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Investigation on the interaction of gliquidone to bovine serum albumin by fluorescence spectroscopy

Qiu-Ju Zhang, Bao-Sheng Liu*, Gai-Xia Li, Rong Han
Key Laboratory of Analytical Science and Technology of Hebei Province, College of Chemistry & Environmental Science, Hebei University, Baoding, 071002, (HEBEI PROVINCE)
E-mail: lbs@hbu.edu.cn

ABSTRACT

Gliquidone interacting with bovine serum albumin in physiological buffer (pH 7.4) was investigated by the fluorescence quenching spectroscopy and synchronous fluorescence spectroscopy. The analysis of the quenching mechanism was done using Stern–Volmer plots which exhibit upward (positive) deviation. The experimental results indicated that the quenching mechanism between bovine serum albumin and gliquidone was static, and the electrostatic interaction played an important role in the interaction. In addition, binding sites *n*, the apparent binding constant *Ka*, the thermodynamic parameters and Hill's coefficients were calculated at different temperatures. The results obtained from the synchronous fluorescence were same with the results from the fluorescence quenching spectroscopy. In addition, synchronous fluorescence spectroscopy provided information about conformational changes of proteins.

KEYWORDS

Fluorescence quenching spectroscopy;
Synchronous fluorescence spectroscopy;
Gliquidone;
Bovine serum albumin;
Interaction

INTRODUCTION

Serum albumins are the most abundant proteins in the circulatory system of a wide variety of organisms, which play a dominant key role in the binding and transport of numerous endogenous and exogenous ligands[1]. They are mainly responsible for the maintenance of blood pH and play an important role in osmotic blood pressure[2]. The drug–albumin complex may be considered as a model to gain fundamental insights into drug–protein interactions and explore its applications[3]. Bovine serum albumin (BSA) and human serum albumin are characterized by a high homology (80%) and similar conformation[4]. Bovine serum albumin plays an important role in binding of numerous drugs in the bloodstream to their target organs for understanding the pharmacokinetics and pharmacodynamics properties of drug candidates[5].

A fluorescence spectroscopy based method is used to study the binding mechanism between drugs and proteins with respect to binding constant, binding site number and thermodynamic parameters, donor-to-acceptor distance, and other information, by examining the change of fluorescence intensity of protein in the maximum emission wavelength before

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and after adding the drugs[6]. It is a useful method in quantitative analysis due to its sensitivity, selectivity, and relatively low cost. Synchronous fluorescence spectroscopy has several advantages over conventional fluorescence spectroscopy, concerning especially simple spectra, which are sharp and narrow[7]. In synchronous fluorescence spectroscopy both the excitation and emission monochromators are scanned simultaneously in such a manner that a constant wavelength interval is kept between emission and excitation wavelengths ($\Delta\lambda$)[8]. It is a powerful tool for exploring conformational changes of proteins.

Type II diabetes mellitus is a chronic disease with high blood glucose levels and many vascular symptoms. Gliquidone, as show in Figure 1, belongs to sulfonylurea-type oral anti-diabetic agent which is one of major type of oral anti-diabetic agents, and it is widely used for type II diabetes[9]. In this article, we described the interaction mechanism of gliquidone and bovine serum albumin using fluorescence quenching spectroscopy and synchronous fluorescence spectrometry, respectively. The results showed that two methods had consistent mechanism, and synchronous fluorescence spectrometry provided the information about conformational changes of bovine serum albumin.

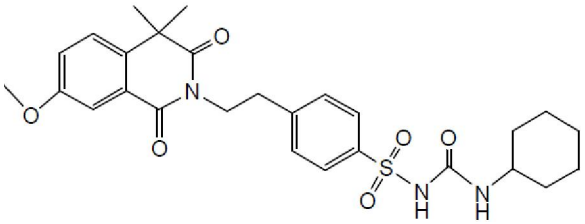

Figure 1 : Chemical structure of gliquidone

EXPERIMENTAL

Reagents and chemicals

Gliquidone (CAS#, 33342-05-1) was of the purity grade inferior 98.5%. Bovine serum albumin was purchased from Sigma Co. and was of the purity grade inferior 99%. Stock solutions of BSA (2.0×10^{-6} mol·L^{-1}) and gliquidone (5.0×10^{-6} mol·L^{-1}) were prepared. Tris-HCl buffer (0.05 mol·L^{-1} Tris, 0.15 mol·L^{-1} NaCl) was used to maintain the pH of the solution at 7.40, and NaCl solution was used to maintain the ionic strength of the solution. Chemicals were all of analytical grade and double-distilled water was used throughout the experiments. All aqueous solutions were stored at 277 K.

