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Spectroscopic studies on the binding of bioactive phenothiazine compounds to human serum albumin

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Abstract

In this paper, the binding characteristics of human serum albumin (HSA) with phenothiazine derivatives (PDS) *viz*., thioridazine hydrochloride (TDH) and triflupromazine hydrochloride (TFP) have been studied by employing different spectroscopic techniques. The Stern–Volmer quenching constant values were found to decrease with increase in temperature thereby indicating the presence of static quenching mechanism in the interactions of PDS with HSA. The number of binding sites, n and the binding constant values, K were noticed to be 1.063 and $(4.46 \pm 0.040) \times 10^4\ L\ M^{-1}$ for TDH and 1.08 and $(5.18 \pm 0.071) \times 10^4\ L\ M^{-1}$ for TFP, respectively at 298 K. The binding distances and the energy transfer efficiency between PDS and protein were determined. The negative value of enthalpy change and positive value of entropy change in the present study indicated that both hydrogen bonding and hydrophobic forces played a major role in the binding of PDS to HSA. The circular dichroism data revealed the conformational changes in secondary structure of protein upon its interaction with PDS. The decreased binding constants of HSA–TDH/TFP in presence of common ions indicated the availability of higher concentration of free drug in plasma.

Keywords: Human serum albumin; Thioridazine hydrochloride (TDH); Triflupromazine hydrochloride (TFP); Fluorescence quenching; Fluorescence resonance energy transfer; Thermodynamic parameters

1. Introduction

Human serum albumin (HSA) is a principal extra cellular protein with a high concentration in blood plasma (40 mg ml^{-1} or 0.6 mM). It is a globular protein and the crystallographic analyses of HSA have revealed that the protein, a 585 amino acid residue monomer, composed of three homologous α-helical domains (I–III), each containing two subdomains (A and B) and stabilized by 17 disulphide bridges [1–3]. Aromatic and heterocyclic ligands were found to bind within two hydrophobic pockets in subdomains IIA and IIIA, namely site I and site II [1–3]. Seven binding sites are localized for fatty acids in subdomains IB, IIIA, and IIIB and on the subdomain interfaces [4]. HSA contains a single tryptophan (Trp 214).

HSA contributes to colloid osmotic blood pressure and is chiefly responsible for the maintenance of blood pH. Moreover, it is known to play an important role in the transport and disposition of endogenous and exogenous ligands present in blood [1]. Its remarkable capacity to bind a variety of drugs results in its prevailing role in drug pharmacokinetics and pharmacodynamics. Its primary pharmacokinetics function is participating in absorption, distribution, metabolism and excretion of drug, of which the drug distribution is the one that HSA controls, because most drugs travel in plasma and reach the target tissues by binding to HSA [5]. Hence, plasma protein after binding to drug has a significant influence on the pharmacokinetics of several drugs. Generally, the weak binding can lead to a short lifetime or poor distribution, while strong binding can decrease the concentrations of free drug in plasma. Because of this, many pharmaceutical firms have developed and standardized screens for HSA binding in the first step of new drug designing. Therefore, the studies on the interactions of a bioactive compound with HSA assume significance in chemistry, life sciences and clinical medicine.

Till now, spectroscopic methods *viz*., fluorescence, UV–vis, CD, FT-IR, and nuclear magnetic resonance (NMR) have been employed to study the interactions of small molecules and protein, and to investigate the conformational changes of protein

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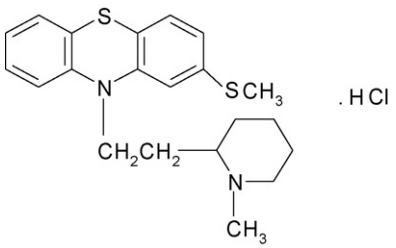

Fig. 1. Molecular structure of thioridazine hydrochloride.

[6–10]. Some techniques such as electrochemical [11], capillary electrophoresis [12] and high-performance liquid chromatography [13] have also been tried for the evaluation of binding parameters. Among these, fluorescence spectroscopy and CD have been widely used owing to their exceptional sensitivity, selectivity, convenience and abundant theoretical foundation.

