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Study of the interaction between doxepin and human serum albumin by spectroscopic methods☆

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Abstract

The binding of doxepin hydrochloride (DH) to human serum albumin (HSA) was investigated by fluorescence, UV–vis absorption and circular dichroism (CD) techniques under simulative physiological conditions. The binding parameters have been evaluated by fluorescence quenching method. Negative enthalpy (ΔH°) and positive entropy (ΔS°) values indicated that both hydrogen bond and hydrophobic forces played a major role in the binding of DH to HSA. The distance r between donor (HSA) and acceptor (DH) was obtained according to the Förster's theory of non-radiation energy transfer. Spectral results revealed that the binding of DH to HSA induced conformational changes in HSA. The effect of common ions on the binding constant of DH–HSA was also examined.
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Keywords: Human serum albumin; Doxepin hydrochloride; Fluorescence quenching; Fluorescence resonance energy transfer; Thermodynamic parameters

1. Introduction

The human serum albumin (HSA) is the most abundant protein constituent of blood plasma and serves as a protein storage component. Recently, the three-dimensional structure of HSA has been determined through X-ray crystallographic measurements [1]. The globular protein consists of a single polypeptide chain of 585 amino acid residues and has many important physiological functions [2]. The globular protein 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. The crystal structure analyses have indicated that the principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA, and the sole tryptophan residue (Trp-214) 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. HSA can bind and carry through the blood stream many drugs, which are poorly soluble in water. 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 effectivity of drugs, and have been an interesting research field in life sciences, chemistry and clinical medicine.

Doxepin hydrochloride (DH) (Fig. 1) is a tricyclic antidepressant used in the treatment of mixed depression, anxiety, panic disorders, sleep disorders and other forms of depression. It is also used occasionally to treat chronic pain, peptic ulcer disease, and some skin conditions.

The molecular interactions are often monitored by spectroscopic techniques because these methods are sensitive and relatively easy to use. They have advantages over conventional approaches such as affinity and size exclusion chromatography, equilibrium dialysis, ultrafiltration and ultracentrifugation, which suffer from lack of sensitivity or long analysis time or both and use of protein concentrations far in excess of the

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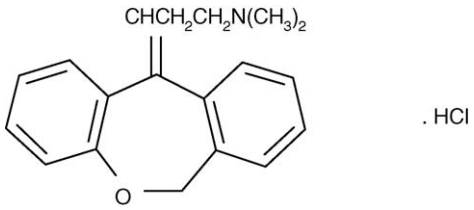

Fig. 1. Structure of doxepin hydrochloride.

dissociation constant for the drug–protein complex [5,6] and for drug–protein interaction studies. In the present paper, we report the mechanism of interaction of DH with HSA by three spectral methods.

2. Experimental

2.1. Apparatus

Fluorescence measurements were performed on a Hitachi spectrofluorimeter Model F-2000 equipped with a 150 W Xenon lamp and slit width of 10 nm. The circular dichroism (CD) measurements were made on a JASCO-J-715 spectropolarimeter using a 0.1 cm cell at 0.2 nm intervals, with three scans averaged for each CD spectrum. The absorption spectra were recorded on a double beam CARY 50-BIO UV–vis spectrophotometer equipped with a 150 W Xenon lamp and slit width of 10 nm. A quartz cell of 1.00 cm was used for measurements of both absorbance and fluorescence.

2.2. Reagents

HSA was obtained from Sigma Chemical Company, St Louis, USA. Doxepin hydrochloride was obtained as gift sample from Torrent Drugs and Chemicals, Ahmedabad, India. The solutions of DH 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. DH–HSA interactions

On the basis of preliminary experiments, HSA concentration was kept fixed at 10 μM and drug concentration was varied from 25 to 150 μM. Fluorescence spectra were recorded at 288, 301 and 309 K in the range of 300–500 nm upon excitation at 280 nm in each case. The absorbances of drug–protein mixtures in the concentration range employed for the experiment did not exceed 0.05 at the excitation wavelength to avoid inner filter effect.

2.3.2. Circular dichroism measurements

The CD measurements of HSA in the absence and presence of DH (1:1, 1:1.7 and 1:2.5) were made in the range of 200–250 nm. A stock solution of 150 μM HSA was prepared.

