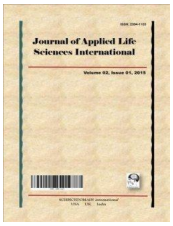

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The Fluorescence Spectroscopic Studies on the Interaction of Diltiazem Hydrochloride with Bovine Serum Albumin

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Authors' contributions

This work was carried out in collaboration between all authors. Author CB performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author JW managed the analyses of the study. Author XT managed the literature searches. Authors XT and QW designed the study and modified the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To explore the interaction of the diltiazem hydrochloride (DTZ) with bovine serum albumin (BSA).
Methodology: Fluorescence and UV-Vis spectroscopic techniques were used to study the interaction between diltiazem hydrochloride (DTZ) and BSA. DTZ is a nondihydropyridine calcium channel blocker used in the treatment of many kinds of diseases. The Stern-Volmer quenching constant (K_{sv}), the quenching rate constant of the bimolecular reaction (K_q), the binding constant (K_a), and a number of binding sites (n) of DTZ with BSA were evaluated.
Results: The results revealed that DTZ quenches the fluorescence intensity of BSA through a static quenching process. The values of ΔS and ΔH indicated that hydrophobic bond interactions played major roles in the binding process and contributed to the stability of the DTZ-BSA complex.

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Based on the Förster's theory of non-radiation energy transfer, the distance between donor (BSA) and acceptor (DTZ) was less than 7 nm, which indicated that energy transfer from BSA to DTZ occurs with high probability.

Keywords: Spectroscopy; diltiazem hydrochloride; bovine serum albumin; thermodynamic parameters.

1. INTRODUCTION

Serum albumins are the most abundant proteins in plasma and play an important role in transporting a variety of compounds [1]. The study of the interaction between drugs with serum albumin at molecular levels could provide useful information about the absorption, distribution, metabolism and excretion properties of drugs [1,2]. While bovine serum albumin (BSA) has two tryptophan residues, i.e., trp-212 and trp-134; human serum albumin (HSA) has only one tryptophan residue, trp-214. BSA has been one of the most extensively studied proteins for its approximately 76% sequence homologous with HSA [3-5]. BSA is suitable for the studies of the drug-albumin complex which could be considered as a model for gaining general fundamental insights into the drug-protein binding.

Many techniques have been used to study the binding of drugs with these proteins, such as fluorescence spectroscopy, UV-Vis absorption spectroscopy, FTIR, etc. [6-10]. Fluorescence spectroscopy has been widely used to monitor changes in the protein conformation, and it allows non-intrusive measurements of substances in low concentration under physiological conditions [11-12].

Diltiazem is a nondihydropyridine calcium channel blocker, used in the treatment of hypertension, angina pectoris, and some types of arrhythmia. It relaxes the smooth muscles in the artery walls, which opens (dilates) the arteries, allows blood to flow more easily, and lowers blood pressure. Additionally, it lowers blood pressure by acting on the heart itself to reduce the rate, strength, and conduction speed of each beat [13-16]. It is also used off-label as an effective preventive medication for migraine [17].

In this paper, attention has been given on studying the biophysical interactions of diltiazem hydrochloride (DTZ) (Fig. 1) with BSA using the fluorescence spectroscopy and UV absorption spectroscopy. Binding constants, quenching rate constants, thermodynamic parameters, and intermolecular distance have been determined. This study provides key information about the structural features which influence the therapeutic effectiveness of this drug. It also can become an important tool in the fields like life sciences, clinical medicine, even in pharmacology and pharmacodynamics.

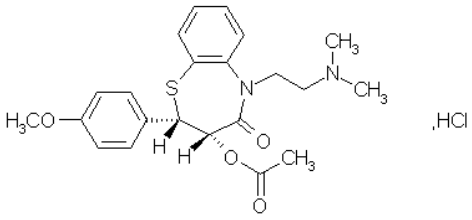

Fig. 1. The structure of diltiazem hydrochloride

2. MATERIALS AND METHODS

2.1 Materials and Apparatus

BSA and DTZ (Sigma) were used without further purification. The chemicals were of reagent grade (purity: 99%). BSA was dissolved in aqueous solution (5×10^{-4} M) containing 0.05 M Tri-HCl buffer (pH 7.4). The BSA solution was prepared based on its molecular weight of 65000. The DTZ solutions were prepared with ultrapure water.