Thioridazine hydrochloride (TDH) (Fig. 1) has a higher incidence of antimuscarinic effects and a lower incidence of extrapyramidal symptoms [14]. It is also known to be a neuroleptic tranquilizer and is commonly used as sedative, antiemetic and anesthetic drug. Triflupromazine hydrochloride (TFP) (Fig. 2) acts on the central nervous system to control violent behavior during acute episodes of psychotic disorders. It can also be used to control severe nausea and vomiting, severe hiccups, and moderate to severe pain in some hospitalized patients [15]. Reports on the interactions of some antihypertensive drugs with HSA are available in the literature [16–19]. However, attempts have not been made so far to investigate the interactions of TDH and TFP with HSA. The present is study focused on biophysical interactions of PDS with HSA. This is the first report on the mechanism of interaction of TDH and TFP with HSA employing fluorescence spectroscopy, spectrophotometry and circular dichroism methods.

2. Experimental

2.1. Apparatus

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. 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.

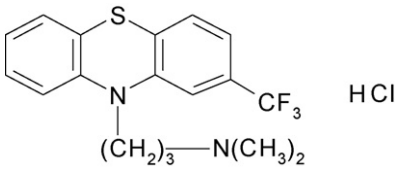

Fig. 2. Molecular structure of triflupromazine hydrochloride.

2.2. Reagents

Human serum albumin (HSA) was obtained from Sigma Chemical Company, St. Louis, USA. Thioridazine hydrochloride (TDH) and triflupromazine hydrochloride (TFP) were obtained as gift samples from Torrent Drugs and Chemicals, India. The solutions of TDH, TFP 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.

2.3. Procedures

2.3.1. Fluorescence investigations

Fluorescence spectra of HSA (12 μM) in presence of TDH (5–45 μM) or TFP (5–50 μM) were recorded at 288, 298 and 308 K in the range of 300–500 nm upon excitation at 280 nm.

2.3.2. Circular dichroism (CD) measurements

The CD spectra of HSA (10 μM) in presence and absence of PDS were made in the range of 200–250 nm. The molar ratios of HSA to drug maintained were 1:1 and 1:4 for TDH, and 1:2, 1:4 and 1:6 for TFP.

2.3.3. Energy transfer between PDS and protein

The absorption spectrum of PDS (12 μM) was recorded in the range of 300–500 nm. The emission spectrum of HSA (12 μM) was also recorded in the range of 300–500 nm. Then, the overlap of the UV absorption spectrum of PDS with the fluorescence emission spectrum of HSA was used to calculate the energy transfer.

2.3.4. Displacement experiments

The displacement experiments were performed using the site probes keeping the concentration of HSA and the probe constant (each of 12 μM). The fluorescence quenching titration was used as before to determine the binding constants of PDS–HSA system in presence of the site probes, warfarin, ibuprofen and digitoxin for sites I, II, and III, respectively [20].

2.3.5. Effects of some common ions

The fluorescence spectra of PDS–HSA were recorded in presence and absence of various common ions *viz.*, SO_4^{2-}, CO_3^{2-}, F^-, Br^-, Mg^{2+}, Cu^{2+}, K^+, Ca^{2+}, and Co^{2+} in the range of 300–500 nm upon excitation at 280 nm. The concentration of HSA was fixed at 12 μM and that of common ion was maintained at 10 μM.

3. Results and discussion

3.1. Interactions between TDH and TFP with HSA

It is reported in the literature that the binding of small molecules to HSA could induce the conformational changes in HSA. This is because the intramolecular forces involved to maintain the secondary structure could be altered and resulted

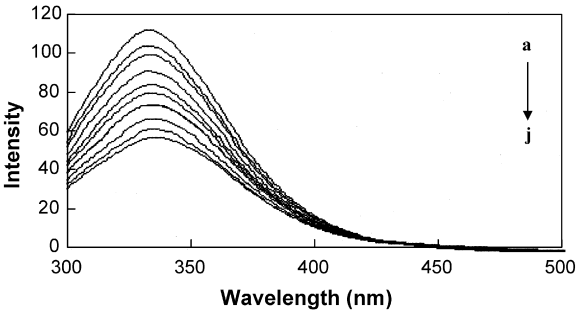

Fig. 3. Fluorescence spectra of HSA in presence of TDH. HSA concentration was fixed at 12 μM (a) and TDH concentration was maintained at 5 μM (b), 10 μM (c), 15 μM (d), 20 μM (e), 25 μM (f), 30 μM (g), 35 μM (h), 40 μM (i) and 45 μM (j).