Apparatus

A Shimadzu RF-5301PC spectrofluorometer was used to record the fluorescence and synchronous fluorescence of BSA. All pH measurements were carried out with a pHS-3C precision acidity meter (Leici, Shanghai, China). All temperatures were controlled by a SYC-15_B superheated water bath (Nanjing Sangli Electronic Equipment Factory). A KQ-250 ultrasonic cleaner (Kun Shan Ultrasonic Instruments Co., Ltd) was used for drug dissolution.

Spectral measurements

Fluorescence spectra of BSA were taken in the absence and presence of gliquidone at different temperatures (298, 310 and 318 K) within the range of 285–450 nm at an excitation wavelength of 280nm and the range of 295–450 nm at an excitation wavelength of 295 nm, respectively. Both excitation and emission slit widths were 5nm. Synchronous fluorescence spectra of BSA were recorded from 280 to 340nm ($\lambda ex - \lambda em = \Delta\lambda = 15nm$) and from 300 to 450nm ($\lambda ex - \lambda em = \Delta\lambda = 60$ nm) at different temperatures.

RESULTS AND DISCUSSION

Analysis of fluorescence quenching

Generally, the fluorescence of protein is caused by three intrinsic fluorophores present in the protein, namely tryptophan, tyrosine, and phenylalanine residues. When bovine serum albumin is excited at 280nm, it mainly reveals the intrinsic fluorescence of tryptophan and tyrosine residues, and the fluorescence of phenylalanine residues can be negligible[10]. Figure 2 is the fluorescence spectra of BSA with varying concentrations of gliquidone at the excitation wavelength of 280nm. From Figure 2, it shows that the fluorescence of BSA regularly decreases with the increasing concentration of gliquidone, indicating that gliquidone could interact with BSA and

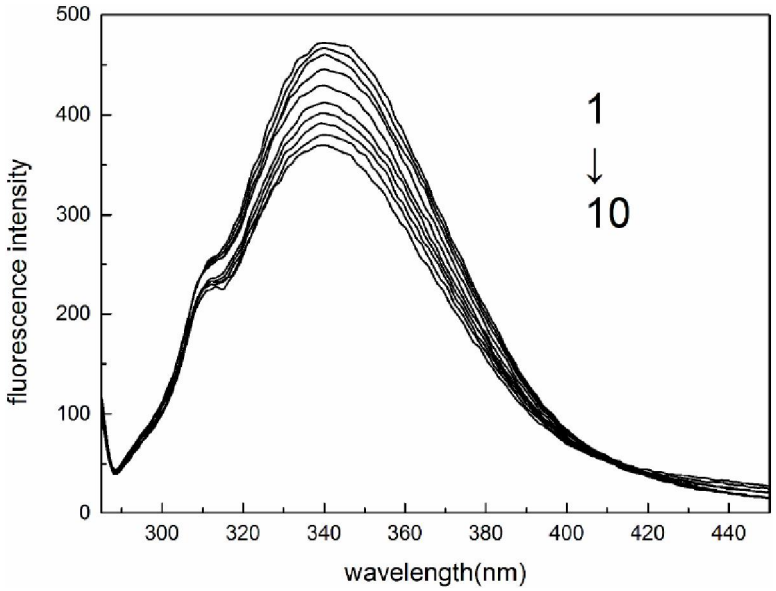

Figure 2 : Fluorescence spectra of gliquidone-BSA system (T =298 K, λ_{ex} = 280 nm); C_{BSA} =2.0× 10^{-7} mol·L^{-1}; 1~10 $C_{Gliquidone}$= (0, 0.15, 0.5,1.0, 1.5, 2.0, 2.5, 3, 3.5) ×10^{-6} mol·L^{-1}

