Spectroscopic Studies on the Interaction between Tilorone and Human Serum Albumin

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Abstract. Under physiological conditions, *in vitro* interaction between the antiviral drug 2,7-bis[2-(diethylamino)ethoxy]-9-fluorenone dihydrochloride (Tilorone, TIL) and human serum albumin (HSA) was investigated at excitation wavelength 280 nm and at different temperatures (298 K and 313 K) by fluorescence emission spectroscopy. TIL showed a strong ability to quench the intrinsic fluorescence of HSA through a static quenching procedure. The binding constant is estimated as $K_A = 7.19 \times 10^4 \text{ L} \cdot \text{mol}^{-1}$ at 298 K. The enthalpy change (ΔH°) and entropy change (ΔS°) were derived to be negative values. A value of 1.63 nm for the average distance r between TIL (acceptor) and tryptophan residues of HSA (donor) was derived from the fluorescence resonance energy transfer.

Introduction

Drug-protein binding has become an important research field in life sciences, chemistry and clinical medicine [1].

Human serum albumin (HSA) serves as the primary transport protein in the human circulation capable of binding reversibly both endogenous and exogenous ligands such as fatty acids, hormones and drugs. It consists of 585 amino acids in a single polypeptide chain [2]. Serum albumin is considered as a model to study the drug-protein interaction *in vitro*. It is a

convenient protein for intrinsic fluorescence measurement due to the presence of tryptophan (Trp) residues which is highly sensitive to its local environment, and can be used to observe changes in the fluorescence emission spectra due to protein conformational changes and binding to substrates [3]. Therefore, study of the interaction between protein and drug molecules will help provide basic information on the pharmacological actions, bio-transformation, bio-distribution of drugs. The binding phenomena will also be useful to explain the

relationship between the structures and functions of proteins.

There are some popular techniques which have been used to investigate the interaction between drugs and proteins. Fluorescence spectroscopy is one of the powerful techniques to study molecular interactions which changes local environment of fluorophore and helps to predict the binding phenomenon of drugs to proteins [4].

There are a number of reports in the literature, where binding of metabolites, drugs, dyes, fatty acids, bio-active-substances have been studied in detail. So the nature of binding of a ligand with HSA (BSA) is different for different ligands [5 - 25].

In this work, we provide fluorescence spectroscopic investigations on the effect of antiviral drug 2,7-bis[2-(diethylamino)ethoxy]-9-fluorenone dihydrochloride (Tilorone, TIL) on the structural and optical properties of human serum albumin. Binding parameters, such as the binding constant, number of sites, binding distance were obtained from the fluorescence data.

Experimental part

Materials

HSA was purchased from Sigma-Aldrich. A $1.0 \cdot 10^{-5}$ mol L⁻¹ solution of HSA was prepared by dissolving 66.5 mg in 100 mL of water. Protein concentration was determined spectrophotometrically by using molar extinction coefficient of $36\,600$ mol⁻¹·L·cm⁻¹ at

280 nm [26].

TIL solution ($1.0 \cdot 10^{-3}$ mol L⁻¹) was prepared by dissolving 0.0484 g of substance in 100 mL of water. The working solutions were obtained by diluting the stock solution with water. TIL (**Scheme 1**) was synthesized as described in [27] and appeared antiviral activity.

Scheme 1. The structure of TIL

Buffer solution was prepared by dissolving 121.14 g tris (tris(hydroxymethyl)aminomethane) (Sigma-Aldrich) in 800 mL H₂O, and adjusting the pH to 7.4 using the appropriate volume of concentrated HCl, diluting to 1000.0 mL with water (1 mol L⁻¹).

All of the used chemicals were of analytical grade or chemically pure. Also high-purity water from Milli-Q system used in the performed.