2.3.3. Effects of some common ions

The fluorescence spectra of DH (25–150 μM)–HSA (12 μM) were recorded in the absence and presence of various common ions (5 μM) viz., SO_4^{2-}, F^-, NO_3^-, I^-, CH_3COO^-, Mg^{2+}, Ca^{2+}, K^+, Ni^{2+} and V^{5+} in the range of 300–500 nm upon excitation at 280 nm.

3. Results and discussion

3.1. Interactions between DH and HSA

It has been 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, which results in the conformational change of protein [7]. 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 sites, 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 [8]. 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 at various concentrations of DH are shown in Fig. 2. It is clear that HSA had a strong fluorescence emission band at 342 nm by fixing the excitation wavelength at 280 nm, while the drug DH had no intrinsic fluorescence. The fluorescence intensity of HSA decreased regularly, and a very slight blue shift was observed for the emission wavelength with increasing DH concentration, indicating that a DH–HSA complex, which could quench the fluorescence of HSA and change the microenvironment of

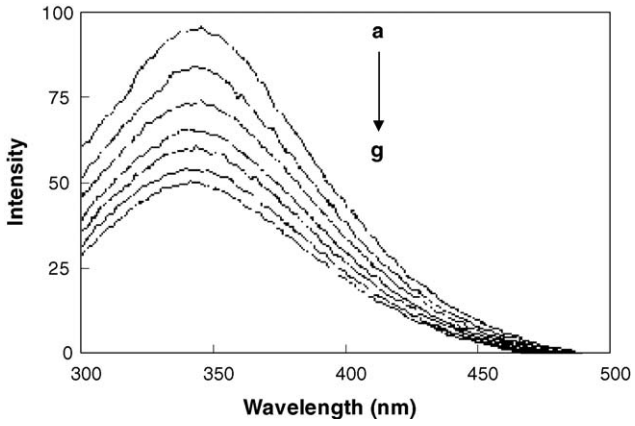

Fig. 2. Fluorescence spectra of HSA in the presence of DH. HSA concentration was 12 μM (a) and DH concentration was at 25 (b), 50 (c), 75 (d), 100 (e), 125 (f), and 150 μM (g).

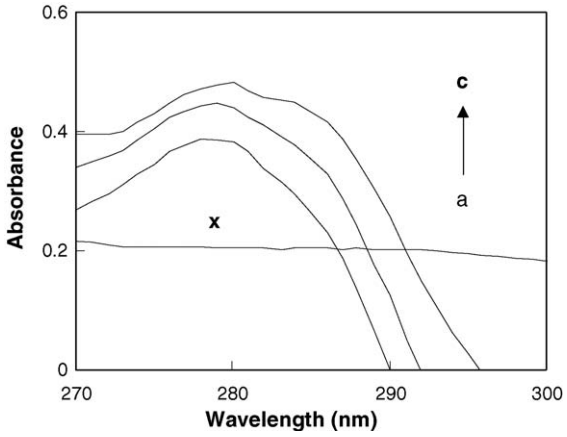

Fig. 3. Absorption spectra of HSA, DH and HSA–DH system. HSA concentration was at 12 μM (a) and DH concentration for DH–HSA system was at 60 (b) and 100 μM (c). A concentration of 12 μM DH (*x*) was used for DH only.

tryptophan residue, was formed. Hence, it is proposed that the binding took place near Trp-214 and led to a conformational change with a local perturbation of the IIA binding site in HSA.

UV–vis absorption measurement is a very simple method and applicable to explore the structural change [9] and to know the complex formation [10]. In the present study, we have recorded the UV absorption spectra of DH, HSA and DH–HSA system (Fig. 3). It is evident that the UV absorption intensity of HSA increased regularly with the variation of DH concentration. The maximum peak position of DH–HSA was shifted slightly towards higher wavelength region. These indicated the structural change of HSA upon interaction with DH.

