results showed that the HSA fluorescence was quenched by TZH through static quenching mechanism. The loss of α-helicity in secondary structure of HSA was observed upon interaction with the drug. Thermodynamic parameters showed that both hydrogen bond and hydrophobic interactions play a role in the binding of TZH to HSA. The distance between Trp-214 of protein and bound TZH was calculated by employing FRET for the first time. The binding force between protein and drug decreased in presence of common ions and shortened the stored time of drug in blood plasma. The biological significance of this work is evident since albumin serves as a carrier molecule for multiple drugs and the interactions of TZH with albumin are not characterized so far.

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