The fluoresence spectra were recorded on RF-5301PC (SHIMADZU) spectrofluorometer equipped with 1.0 cm quartz cells. A UV-vis spectrophotometer (UV-1800, SHIMADZU) was used for scanning the UV spectrum equipped with 1.0 cm quartz cells.

2.2 Procedures

Fluorescence spectra were recorded from 300 nm to 450 nm with the excitation wavelength at 285 nm. Both excitation and emission slit widths were 3 nm. Stock solutions of DTZ 0.01 M in buffer (pH 7.4) were prepared at room temperature (15°C). Samples containing 2 ml of

the BSA solution and DTZ solutions were mixed to obtain final DTZ concentrations of 2.5×10^{-4}-10×10^{-4} M with constant BSA content 1×10^{-6} M. All experiments were measured at three temperatures.

The absorption spectra of protein in the presence and absence of DTZ were recorded in the range of 200-450 nm. The concentrations of protein and drug maintained were the same as those mentioned above.

3. RESULTS AND DISCUSSION

3.1 Quenching Mechanism of BSA Fluorescence by Diltiazem Hydrochloride

The change of intrinsic fluorescence intensity of BSA was due to tryptophan residue when small molecules bound to BSA [18]. Fluorescence quenching can be induced by a variety of molecular interactions of a fluorophore with quencher molecule, including ground-state complex formation, energy transfer, molecular rearrangement, excited-state reactions, and collisional quenching [19]. BSA has three fluorophores which are tryptophan, tyrosine, and phenylalanine [20]. The fluorescence of tyrosine is almost quenched due to the effect of the nearest amino group, a carboxyl group, or a tryptophan residue. Phenylalanine has a very low fluorescence quantum yield [21]. The effect of DTZ on the BSA fluorescence spectra is shown in Fig. 2. The fluorescence intensity of BSA gradually decreased upon increasing the concentration of DTZ. The decrease in fluorescence intensity is known as quenching which indicates that there are interactions between DTZ and BSA. A slightly red shift in the maximum emission wavelength of BSA was observed upon the addition of diltiazem hydrochloride. The slight red shift can be explained by an increasing polarity of the region surrounding tryptophan [22-24].

The fluorescence intensity of a compound can be decreased by a variety of molecular interactions, for example, molecular rearrangements and collisional quenching [25]. There was no fluorescence emission from diltiazem hydrochloride at the range of 300-450 nm. Fluorescence quenching is described by the Stern-Volmer equation [26]:

$$F_0 / F = 1 + Kq\,\tau_0[Q] = 1 + Ksv[Q] \qquad (1)$$

Where, F_0 and F are the relative fluorescence intensities in the absence and presence of the quencher, Kq, Ksv, τ_0 and [Q] are the bimolecular quenching rate constant, the Stern-Volmer quenching constant, the average lifetime of the biomolecule without quencher ($\tau_0=10^{-8}$ s) [27] and the concentration of the quencher, respectively. As shown in Fig. 3, the curves of F_0/F versus [Q] were plotted according to Equation (1).

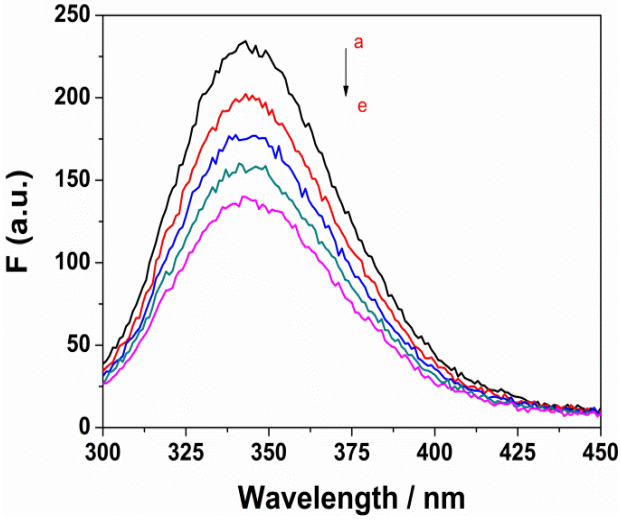

Fig. 2. Fluorescence spectra of DTZ-BSA systems in (a) free BSA: (1×10^{-6} M), pH 7.4 buffer at 298 K, (b–e) with DTZ at 2.5×10^{-4}, 5×10^{-4}, 7.5×10^{-4}, 10×10^{-4} M