CD, a sensitive technique to monitor the conformational change in the protein was employed in the present study. The albumin structure is predominantly α-helical. Approximately, 67% of HSA is α-helical [11]. In this work, the 1:1, 1:1.7 and 1:2.5 molar ratios of drug to HSA was used for the CD measurements. The CD spectra of HSA in the absence (line a) and presence (lines b–d) of DH were shown in Fig. 4. The CD spectra of HSA exhibited two negative bands in the UV region at 208 and

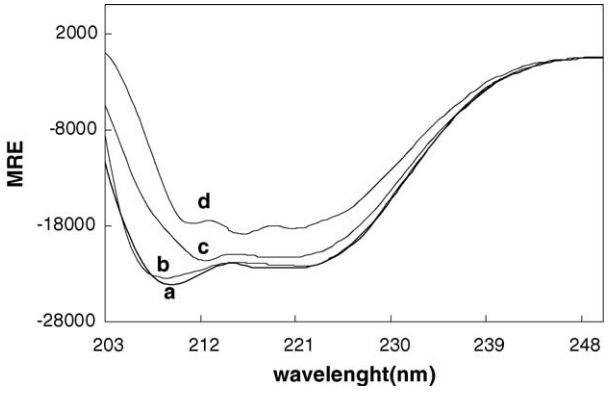

Fig. 4. The CD spectra of the HSA–DH system obtained in 0.1 M phosphate buffer of pH 7.4 at room temperature. HSA concentration was kept fixed at 10 μM (a) and in HSA–DH system, the DH concentration was 10 (b), 17 (c) and 25 μM (d).

218 nm, characteristic of an α-helical structure of protein [12]. The CD results were expressed in terms of mean residue ellipticity (MRE) in degree cm^2 $dmol^{-1}$ according to the following equation [13]:

$$\text{MRE} = \frac{\text{Observed CD(m degree)}}{C_P n l \times 10}$$

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

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

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 68.06% in free HSA to 58.96% in the HSA–DH complex, which was indicative of the loss of α-helicity upon interaction. The percentage of protein α-helix structure decreased indicated that the drug, DH bound with the amino acid residue of the main polypeptide chain of protein and destroyed their hydrogen bonding networks [15]. Under physiological conditions, fatty acids are present and normally can stabilize the conformation of HSA; furthermore, they occupy various binding sites of HSA [16]. However, under the simulative physiological conditions of this study, the contents of fatty acids are very low and HSA is rather flexible. After added to HSA solution, DH can bind to HSA and the binding of DH with HSA was hardly interfered by existing fatty acids. So, we concluded that the decrease of α-helical content of HSA was the result of DH bound to HSA.

3.2. Binding mechanism and binding parameters

The fluorescence quenching data at 288, 301 and 309 K were analyzed by the Stern–Volmer equation, $F_0/F = 1 + K_{SV}[Q]$ 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 and $[Q]$ is the concentration of quencher (DH). The results are shown in Fig. 5. The K_{SV} and the Pearson's correlation coefficient values, R^2 (the linear relationship between F_0/F and $[Q]$) obtained at different temperatures are shown in Table 1. From these results, we can see that the K_{SV} values decreased with increasing temperature, which was consistent with the static type of quenching mechanism. So, the possible quenching mechanism between DH and HSA was suggested as static quenching but not dynamic quenching. That is, DH bound to HSA and a DH–HSA complex was formed, which resulted in a fluorescence quenching of the fluorophore.

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 [17], $\lg(F_0 - F)/F = \lg K + n \lg[Q]$ where K and n are the binding

Table 1
Thermodynamic parameters of HSA–DH system

T (K)	$K_{SV} \times 10^{-3}$ (L mol^{-1})	R^2	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
288	6.51 ± 0.051	0.9975	−21.99		
301	6.03 ± 0.043	0.9986	−22.10	−15.75	21.60
309	5.52 ± 0.024	0.9982	−22.44		

constant and the number of binding sites, respectively. Thus, a plot of $\lg(F_0 - F)/F$ versus $\lg[Q]$ can be used to determine K as well as n. The values of K were found to be $(9.73 \pm 0.025) \times 10^3\ M^{-1}$; $(7.06 \pm 0.032) \times 10^3\ M^{-1}$ and $(6.21 \pm 0.041) \times 10^3\ M^{-1}$ and those of n were noticed to be 1.04 ± 0.004, 1.02 ± 0.005 and 1.01 ± 0.003, respectively, at 288, 301 and 309 K. It was found that the binding constant decreased with the increasing of temperature, resulting in a reduction of the stability of the DH–HSA complex. Meanwhile, from the data of n, we may infer that there was one independent class of binding sites on HSA for DH. Hence, DH most likely binds to the hydrophobic pocket located in subdomain IIA; that is to say, Trp-214 is near or within the binding site.