in conformational changes of protein [21]. The fluorescence quenching spectra of HSA in presence of different concentrations of a representative drug, TDH are shown in Fig. 3. HSA has strong fluorescence emission with a peak at 335 nm upon excitation at 280 nm. From Fig. 3, it is clear that the λ_{max} of tryptophan fluorescence of HSA shifted to longer wavelength (from 335 to 338 nm) upon the addition of 5–45 μM TDH to HSA (12 μM). The shift in λ_{max} towards longer wavelength and decrease in fluorescence intensity revealed the increased polarity of the solvent. Under the experimental conditions, the TDH did not show any fluorescence intensity. TFP caused a concentration dependent quenching of the intrinsic fluorescence of HSA. The occurrence of an isoactinic point at 398 nm indicated the existence of bound and free TFP in equilibrium [22].

UV absorption spectra of drug, protein and drug–HSA systems were recorded and the absorption spectra of a representative drug are shown in Fig. 4. The UV absorption intensity of HSA increased with the variation of concentration of both PDS. Further, a slight blue shift of maximum peak position was noticed in both cases that could be due to formation of a complex between the drug and HSA [23]. This also indicated that the peptide strands of protein molecules extended more upon the addition of PDS to HSA [24].

CD, a sensitive technique to monitor the conformational changes in the protein was employed in the present study. The

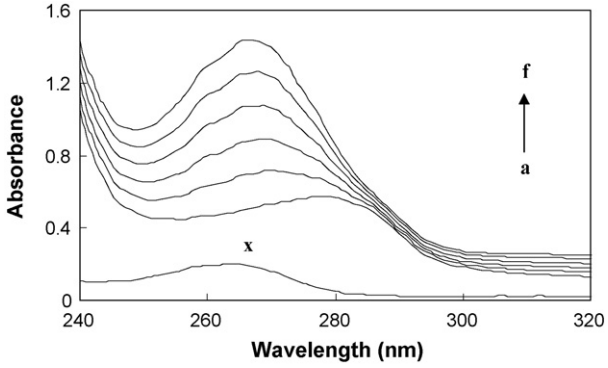

Fig. 4. Absorption spectra of HSA, TDH and HSA–TDH system. HSA concentration was at 12 μM (a). TDH concentration for TDH–HSA system was maintained at 5 μM (b), 10 μM (c), 15 μM (d), 20 μM (e) and 25 μM (f). A concentration of 12 μM TDH (x) was used for TDH only.

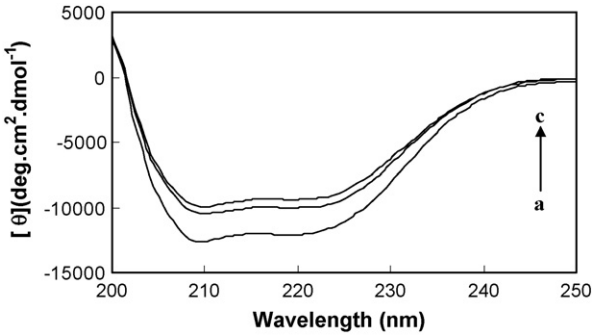

Fig. 5. The CD spectra of HSA-TDH system. HSA concentration was kept fixed at 10 μM (a). In HSA–TDH system, the TDH concentration was 10 μM (b) and 40 μM (c).

CD spectra of HSA recorded in presence of lower concentrations of PDS did not exhibit appreciable changes in the conformation of HSA in terms of α-helicity. However, appreciable changes in α-helicity values were noticed in presence of higher concentrations of both PDS. The CD spectra of HSA in the absence and presence of a representative drug, TDH are shown in Fig. 5. 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 [25]. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ according to equation,

$$\text{MRE} = \frac{\text{observed CD (mdeg)}}{C_P nl \times 10} \quad (1)$$

and the α-helical contents of free and combined HSA were calculated from MRE values at 208 nm using the equation [25],

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

The results revealed that the α-helicity was decreased from 35.92% in free HSA to 26.56% in HSA–TDH complex, and from 35.2% in free HSA to 29.4% in HSA–TFP complex. The CD spectra of HSA in presence and absence of PDS were observed to be similar in shape indicating that the structure of HSA is also predominantly α-helical [26] even after binding to PDS.