Fig. 3 depicts that each plot exhibited a good linear relationship during the addition of DTZ 2.5×10^{-4}-10×10^{-4} M and the slope of the Stern-Volmer plot decreases with increasing temperature. The dynamic Ster-Volmer quenching constants (K_{sv}) were achieved by the slope of regression curves in the linear range, and quenching rate constants K_q were calculated based on the fluorescence lifetime of biopolymers (Table 1). Generally, the maximum collisional quenching constant of various kinds of quenchers with biomolecule is 2.0×10^{10} $M^{-1}\cdot s^{-1}$ [28-29]. The bimolecular quenching rate constants (K_q) are larger than the maximum collisional quenching constant and also the static quenching constants decreased with the increase of temperature (Table 1). The presence of the occurrence of a static quenching interaction between BSA and DTZ has been proposed in the current study.

Small molecules are assumed to bind independently to a set of equivalent sites on a macromolecule [20]. The number of binding sites and the binding constant of the interaction between DTZ and BSA were described with the following equation [30].

$$\log[(F_0 - F)/F] = \log Ka + n\log[Q] \quad (2)$$

Where n is the number of binding sites, Ka is the binding constant. A plot of log $[(F_0\text{-}F)/F]$ versus log [Q] gives a straight line, whose slope equals to binding sites and the intercept on Y-axis equals to log K_a (Fig. 4). The values of Stern-Volmer constants are given in Table 1. The values of n were approximately equal to 1, indicating that there was one binding site in NG to BSA. BSA most likely binds to the hydrophobic pocket located in sub-domain IIA [31]. The value of K_a is significant to understand the distribution of the drug in plasma [32]. Larger values of K_a observed in the present study proposes the presence of strong binding between DTZ and BSA. It was also found that the binding constant values decreased with increasing temperature due to the reduction of stability of the DTZ-BSA complex and it was consistent with the static quenching mechanism obtained for the interaction of DTZ with BSA [18]. As expected, the value of K_{SV} is comparable with our previous work [29,32-36].

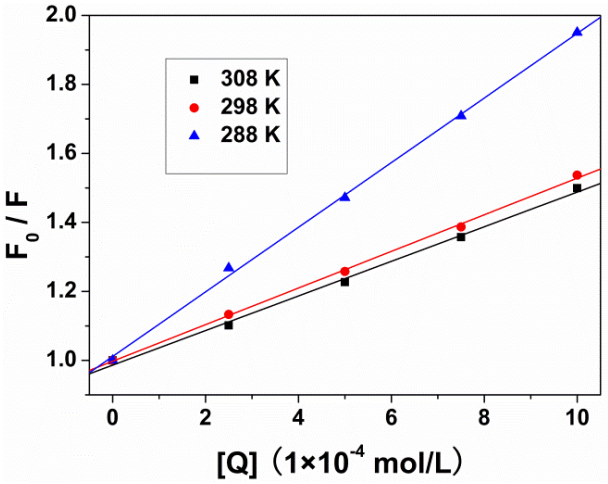

Fig. 3. The Stern-Volmer curves of DTZ-BSA systems

Table 1. The values of Stern-Volmer constants: Bimolecular quenching rate constant, quenching constant, binding constant, binding sites and correlation coefficients and the correlation coefficients (R^2) at different temperatures for DTZ-BSA system

T(K)	K_q (× 10^{12} L·mol^{-1} ·S^{-1})	K_{sv} (× 10^4 L·mol^{-1})	K_a (×10^4 L·mol^{-1})	*n*	R^2
288	0.0937	0.0937	1.381	1.1473	0.9965
298	0.0531	0.0531	0.518	0.9983	0.9989
308	0.0502	0.0502	0.504	0.9122	0.9987