3.3. Types of interaction force between HSA and DH

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. Therefore, the thermodynamic parameters dependent on temperatures were analyzed in order to further characterize the acting forces between DH and HSA. The acting forces between a small molecule substance and macromolecule mainly include hydrogen bond, van der Waals force, electrostatic force, hydrophobic interaction force, and so on. The thermodynamic parameters, enthalpy (ΔH°), entropy (ΔS°) and free energy change (ΔG°), are the mainly evidence to estimate the binding mode. The thermodynamic parameters were evaluated using the equations, $\lg K = -\Delta H^\circ/2.303RT + \Delta S^\circ/2.303R$ and $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. The results obtained are shown in Table 1.

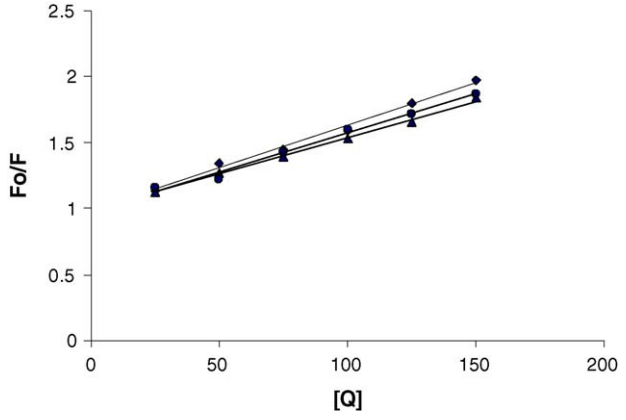

Fig. 5. The Stern–Volmer curves for the binding of DH with HSA at 288 (♦), 301 (●) and 309 K (▲). Excitation wavelength was 280 nm and [HSA] = 12 μM.

Large hydrophobic species such as proteins avoid the water molecules in aqueous solution as far as possible by associating into micelle-like structures with the non-polar portions in contact in the inner regions of the micelles, the polar ends facing the water molecules. This attraction of hydrophobic species, resulting from their unwelcome reception in water, is known as hydrophobic bonding or better, hydrophobic interaction. For drug–protein interaction, the positive ΔS° is frequently taken as evidence for the hydrophobic interaction [18] because the water molecules that are arranged in an orderly fashion around the ligand and protein acquire a more random configuration. In this way, the destruction of the water structure was induced by hydrophobic interactions. The negative ΔH° value (-15.75 kJ mol^{-1} or 3.76 kcal mol^{-1}) observed in the present study cannot be attributed to electrostatic interactions since for electrostatic interactions ΔH° is very small, almost zero [19,20]. Negative ΔH° value is observed whenever there is hydrogen bonding in the binding [20]. The negative ΔH° and positive ΔS° values, therefore, showed that both hydrogen bond and hydrophobic interactions play a role in the binding of DH to HSA [21,22].

3.4. Energy transfer between DH and HSA

The studies on fluorescence studies proved that HSA could form a complex with DH. HSA has a single tryptophan residue and the fluorescence of HSA mainly comes from Trp-214. So, the distance between the Trp-214 and the bound DH could be determined using fluorescence resonance energy transfer (FRET). FRET occurs whenever, the emission spectrum of fluorophore (donor) overlaps with the absorption spectrum of another molecule (acceptor). The overlap of the UV absorption