3.2. Binding mechanism and binding parameters

A possible quenching mechanism is evident from the Stern–Volmer plot of a representative system, HSA–TDH (Fig. 6) at different temperatures (288, 298 and 308 K). The Stern–Volmer plots for both TDH–HSA and TFP–HSA systems were found to be linear with the slopes decreasing with increase in temperature. The values of K_{SV} and R^2 at different temperatures for both systems are shown in Table 1 and these results indicated the occurrence of a static quenching mechanism between PDS and HSA.

In order to invoke this possibility, the mechanism was assumed to involve dynamic quenching. The quenching equation [27] is represented by

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

Table 1
Thermodynamic and binding parameters of PDS–HSA systems

	T (K)	K_{SV} ($\times 10^{-4}$ L mol^{-1})	R^2	Binding constant, K ($\times 10^{-4}$ L M^{-1})	n	K_q ($\times 10^{-12}$ L M^{-1} S^{-1})	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
TDH	288	5.58 ± 0.051	0.9975	9.14 ± 0.073	1.125	5.58 ± 0.051	−26.40	−20.74	19.68
	298	5.00 ± 0.043	0.9986	4.46 ± 0.040	1.063	5.00 ± 0.043	−26.60		
	309	4.50 ± 0.024	0.9982	3.48 ± 0.036	1.044	4.50 ± 0.024	−26.80		
TFP	288	5.41 ± 0.046	0.9985	7.91 ± 0.067	1.126	5.41 ± 0.046	−26.78	−24.11	9.28
	298	4.91 ± 0.028	0.9976	5.18 ± 0.071	1.080	4.91 ± 0.028	−26.88		
	309	3.18 ± 0.039	0.9980	3.63 ± 0.058	1.068	3.18 ± 0.039	−26.972		

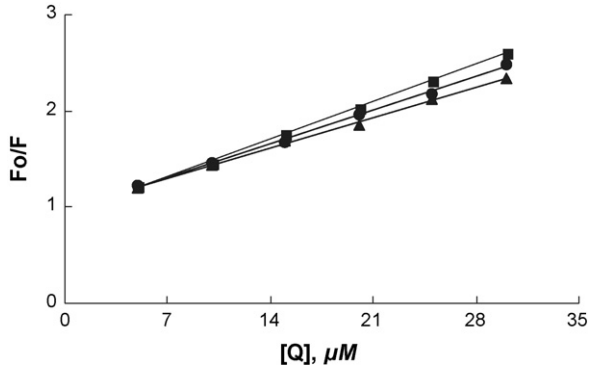

Fig. 6. Stern–Volmer plot for the binding of TDH to HSA at 288 K (■), 298 K (●) and 308 K (▲).

where K_{SV} is the slope of linear regression and $K_{SV} = K_q \tau_0$. The results obtained for a representative system, TDH–HSA are shown in Fig. 6. The fluorescence lifetime of the biopolymer [28] is known to be 10^{-8} s. In the present study, the quenching rate constant values, K_q decreased with increase in temperature (Table 1) indicating the presence of static quenching mechanism in the binding of PDS to protein. Further, the order of magnitude of K_q was calculated to be 10^{12} in the present work. The maximum scatter collision quenching constant, K_q of various quenchers with the biopolymer [23] is reported to be 2×10^{10} L mol^{-1} s^{-1}. So, the rate constants of the protein quenching procedure initiated by TDH and TFP are greater than the value of K_q for the scatter mechanism. This implied that the quenching was not initiated by dynamic collision but originated from the formation of a complex.

3.3. Analysis of binding equilibria

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation [24]

$$\frac{\log(F_0 - F)}{F} = \log K + n \log[Q] \quad (4)$$

where K and n are the binding constant and the number of binding sites, respectively. The values of K and n for TDH–HSA system and TFP–HSA system at 288, 298 and 308 K were calculated and the values are summarized in Table 1. It was noticed that the binding constant values decreased with increase in temperature, resulting in reduction of the stability of PDS–HSA complexes. The values of n for TDH–HSA and TFP–HSA were noticed to be almost equal to unity indicating that there was one independent class of binding sites on HSA for both PDS. Hence, the PDS most likely bound to the hydrophobic pocket located in subdomain IIA; that is to say, Trp 214 is near or within the binding site [25].