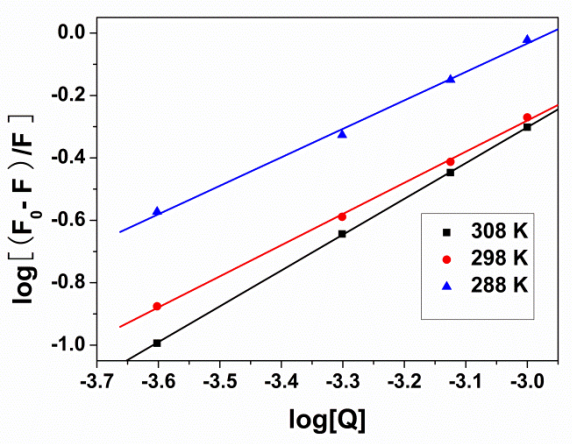

Fig. 4. The curves of lg[(F_0-F)/F] VS lg [Q] at different temperatures

3.2 Thermodynamic Parameters

There are many different mechanisms about the interaction of small molecules and macromolecules, such as electrostatic interactions, hydrogen bonds, hydrophobic forces, and Van der Waals interactions [33]. In order to clarify the interaction of DTZ with BSA, the thermodynamic parameters, Gibbs free energy changes (*ΔG*), enthalpy changes (*ΔH*), and entropy change (*ΔS*) were calculated by the Van't Hoff equation [34-37]:

$$\ln Ka = -\Delta H / RT + \Delta S / R \quad (3)$$

$$\Delta G = -RT \ln Ka \quad (4)$$

$$\Delta S = (\Delta H - \Delta G) / T \quad (5)$$

Where *R* and *T* are the gas constant and temperature in Kelvin scale, respectively. *Ka* is the equilibrium constant at temperature *T*. According to the Van't Hoff equation, *ΔH* and Δ*S* can be obtained from the slope and intercept of the plot of ln K_a versus 1/*T*, respectively. The value of *ΔG* can be calculated using Equation (4). The results are presented in Table 2.

Table 2. Thermodynamic parameters for DTZ-BSA system

T(K)	*ΔH* (KJ/mol)	*ΔS* (J/mol)	*ΔG* (KJ/mol)
288	-37.547	-71.490	-17.313
298			-15.484
308			-15.934

Researchers have proved the sign and magnitude of the thermodynamic parameters associated with various individual kinds of interaction that may take place in the ligand-protein binding process [29]. The negative sign for *ΔG* means that the interaction process is spontaneous for the DTZ. The negative *ΔH* and *ΔS* indicate an interaction between DTZ and biomolecule was Van der Waals forces and hydrogen bonds [38].

3.3 Energy transfer between DTZ and BSA

According to the Förster non-radiative energy transfer theory, fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the different electronic excited states of molecules [31]. Usually, FRET occurs whenever the emission spectrum of donor overlaps with the absorption spectrum of another acceptor and the distance between the donor and the acceptor is not longer than 7 nm [39]. Fig. 5 displays the overlap for the investigated donor-acceptor pair. According to the Förster theory, the energy transfer efficiency E is defined by the following equation (6).

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

Where *r* is the distance from the donor to the acceptor and R_0 is the Förster critical distance where the efficiency of energy transfer is 50%. R_0 can be calculated from the donor emission and acceptor absorption spectra using the Förster formula.

$$R_0^{\ 6} = 8.8\times10^{-25} K^2 \Phi N^{-4} J \quad (7)$$

$$J = [\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda]/[\sum F(\lambda)\Delta\lambda] \quad (8)$$

In Equation (7), K^2 is the orientation factor related to the geometry of the dipoles of the donor and acceptor and K^2=2/3 for the random orientation as in fluid solution. N is the average refractive index of the medium in the wavelength range where the spectral overlap is significant. In Equation (8), F is the corrected fluorescence intensity of the donor in the wavelength range λ, $\varepsilon(\lambda)$ is the molar absorptivity of the acceptor at wavelength λ. J is the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, which could be calculated by integrating the overlap portion of the spectra (Fig. 4). Fig. 4 shows the spectral overlap between the absorption spectrum of DTZwith the fluorescence spectrum of BSA in the wavelength range of 300-450 nm. It was reported that K^2= 2/3, F= 0.118 and n= 1.336 for BSA [40]. As shown in Table 3, the donor to acceptor distance in the complex is less than 7 nm, which indicates that energy transfer from BSA to DTZ occurs with high probability. BSA has two tryptophan residues: trp-212 and trp-134. The docking for DTZ was not studied. Therefore, the distance calculated here, is the average distance between DTZ and the two tryptophan residues in BSA.