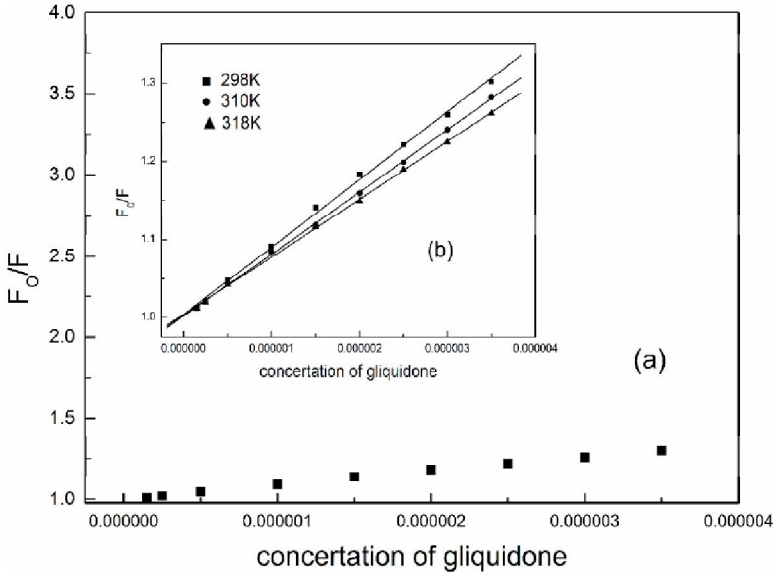

Figure 3 : Stern-Volmer plots of BSA- gliquidone system at different temperatures (λex =280 nm); (a: 298K, b: comparison of three temperatures)

quench its intrinsic fluorescence. However, no significant shift of the maximum emission wavelength was observed.

Quenching can occur by different mechanisms, which are usually classified as dynamic and static quenching[11]. Dynamic quenching is based on collisions between two substances. Static quenching is based on the interaction between two substances occurring during the formation of a ground-state complex[12]. In general, static and dynamic quenching can be distinguished by difference sin their temperature dependence. For dynamic quenching, because higher temperatures can lead to higher energy and stronger collisions, the bimolecular quenching constant is expected to become larger with increasing temperatures. By contrast, increasing temperature is likely to decrease the stability of the complex and thus lower the value of the static quenching constant[13].

In order to confirm the type of BSA fluorescence quenching, the fluorescence intensity data of BSA at 298, 310 and 318 K was analyzed according to the Stern–Volmer equation[14]:

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

Where, F_0 and F are the steady-state fluorescence intensities with and without the quencher, respectively; K_{SV} is the Stern-Volmer quenching constant; K_q is the quenching rate constant of the bimolecular; τ_0 is the average lifetime of the molecule without quencher; and [D] is the quencher concentration. Figure 3 is the plot of [F_0/F] versus [D]. Both quenching constant and quenching rate constant are shown in TABLE 1. K_q value, calculated by considering fluorescence lifetime of the biopolymer to be equal to 10^{-8} s, is of the order of 10^{12} L·mol^{-1}·s^{-1}. It is known for the case of dynamic quenching that the highest quenching rate constant of various quenchers with biopolymers is observed to be 2×10^{10} L·mol^{-1}·s^{-1}[15]. Therefore, the static quenching could be the main mechanism of the fluorescence quenching of BSA by gliquidone.

Binding constant and binding sites

For static quenching, the relation between fluorescence intensity and the amount of quencher can be defined using the 'modified' Stern–Volmer equation (Eq. 2)[16]:

$$(A_0 - A)^{-1} = A_0^{-1} + K_b^{-1} A_0^{-1} [L]^{-1} \quad (2)$$

Where, F_0 and F are the fluorescence intensities before and after the addition of quencher, respectively. [Dt] is the total drug concentration and [Bt] is the total protein concentration. From the plot of lg [(F_0-F)/F] versus lg([Dt]-n[Bt](F_0-F)/F_0), binding constants Ka and the number of binding sites n are calculated from the intercept and slope. The values of Ka and n at different temperatures are listed in TABLE 2. From TABLE 2, the values of n are almost equal to 1, which shows that there is one binding site for gliquidone in BSA; a decrease in the binding constant Ka is observed with an increase in temperature, which once again shows that the type of quenching was static[17]. According to TABLE 1 and 2, in the presence of gliquidone, the K_q and Ka value at λex = 280nm is greater than its counterpart at λex =295nm, suggesting that tyrosine and tryptophan residues played an important role in the interaction between gliquidone and BSA.