Apparatus

Absorption spectra were recorded at room temperature on a UV-2401 PC UV-Vis spectrophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence spectrometer (Varian, Australia) equipped with a 150-W xenon lamp in a 1-cm quartz cell using an excitation wavelength of 280 nm over a wavelength range of 285–475 nm at two temperatures (298, 313 K). The

excitation and emission bandwidths were 10 nm.

For different temperatures a thermostat bath (WNB 14 Memmert GmbH, Germany) was used. The pH values of solutions were measured on Lab 850 pH meter (Schott Instruments GmbH, Germany) with a glass electrode.

Data were analyzed using Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA).

Procedures

1.00 mL Tris-HCl buffer, 1.0 mL of $1.0 \cdot 10^{-5}$ mol L⁻¹ HSA solution and a appropriate quantities of the working TIL solution ($1 \cdot 10^{-4}$ mol L⁻¹, $1 \cdot 10^{-5}$ mol L⁻¹) were transferred to a 10 mL flask. Then the solution was diluted to 10 mL with high-purity water. All solutions were mixed thoroughly and kept 5 min before measurements.

The UV/vis absorbance spectra were recorded at room temperature. 1.00 mL Tris-HCl buffer, 5.0 mL of $1.0 \cdot 10^{-5}$ mol L⁻¹ HSA solution and a appropriate quantities of the working TIL solution ($1 \cdot 10^{-4}$ mol L⁻¹, $1 \cdot 10^{-5}$ mol L⁻¹) were transferred to a 10 mL flask. Then the solution was diluted to 10 mL with high-purity water. A blank system for absorbance spectra of TIL and HAS containing only 1.00 mL Tris-HCl buffer and diluted to 10 mL with high-purity water. A blank systems for absorbance spectra of (HSA + TIL) containing 1.00 mL Tris-HCl buffer, appropriate concentration of tilorone and diluted to 10 mL

with high-purity water.

Results and discussion

UV-vis absorption spectra experiments

UV-vis absorption measurement is a simple and pertinent method that is used to investigate structural changes and to explore complex formation [28]. TIL exhibited one evident peak at 270 nm (**Figure 1, a**). To initially verify the quenching mechanism, the UV absorption spectra of (HSA) and difference absorption spectrum between ((HSA + TIL) - TIL) were measured and recorded. As shown in **Figure 1, b**, HSA possessed two absorption peaks at 220 nm and 280 nm. The strong absorption peak at around 220 nm reflected the absorption of the backbone of HSA, while the weak absorption peak at around 280 nm resulted from the aromatic amino acids (Trp, Tyr, and Phe). With the addition of TIL, the intensity of the peak at 220 nm was changed with a red shift of about 2 nm, indicating disturbances to the microenvironment around the amide bonds in the protein. The results demonstrated the existence of an interaction between TIL and HSA. The absorption spectrum at around 280 nm did not shift, indicating that the microenvironment around the aromatic acid residues was not exposed to any change upon HSA-TIL complexation.

Fluorescence quenching spectra

Any process, which decreases the fluorescence intensity of a sample, is called fluorescence quenching.

Figure 1. Absorption spectrum of TIL ($c_{\text{TIL}} = 2.0 \times 10^{-5} \text{ mol L}^{-1}$) (a); effect of TIL on UV absorption spectra of HSA (b) ($T = 298 \text{ K}$, $\text{pH } 7.4$, $c_{\text{HSA}} = 5 \cdot 10^{-6} \text{ mol L}^{-1}$, $c_{\text{TIL}} \times 10^{-6} \text{ mol L}^{-1}$, 1-7: 0; 1.0; 2.0; 5.0; 7.0; 10.0; 20.0 (2-7 - difference absorption spectrum between TIL—HSA and TIL)

The basic principles like excited state reactions, molecular rearrangements, energy transfer, ground state complex formation, and collisional quenching involves in molecular interaction, which can result in quenching.