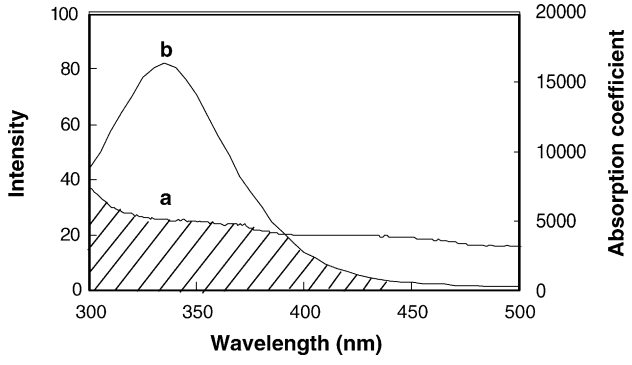

Fig. 6. The overlap of the fluorescence spectrum of HSA and the absorbance spectrum of DH (λ_{ex} = 280 nm, λ_{em} = 342 nm and [HSA]/[DH] = 1:1). The fluorescence spectrum of HSA (b) and the absorption spectrum of DH (a).

Table 2
Effects of common ions on binding constants of HSA–DH

System	Association constant (M^{-1})
HSA + DH	$(7.06 \pm 0.061) \times 10^3$
HSA + DH + V^{5+}	$(1.86 \pm 0.024) \times 10^2$
HSA + DH + K^+	$(1.35 \pm 0.013) \times 10^2$
HSA + DH + Ni^{2+}	$(1.25 \pm 0.033) \times 10^2$
HSA + DH + Ca^{2+}	$(1.16 \pm 0.046) \times 10^2$
HSA + DH + Mg^{2+}	$(0.75 \pm 0.017) \times 10^3$
HSA + DH + $SO_4{}^{2-}$	$(3.39 \pm 0.091) \times 10^3$
HSA + DH + CH_3COO^-	$(1.56 \pm 0.024) \times 10^3$
HSA + DH + $NO_3{}^-$	$(3.41 \pm 0.083) \times 10^3$
HSA + DH + I^-	$(1.93 \pm 0.026) \times 10^3$
HSA + DH + F^-	$(1.69 \pm 0.034) \times 10^3$

spectrum of DH with the fluorescence emission spectra of HSA is shown in Fig. 6. 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 can be calculated according to Förster's theory [23]. The efficiency of energy transfer, E, is calculated using the equation:

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

where F and F_0 are the fluorescence intensities of HSA in the presence and absence of DH; r, the distance between acceptor and donor; and R_0 is the critical distance when the transfer efficiency is 50%.

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

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 the acceptor. J is given by:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (3)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength λ; and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, $K^2 = 2/3$, $N = 1.36$ and $\Phi = 0.15$ [24]. From Eqs. (1)–(3), we could able to calculate that $J = (1.18 \pm 0.007) \times 10^{-15}\ cm^3\ L\ mol^{-1}$, $R_0 = 1.76 \pm 0.003$ nm, $E = 0.2 \pm 0.001$ and $r = 2.15 \pm 0.004$ nm. The donor-to-acceptor distance, $r < 8$ nm [10,17] indicated that the energy transfer from HSA to DH occurs with high possibility.

3.5. The effect of common ions on the binding constant

The effect of common ions viz., $SO_4{}^{2-}$, F^-, $NO_3{}^-$, I^-, CH_3COO^-, $PO_4{}^{3-}$, Mg^{2+}, Co^{2+}, K^+, Ni^{2+} and V^{5+} on the binding constant of DH–HSA system was investigated at 300 K by recording the fluorescence intensity in the range 300–500 nm upon excitation at 280 nm. The fluorescence emission spectrum of DH in the presence of common ion shows that there is no interaction between the common ion and DH. But, there is a binding reaction between the common ion and protein, and thus the presence of common ion directly affects the binding between DH and HSA. As evident from the Table 2, the presence of common ions reduced the DH–HSA binding, causing DH to be quickly cleared from the blood, which may lead to the need for more doses of DH to achieve the desired therapeutic effect.

4. Conclusions

This paper provided an approach for studying the interactions of fluorescent protein with DH using absorption, fluorescence and CD techniques. The results showed that HSA fluorescence quenched by DH through static quenching mechanism. This work gave a more comprehensive study and the distance between Trp-214 and bound DH was calculated using FRET for the first time. The biological significance of this work is evident since albumin serves as a carrier molecule for multiple drugs and the interactions of DH with albumin are not characterized so far. Hence, this report has a great significance in pharmacology and clinical medicine as well as methodology.

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