The primary binding site studies

Upon excitation at 280nm, both tryptophan and tyrosine are readily excited, while at an excitation wavelength of 295nm, only the tryptophan emits fluorescence[18]. Based on the Stern–volmer equation, comparing the fluorescence quenching constant ex-

TABLE 1 : Stern–volmer quenching constants for BSA–gliquidone system at different temperatures

λ_{ex}/(nm)	T/K	K_q/(L·mol^{-1}·s^{-1})	K_{sv}/(L·mol^{-1})	r_1
280	298	8.24×10^{12}	8.24×10^{4}	0.9967
	310	7.95×10^{12}	7.95×10^{4}	0.9998
	318	7.54×10^{12}	7.54×10^{4}	0.9991
295	298	7.53×10^{12}	7.53×10^{4}	0.9976
	310	7.03×10^{12}	7.03×10^{4}	0.9993
	318	6.36×10^{12}	6.36×10^{4}	0.9974

K_q is the quenching rate constant; r_1 is the linear relative coefficient of F_0/F~[D].

TABLE 2 : Binding constants for BSA–gliquidone system at different temperatures

λ_{ex}/(nm)	T/K	Ka/(L·mol^{-1})	n	r_2
280	298	9.23×10^{4}	1.01	0.9970
	310	8.03×10^{4}	0.99	0.9994
	318	7.69×10^{4}	0.99	0.9992
295	298	7.49×10^{4}	1.01	0.9979
	310	7.22×10^{4}	1.00	0.9965
	318	6.81×10^{4}	0.98	0.9943

Ka is the binding constant; n is the number of binding site. r_2 is the linear relative coefficient of lg(F_0-F)/F~lg{[D_t]-n[B_t](F_0-F)/F_0}.

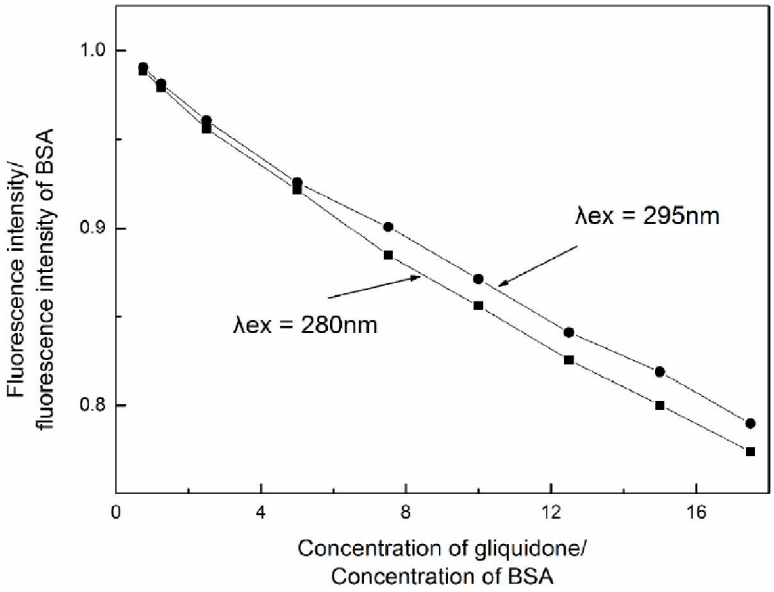

Figure 4 : Quenching curves of BSA-gliquidone system at λ_{ex} = 280nm and 295nm; C_{BSA} =2.0 × 10^{-7} mol·L^{-1}; 1~10 $C_{Gliquidone}$ =(0, 0.15, 0.5,1.0, 1.5, 2.0, 2.5, 3, 3.5) × 10^{-6} mol·L^{-1}