3.4. Binding mode and binding site

Binding studies were carried out at 288, 298 and 308 K at which HSA does not under go any structural degradation. The

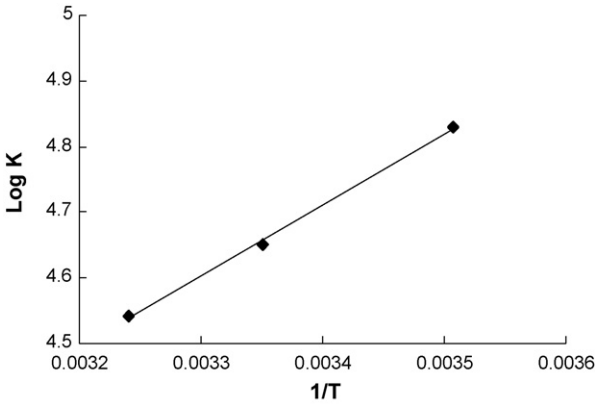

Fig. 7. log K *vs.* $1/T$ plot for TDH–HSA system.

thermodynamic parameters were evaluated using the equations

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

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (6)$$

The plot of log K *versus* $1/T$ enabled the determination of the values of ΔH° and ΔS°. The plot for a representative system, TDH–HSA is shown in Fig. 7. The results of both systems are recorded in Table 1. Ross and Subramanian [29] have characterized the sign and magnitude of the thermodynamic parameters associated with various individual kinds of interaction. Accordingly, a positive ΔS° value is frequently taken as a typical evidence for hydrophobic interaction from the point of view of water structure. Negative ΔH° values observed in the present study could not be attributed to electrostatic interactions since for electrostatic interactions ΔH° is very small, almost zero [29,30]. The negative ΔH° and positive ΔS° values obtained in case of both TDH and TFP therefore indicated that the hydrogen bonding and hydrophobic interactions play a role in the binding of PDS to HSA [31,32].

Sudlow et al. [20] have suggested two distinct binding sites on HSA namely site I and site II. Site I of HSA showed affinity for warfarin, phenylbutazone, etc., while site II exhibited affinity for ibuprofen, flufenamic acid, etc. The binding of digitoxin was found to be independent of sites I and II [33,34]. In order to determine the specificity of the PDS binding, competition experiments were performed with warfarin, ibuprofen and digitoxin as per Sudlow's classification of the binding sites. Table 2 shows the binding constants of PDS–HSA in presence of different site markers. As evident from Table 2, PDS were not significantly displaced by ibuprofen or digitoxin. However, warfarin (site I) showed a significant displacement of PDS suggesting that the PDS binding site on HSA is site I. Hence, the site I located in subdomain IIA near Trp 214 was considered to be the main binding site for PDS in HSA.

3.5. Energy transfer between PDS and HSA

The spectral studies have revealed that the HSA could form a complex with PDS. HSA has a single tryptophan residue (Trp 214) and the fluorescence of HSA mainly comes from Trp 214. So, the distance between Trp 214 and the bound PDS could be determined using fluorescence resonance energy transfer theory (FRET). The overlap of the UV absorption spectrum of a representative drug, TDH with the fluorescence emission spectrum of HSA is shown in Fig. 8. The distance between the donor and acceptor and extent of spectral overlaps determines the extent of energy transfer. The distance between the donor and acceptor was calculated according to Förster's theory [35]. The efficiency of energy transfer, E, was calculated using the equation,

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (7)$$

where F and F_0 are the fluorescence intensities of HSA in presence and absence of PDS, r the distance between acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50%. The value of R_0 was calculated using the equation,

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \quad (8)$$

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 is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of

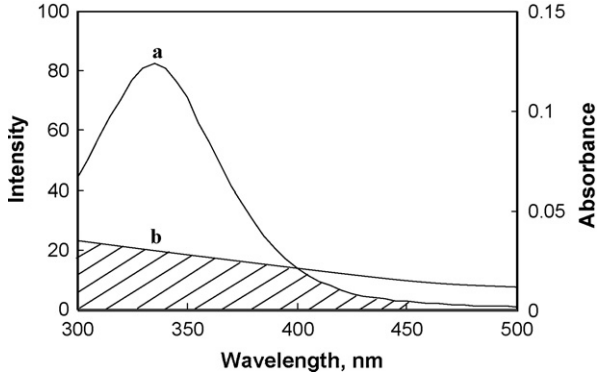

Fig. 8. The overlap of the fluorescence spectrum of HSA (a) with the absorbance spectrum of TDH (b). {c(BSA)/c(TDH) = 1:1}.