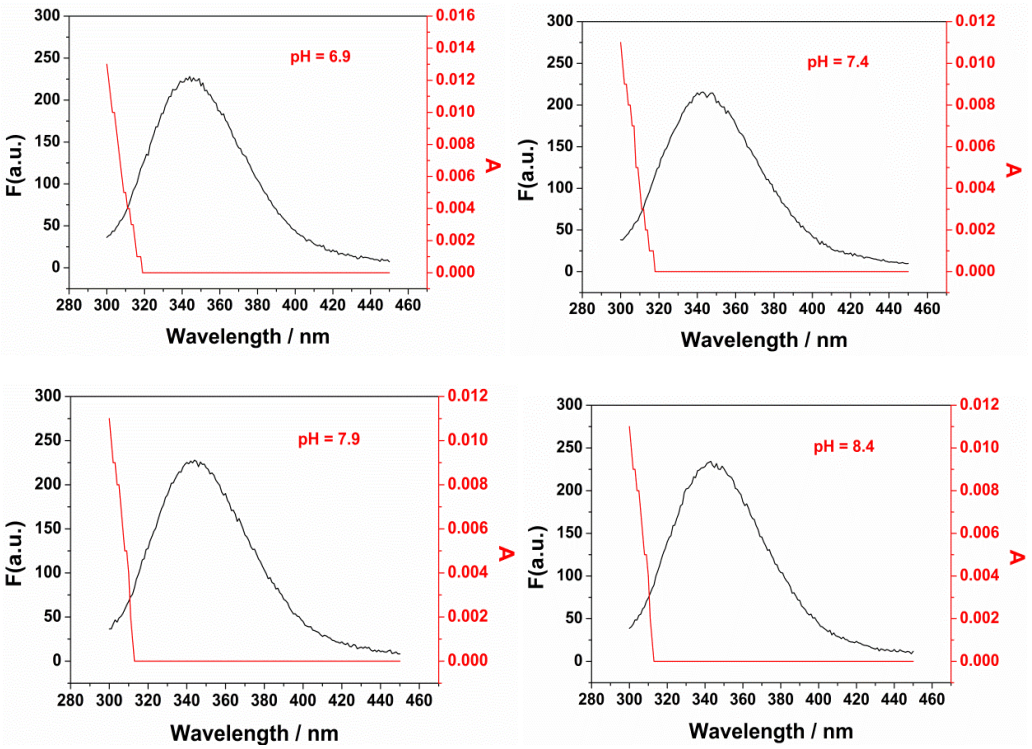

Fig. 5. The spectral overlap between the absorption spectrum of diltiazem hydrochloride with the fluorescence spectrum of BSA under different pH conditions

Table 3. Parameters of E, J, r, R_0 between diltiazem hydrochloride and BSA under different pH conditions

pH	*E*	*J*(×10^{-14} cm^3·L/mol)	R_0(nm)	*r*(nm)
6.9	0.087	3.144	2.022	2.992
7.4	0.092	2.378	1.953	2.861
7.9	0.121	2.390	1.932	2.688
8.4	0.132	2.526	1.949	2.668

4. CONCLUSION

Fluorescence and Ultraviolet-Visible methods for the determination of the interaction between DTZ and BSA were provided in the present study. The results showed that DTZcould quench the fluorescence of BSA. Based on the Stern-Volmer equation, the quenching rate constants were evaluated and their values suggested that the fluorescence quenching was a static process. The binding constant K_a and the number of binding site n were calculated according to the fluorescence quenching results. The thermo dynamic parameters suggested that the Van der Waals forces and hydrogen bonds play an important role in stabilising the DTZ-BSA system. Based on the mechanism of energy transfer, the donor-acceptor distance of the DTZand BSA was calculated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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