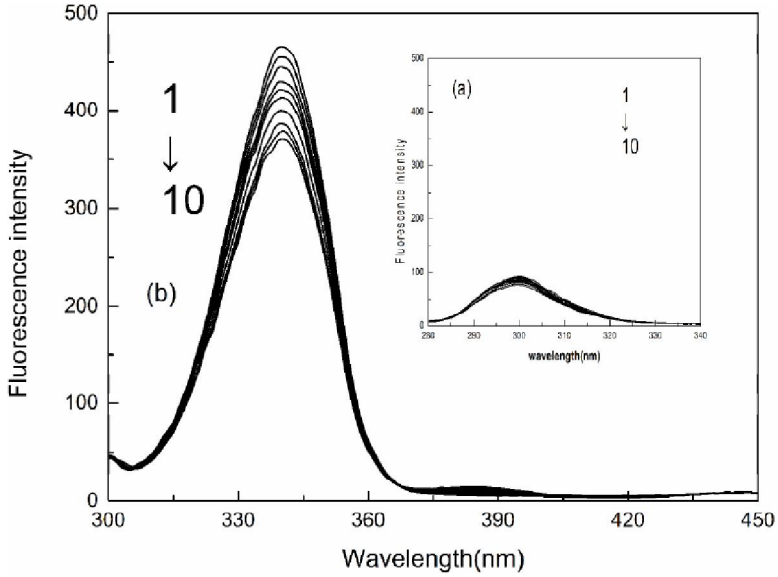

Figure 5 : Synchronous fluorescence spectra of BSA-gliquidone system; (*T* = 298 K, a: Δλ=15nm b: Δλ=60nm); C_{BSA} =2.0 × 10^{-7} mol·L^{-1}; 1~10 $C_{Gliquidone}$ =(0, 0.15, 0.5,1.0, 1.5, 2.0, 2.5, 3, 3.5) × 10^{-6} mol·L^{-1}

cited at 280nm and 295nm allows us to estimate the participation of tryptophan and tyrosine groups in the system. A comparison of the quenching curves obtained at 280nm and 295nm excitation for BSA-gliquidone is shown in Figure 4. It can be clearly seen that they do not overlap and that the quenching curves at 295nm were higher than those at 280nm. This phenomenon shows that in the interaction of gliquidone with BSA, both the tryptophan and tyrosine groups took part. In this way, it could confirm that the primary binding site for gliquidone was located in sub-domain IIA of BSA.

Synchronous fluorescence spectra studies

Synchronous fluorescence spectroscopy is a powerful tool for exploring conformational changes of proteins. When the wavelength interval between excitation and emission is 15 or 60nm, the synchronous fluorescence spectra give information about the molecular environment in the vicinity of tyrosine or tryptophan residues, respectively[19]. A shift in the maximum of emission wavelength is related to a

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change in the polarity of the surrounding environment. The effect of gliquidone on the synchronous fluorescence spectra of BSA at Δλ=15 and Δλ=60nm are presented in Figure 5. As seen in Figure 5, when Δλ is fixed at 60nm and15nm, no shift of λ_{max} is apparent. This suggests that the polarity and hydrophobicity around tyrosine and tryptophan microenvironment was not affected by gliquidone.

The corresponding results for Δλ =60nm, according to equations (1) and (2), are shown in TABLE 3. From TABLE 3, it can be seen the values of K_{SV} decreased with increasing temperature, implying that the static quenching was dominant in the system. Obviously, the rate constants K_q at three temperature are greater than the maximum scatter collision quenching constants of various quenchers with the biomolecule (2×10^{10}L·mol^{-1}·s^{-1}), which indicates that the nature of quenching was not dynamic but probably static, resulting from the formation of gliquidone-BSA complex.

In addition, from the values of *n*, it can be inferred that there is one independent class of binding sites on BSA for gliquidone. The *Ka* values decreases with the increasing temperature, which further evidences that the fluorescence quenching was a static quenching process. The quenching mechanism obtained by synchronous fluorescence method is coincident with that obtained by fluorescence method.

Thermodynamic parameters and nature of the binding forces

Small molecules are bound to macromolecules by four binding modes: hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interactive forces[20]. The enthalpy change (ΔH) and entropy change (ΔS) of binding reaction are the main evidence for confirming binding modes[21]. From a thermodynamic standpoint, ΔH>0 and ΔS>0 suggest a hydrophobic interaction; ΔH<0 and ΔS<0 reflect van der Waal's forces or hydrogen bond formation; and ΔH≈0 and ΔS>0 suggest an electrostatic force[22]. Because the effect of the temperature is pretty small, the enthalpy change of the interaction can be regarded as a constant if the temperature range is not too wide[23]. The thermodynamic parameters can be calculated on the basis of equations (3) and (4)[24]:

$$R\ln K = \Delta S - \Delta H / T \quad (3)$$

$$R\ln K = \Delta S - \Delta H / T \quad (4)$$

Where, *K* is the binding constant at the correspond-

TABLE 3 : Quenching reactive parameters of gliquidone and BSA at different temperatures by synchronous fluorescence spectroscopy (Δλ=60nm)

T/(K)	K_{q1} /(L·mol^{-1}·s^{-1})	K_{sv1} / (L·mol^{-1})	r_3	K_{a1} /(L·mol^{-1})	*n*	r_4
298	7.04×10^{12}	7.04×10^{4}	0.999 3	7.91×10^{4}	1.03	0.998 5
310	6.70×10^{12}	6.70×10^{4}	0.998 9	7.23×10^{4}	1.03	0.998 7
318	6.28×10^{12}	6.28×10^{4}	0.998 8	6.66×10^{4}	1.01	0.998 5

K_q is the quenching rate constant; *Ka* is the binding constant; *n* is the number of binding site. r_3 is the linear relative coefficient of F_0/F~[*D*]; r_4 is the linear relative coefficient of $\lg(F_0-F)/F$~$\lg\{[D_t]-n[B_t](F_0-F)/F_0\}$.