The interaction of TIL with HSA was evaluated by monitoring the intrinsic fluorescence intensity changes of proteins upon addition of TIL (**Figure 2**). The emission of HSA was characterized by a broad emission band at 346 nm. HSA fluorescence intensity decreased remarkably as TIL concentrations increased. TIL produced ~50% quenching in HSA fluorescence intensity, which was

accompanied by a 2 nm blue shift in the emission maximum at the same ligand concentration. The shift in the emission maximum of HSA towards shorter wavelength suggested increased hydrophobicity in the microenvironment of the protein fluorophores upon interaction with this compound [29]. This indicated that TIL could interact with HSA.

Analysis of the emission change of HSA with various amounts of TIL was carried out with the Stern–Volmer equation:

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

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher,

respectively, Q the quencher concentration, and k_q the quenching rate constant for a biomolecular reaction, K_{SV} the Stern–Volmer quenching constant and τ_0 is the average lifetime for fluorophore in the absence of quencher evaluated at 10^{-8} s [12].

Figure 2. Fluorescence emission spectra of HSA in the presence of TIL various amounts (c_{TIL} ($\mu\text{mol L}^{-1}$), curves (1-10): 0, 1.0, 2.0, 5.0, 7.0, 10.0, 20.0, 25.0; 30.0, 35.0; pH 7.4, $\lambda_{ex} = 280$ nm, $c_{HSA} = 1 \cdot 10^{-6}$ mol L^{-1} , $T = 298$ K)

Figure 3 shows plots of F_0/F versus TIL concentrations at different temperatures. They exhibit good linear correlations. It is clear that plots in Fig. 3 are approximately consistent with the Stern–Volmer equation, from which the Stern–Volmer quenching constant K_{SV} are derived and are listed in **Table 1**.

A quenching process can be usually induced by a collisional process and/or a formation of a complex between quencher and fluorophore. The values for quenching rate constants in Table 1 are of the magnitude of $10^{12} \text{ L} \times \text{mol}^{-1} \text{ s}^{-1}$.

Figure 3. Stern-Volmer plots for quenching of HSA fluorescence by TIL at different temperatures (pH 7.4, $\lambda_{ex} = 280$ nm, $c_{HSA} = 1 \cdot 10^{-6}$ mol L^{-1})

Table 1. The Stern-Volmer constants, and quenching constants of the HSA by TIL at different temperatures

$T(K)$	$K_{SV} \times 10^{-4}$ ($\text{L} \cdot \text{mol}^{-1}$)	R^a	$k_q \times 10^{-12}$ ($\text{L} \cdot \text{mol}^{-1} \text{ s}^{-1}$)
298	3.23	0.9978	3.23
313	2.07	0.9949	2.07

^a correlation coefficient

Obviously, the quenching rate constant k_q for TIL–HSA is greater than that of a scatter process. It may be attributed a quenching process initiated by bio-active substances. So it shows that the above quenching was not initiated by dynamic collision but forms compound, it was static quenching.

Furthermore, linear Stern–Volmer plots may either reveal the occurrence of just a binding site for quencher in the proximity of the fluorophore or indicate the existence of a single

type of quenching [31].

When small molecules are bound independently to a set of equivalent sites on a macromolecule, the binding constant (K_A) and sites (n) can be derived from the Eq. (2) [16]:

$$\log \frac{F_0 - F}{F} = \log K_A + n \log [Q] \quad (2),$$

where F_0 and F are the fluorescence intensity in the absence and presence of bio-active substance, respectively.

They still display good linear correlations. The values for K_A and n derived from plots in Fig. 3 according to Eq. (2) are collected in Table 2.