Table 2
Comparison of binding constant of PDS–HSA before and after the addition of the site probe

	K, without the site probe ($\times 10^{-4}$ L M^{-1})	K, with warfarin ($\times 10^{-4}$ L M^{-1})	K, with ibuprofen ($\times 10^{-4}$ L M^{-1})	K, with digitoxin ($\times 10^{-4}$ L M^{-1})
TDH	5.00 ± 0.040	2.31 ± 0.018	4.26 ± 0.053	4.30 ± 0.061
TFP	4.91 ± 0.071	3.14 ± 0.035	4.98 ± 0.046	5.04 ± 0.024

Table 3
Effects of common ions on binding constants of PDS–HSA systems

System	Association constant (M^{-1})	
	TDH	TFP
HSA	$(4.46 \pm 0.040) \times 10^4$	$(5.18 \pm 0.071) \times 10^4$
HSA + Co^{2+}	$(1.47 \pm 0.065) \times 10^4$	$(1.10 \pm 0.065) \times 10^4$
HSA + K^+	$(1.97 \pm 0.044) \times 10^4$	$(1.52 \pm 0.016) \times 10^4$
HSA + Cu^{2+}	$(1.39 \pm 0.006) \times 10^4$	$(1.10 \pm 0.022) \times 10^4$
HSA + Ca^{2+}	$(1.64 \pm 0.028) \times 10^4$	$(9.24 \pm 0.069) \times 10^3$
HSA + Mg^{2+}	$(2.76 \pm 0.034) \times 10^4$	$(8.83 \pm 0.068) \times 10^3$
HSA + $SO_4{}^{2-}$	$(6.86 \pm 0.043) \times 10^3$	$(2.69 \pm 0.039) \times 10^4$
HSA + CH_3COO^-	$(7.58 \pm 0.067) \times 10^3$	$(3.91 \pm 0.025) \times 10^4$
HSA + $CO_3{}^-$	$(1.22 \pm 0.005) \times 10^4$	$(2.04 \pm 0.026) \times 10^4$
HSA + Br^-	$(1.12 \pm 0.025) \times 10^4$	$(3.09 \pm 0.037) \times 10^4$
HSA + F^-	$(1.69 \pm 0.006) \times 10^4$	$(2.18 \pm 0.035) \times 10^4$

the acceptor. J was given by

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (9)$$

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 $\Phi = 0.15$ [36]. From Eqs. (7)–(9), we could able to calculate that $J = 1.17 \times 10^{-15}\ cm^3\ L\ mol^{-1}$, $R_0 = 1.76$ nm, $E = 0.26$ and $r = 2.13$ nm for TDH–HSA system and $J = 1.22 \times 10^{-15}\ cm^3\ L\ mol^{-1}$, $R_0 = 1.78$ nm, $E = 0.23$ and $r = 2.86$ nm for TFP–HSA system. The donor to acceptor distance, $r < 8$ nm [23] indicated that the non-radiative energy transfer took place from HSA to PDS with greater possibility. This again indicated the presence of static quenching mechanism in the interaction between HSA and PDS.

3.6. The effect of common ions on the binding constant

From the fluorescence emission spectrum of PDS in presence of common ions it was evident that there was no interaction between common ions and PDS. However, there was interaction between common ions and protein and hence the presence of these ions was expected to directly affect the binding of PDS to HSA. As evident from Table 3, the presence of common ions decreased the binding constants of TDH–HSA and TFP–HSA. This revealed that the binding force between protein and drug decreased and shortened the stored time of drug in blood plasma. This led to the need for more doses of PDS to achieve the desired therapeutic effect [37].

4. Conclusions

This work is an example of rarely encountered study wherein the interactions of HSA with selected PDS have been investigated by employing fluorescence, absorption and circular dichroism techniques. The biological significance of this work is evident since albumin serves as a carrier molecule for multiple drugs and the interactions of the potentially active PDS with HSA are not characterized so far. Both PDS quenched the fluorescence of HSA through static quenching mechanism. The distance between the donor (protein) and the acceptor (TDH/TFP) was also calculated using FRET. The shape and intensity of negative CD bands at 208 and 218 nm revealed the changes in the conformation of protein in presence of PDS.

Acknowledgements

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