TABLE 4 : The thermodynamic parameters of gliquidone-BSA at different temperatures

		T/(K)	*Ka*/(L·mol^{-1})	Δ*H*/(KJ·mol^{-1})	Δ*S*/(J·mol^{-1}·K^{-1})	Δ*G*/(KJ·mol^{-1})
Fluorescence quenching spectra	λex= 280nm	298K	9.23×10^{4}	-7.35	70.39	-28.33
		310K	8.03×10^{4}		70.18	-29.11
		318K	7.69×10^{4}		70.42	-29.74
	λex= 295nm	298K	7.49×10^{4}	-5.99	73.21	-27.81
		310K	7.22×10^{4}		73.69	-28.83
		318K	6.81×10^{4}		73.69	-29.42
Synchronous fluorescence spectroscopy	Δλ= 60nm	298K	7.91×10^{4}	-6.68	71.35	-27.94
		310K	7.23×10^{4}		71.47	-28.84
		318K	6.66×10^{4}		71.33	-29.36

TABLE 5 : Hill's coefficient of gliquidone-BSA systems at different temperatures

T /(K)	Fluorescence quenching spectroscopy				Synchronous fluorescence spectroscopy	
	λex= 280nm		λex= 295nm		Δλ= 60nm	
	n_H	r_5	n_H	r_5	n_H	r_5
298K	1.057	0.9981	1.070	0.9973	1.026	0.9982
310K	1.044	0.9995	1.028	0.9968	1.089	0.9983
318K	1.044	0.9994	1.059	0.9968	1.080	0.9989

ing temperature, *T* is the experimental temperature and *R* is the gas constant. The enthalpy change (ΔH) is calculated from the slope of the plot of ln*K* versus 1/*T*. The free energy change ΔG and entropy change ΔS are calculated from Eq. (4), respectively. The calculated results are given in TABLE 4. From TABLE 4, the negative sign for ΔG indicates that the binding process for BSA and gliquidone is spontaneous[25]. In addition, ΔH is almost zero, ΔS is positive, implying electrostatic force plays a major role in the binding between gliquidone and BSA.

Hill's coefficient of BSA-gliquidone system

In biochemistry, the binding of a ligand molecule at one site of a macromolecule often influences the affinity for other ligand molecules at additional sites. This is known as cooperative binding. Hill's coefficient is calculated graphically on the basis of the following equations[26]:

$$\lg\frac{Y}{1-Y} = \lg K + n_H \lg[D] \quad (5)$$

Where, *Y* is the fractional binding saturation; *K* is the binding constant and n_H is Hill's coefficient. A value of Hill's coefficient >1 indicates positive cooperativity. Conversely, a values of Hill's coefficient<1 exhibits negative cooperativity. A coefficient = 1 indicates a non-cooperative reaction.

$$\frac{Y}{1-Y} = \frac{Q}{Q_m - Q} \quad (6)$$

For fluorescence measurements:

$$Q = \frac{F_0 - F}{F_0} \quad (7)$$

Where, $1/Q_m$ is the intercept of the plot 1/*Q* versus 1/ [*D*]. Hill's coefficients are given in TABLE 5. The values of n_H are equal to 1 at different temperatures, indicating there is non-cooperative reaction between BSA and gliquidone.

CONCLUSION

In this paper, the interaction between BSA and gliquidone was investigated by fluorescence quenching and synchronous fluorescence spectroscopy at pH7.40. The results indicated that the quenching mechanism of gliquidone with BSA was static; there is one binding site for gliquidone in BSA; the primary binding site for gliquidone was located in subdomain IIA of BSA; the binding reaction was spontaneous and electrostatic force played major role in the reaction; there is non-cooperative reaction between BSA and gliquidone. In addition, synchronous spectroscopy showed that the conformation of BSA did not changed in the presence of gliquidone. The investigation between drug and protein is extremely important to understand pharmaceutics, pharmacokinetics and toxicity of the drug as well as the relationship of structure and function of the protein.

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