Table 2. Binding constant K_A , the number of binding sites n and distance between acceptor and donor r at different temperatures and thermodynamic parameters of TIL-HSA interaction at pH 7.4 ($\lambda_{\text{ex}} = 280 \text{ nm}$)

$T(K)$	$K_A \times 10^{-4} (L \cdot mol^{-1})$	n	r	$\Delta H^\circ (kJ \cdot mol^{-1})$	$\Delta G^\circ (kJ \cdot mol^{-1})$	$\Delta S^\circ (J \cdot mol^{-1} \cdot K^{-1})$
298	7.19	1.08	1.63	-33.64	-27.71	-19.91
313	3.75	1.05	1.66		-27.41	

They indicate that the amount of TIL will affect the pattern of TIL binding to HSA. The influence of temperatures on the TIL binding to HSA is significant. It is obvious that higher temperature will lead to a rapid collision, whereas results in a decrease in the binding strength. As a result, K_A and n will decrease with raising temperature.

Furthermore, the value for n close to one, we may infer that there exists only one kind of binding site on HSA for TIL binding (determined by this method).

Collectively, the fluorescence quenching of HSA by drug was caused by the static quenching, which supported the HSA UV spectral shifts [30].

Binding mode

The interaction of BSA and small drug molecules may involve hydrophobic forces, electrostatic forces, van der Waals, hydrogen

bonds and other interactions. The signs and magnitudes of thermodynamic parameters for protein reactions can be accounted for the main forces contributing to protein stability. The values for the enthalpy change (ΔH°) and entropy change (ΔS°) will be derived from the van't Hoff equation by considering ΔH° not varying significantly over the experimental temperature range:

$$\ln K_A = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (3),$$

where K_A is the binding constant at a definite temperature T and R is the gas constant. Consequently, the amount of free energy change ΔG° required for the binding is estimated from the following equation:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (4)$$

From the enthalpy change (ΔH°) and entropy (ΔS°), the mode of interaction can be assessed. It is suggested that if $\Delta H^\circ > 0$ and ΔS°

> 0 , hydrophobic interaction occurs, and if $\Delta H^\circ < 0$ and $\Delta S^\circ < 0$, Van der Waals and hydrogen bonds dominates, and if $\Delta H^\circ < 0$ and $\Delta S^\circ > 0$, is due to electrostatic forces [32]. The data for ΔH° and ΔS° of TIL binding to HSA are listed in Table 2. With respect to recognized literature data on analogous systems [19,33], the negative values of ΔH° and ΔS° can be attributed in part to van der Waals forces and in part to the formation of hydrogen bonds.

Energy transfer from HSA to TIL

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the different electronic excited states of molecules. In this interaction, excitation energy is transferred from one molecule (donor) to another (acceptor) through direct electrodynamic interaction, without emission of a photon from the former molecular system [34]. Energy transfer may occur under the following conditions: when the donor can produce fluorescent light; when there is an overlap between the fluorescence emission spectrum of the donor and the absorbance spectrum of the acceptor; and when the distance between the donor and the acceptor is less than 8 nm. The efficiency of energy transfer between TIL and the HSA Trp214 residue could be used to evaluate the distance between the two using FRET. The overlap of the absorbance spectrum of TIL with the fluorescence emission spectrum of HSA has been shown in **Figure 4**.

According to Forster's non-radiative resonance energy transfer theory [35], energy transfer efficiency E is related not only to the distance (r) between the bound bio-active molecule (acceptor) and the protein residue (donor), but also to the critical energy transfer distance (R_0).

Figure 4. Overlap of the absorption spectrum of TIL (1) with the fluorescence emission spectrum of HSA (2) ($c_{\text{HSA}}/c_{\text{TIL}} = 1:4$, $5 \cdot 10^{-6} \text{ mol L}^{-1}$; $T = 298 \text{ K}$; $\lambda_{\text{ex}} = 280 \text{ nm}$)

By Forster's theory, the efficiency of energy transfer (E) can be calculated according to the following equation:

$$E = 1 - F / F_0 = R_0^6 / (R_0^6 + r^6) \quad (5),$$

where r is the distance between acceptor (TIL) and donor (HSA), and R_0 is the critical distance when the transfer efficiency is 50%. The value of R_0 is calculated using the following equation:

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J \quad (6),$$

where K^2 is the spatial orientation factor of the dipole; n is the refractive index of the medium; ϕ is the fluorescence quantum yield of donor; and J is the spectral overlap between the emission spectrum of donor and the absorption spectrum of acceptor (Figure 4), given by:

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda)d\lambda} \quad (7),$$

where $F(\lambda)$ is the normalized fluorescence intensity of the donor at wavelength λ , and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ . In the present case, $K^2 = 2/3$, $n = 1.336$, and $\varphi = 0.118$ [36]. Hence, from equations (5)–(7), we could calculate the following parameters are listed in **Table 3**. In conclusion, the distance (r) between HSA

Trp214 and bound TIL was much less than 8 nm, and accorded with the relationship $0.5R_0 < r < 1.5R_0$. This implied that the non-radiative energy transfer from HSA to TIL occurred with high possibility, which was in accordance with the occurrence of a static quenching mechanism. This result indicated that the binding obeyed the conditions of Forster's energy transfer. A comparison of the J , R_0 and r values of different ligands bound to proteins is given in Table 3.

Table 3. Comparative assessment of the TIL (ligand) distance to protein's fluorophore (HSA) measured by Forster's nonradiative energy transfer with other ligands bound to proteins

Ligand	Protein	J (cm ³ L mol ⁻¹)	R_0 (nm)	r (nm)	References
mitoxantrone	HSA	1.67×10^{-15}	0.48	2.64	[5]
imidacloprid	HSA	3.17×10^{-15}	1.52	2.10	[6]
captopril	HSA	1.09×10^{-18}	2.37	1.05	[7]
pyrimidine-annulated spiro-dihydrofuran	BSA	9.14×10^{-16}	1.71	2.59	[8]
eupatorin	BSA	1.79×10^{-14}	1.56	1.64	[9]
erlotinib hydrochloride	BSA	3.89×10^{-21}	2.00	2.20	[10]
sanguinarine	BSA	1.29×10^{-14}	2.63	2.38	[11]
carbamazepine	BSA	3.24×10^{-15}	2.11	3.60	[12]
naringin	BSA	3.24×10^{-15}	2.32	3.25	[13]
3-carboxyphenoxathiin	BSA	1.63×10^{-15}	1.86	1.86	[14]
paclobutrazol	HSA	1.35×10^{-14}	2.58	4.41	[15]
pazufloxacin mesilate	BSA	9.93×10^{-15}	2.45	4.04	[16]
benzidine	trypsin	3.92×10^{-15}	2.67	2.97	[17]
nevadensin	lysozyme	8.17×10^{-15}	2.41	2.28	[18]
farrerol	lysozyme	5.19×10^{-14}	2.07	2.67	[19]
fosfomycin	BSA	1.87×10^{-14}	2.83	4.99	[24]
sulfamethoxazole	HSA	1.70×10^{-16}	2.79	3.89	[25]
tilorone (presented study)	HSA	7.88×10^{-16}	1.67	1.63	this work

Conclusions

In this work, the interaction between TIL and serum albumin (HSA) have been investigated by fluorescence method combined

with UV-vis spectroscopy techniques under simulative physiological condition. The experiment results indicate that TIL quenches the intrinsic fluorescence of proteins through

static quenching mode of TIL-HSA system, which supported the HSA UV spectral shifts and the decreasing binding constant for TIL and HSA complex with increasing temperature indicates the decomposition of the system.

The values of binding constant and the number of binding sites of the TIL-HSA system are determined using the Stern-Volmer equation. The average binding distance between donor and acceptor molecules is found to be 1.63 nm for HSA - TIL systems from the Forster non-radioactive resonance energy transfer theory.

Since, the pharmaceutical firms need standardized screens for protein binding in the first step of new drug design, this kind of study of interaction between HSA with TIL would be useful in pharmaceutical industry and clinical medicine.

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