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18β-Glycyrrhetinic acid interaction with bovine serum albumin

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Abstract

The interaction of 18β-glycyrrhetinic acid (GA): The metabolite of glycyrrhizic acid which is the main active component of a commonly used traditional Chinese medicine (TCM) *Glycyrrhiza Uralensis Fisch* with bovine serum albumin (BSA) has been investigated. Fluorescence emission spectra of serum albumin in the presence of GA, recorded at the excitation wavelength 280 nm, clearly show that GA act as quencher and have different quenching mechanism at a pH below or above the isoelectric point (p*I*). The binding sites number *n* and apparent binding constant *K* were measured. The thermodynamic parameters ΔH°, ΔG°, ΔS° at different temperatures were calculated. The effects of some common metal ions on binding are considered. Synchronous fluorescence and UV–vis spectra were used to study protein conformation. Energy transfer between GA and HSA was calculated by Förster's theory and the binding site was suggested to be site II. The binding of monoammonium glycyrrhizinate (GL) to BSA is also compared.

Keywords: 18β-Glycyrrhetinic acid; Serum albumin–drug interactions; Fluorescence quenching; Bovine serum albumin; Binding sites

1. Introduction

Glycyrrhiza Uralensis Fisch is the most common traditional Chinese medicines (TCMs) [1]; it appears in many Chinese herbal formulations. Glycyrrhizic acid is the main active ingredient of it and GA is the in vivo metabolite of this acid. GA is an inhibitor of some cancer cell and enzyme, such as choriocarcinoma [2], melanoma [3], mastocytoma [4] and 11β-hydroxysteroid dehydrogenase [5,6]. GA is a potent inducer of mitochondrial permeability transition and can trigger the pro-apoptotic pathway [7]. It can enhance the activity of hydrocortisone [8] and retard the development of autoimmune disease [9]. Also it possesses hepatoprotective effects and can protect hepatoinjury from carbon tetrachloride [10] and retrorsine [11].

Serum albumin is the most abundant protein in animal's including human circulatory system. It is in charge of the transport of a variety of endogenous and exogenous substances in body and plays an important role in the distribution and deposition of these substances [12]. When drugs are absorbed, they enter into the circulatory system and extensively and reversibly bind to serum albumin [13]. An important aspect of a drug's biodisposition profile is the extent to which it binds to plasma proteins [14]. Drug–protein interaction has significance in pharmacology. It can affect the biological activity [15,16] and toxicity [17–19] of drug. The binding parameters are helpful in the study of pharmacokinetics and the design of dosage forms [20,21].

The tryptophan residues in serum albumin have intrinsic fluorescence. Information about the protein can be obtained by the measurement of intrinsic fluorescence intensity of the tryptophan residues before and after the addition of the drug. Many fluorescent methods have been developed for investigating the interaction of drug-macromolecule and most them are collected in corpora of principles of fluorescence of Lakowicz [22]. Among them, 8-anilino-1-naphthalenesulfonate (ANS) is the most commonly used fluorescent probe. ANS was applied for studies of salvation dynamics in a protein, resonance energy transfer and other binding parameters [23–25]. In recent year, there are increasing studies on the interaction between serum albumin and active components in TCMs, such as Jatrorrhizine in Tinospora cordifolia [26], Curcumin from *Curcuma longa*

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L. [27], Wogonin in Scutellariae [28], Alpinetin from Amomum alnus [29] and so on. As glycyrrhiza appears in many TCM prescriptions, binding of its primary bioactive ingredient GA to serum albumin is worthy of investigation. In this paper, fluorescence emission spectra of serum albumin in the presence of GA below and above protein p*I* clearly show that GA acts as quencher and have different quenching mechanism at a pH below or above the isoelectric point (p*I*). The binding sites number *n* and apparent binding constant *K* were measured. The thermodynamic parameters at different temperatures were calculated. Synchronous fluorescence and UV–vis spectra were used to study protein conformation. The binding of monoammonium glycyrrhizinate to bovine serum albumin (BSA) is also compared and it shows a difference result from GA. In addition, a comparative study of BSA interaction with GA and phenylbutazone (PB), fluofenamic acid (FA) and ibuprofen (IB) should help understand preferential binding at the molecular level. Through this work, we hope we can provide some clues on the pharmacology of glycyrrhiza.

2. Experimental

2.1. Materials

All fluorescent measurements were carried out on an F-4500 fluorescence spectrophotometer (Hitachi, Kyoto, Japan) equipped with a xenon lamp source and 1.0 cm cell. A TU-1800SPC recording spectrophotometer (Puxi Ltd. Of Beijing, China) equipped with 1.0 cm quartz cells was used for the UV spectrum scanning. All pH measurements were made with a pHS-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. The mass of sample was accurately weighed with a microbalance (Sartorius).

2.2. Apparatus and methods

BSA (≥98%, Roche) was used without further purification and its molecular weight was assumed to be 66,500. BSA stock solution (2×10^{-5} mol L^{-1}) was prepared with doubly distilled water and was kept in the dark at 4 °C. 18β-Glycyrrhetinic acid (≥97%, Fluka, product of Spain), glycyrrhizic acid ammonium salt (≥95%, Fluka, product of Japan), phenybutazon (PB), fluofenamic acid (FA) and ibuprofen were of analytical grade, and the stock solutions were prepared in 90% ethanol and their final concentration was equal to BSA's, namely 410^{-6} mol L^{-1}. NaCl (0.5 mol L^{-1}) solution was used to maintain the ionic strength. Buffer (pH 7.40) consists of Tris (0.1 mol L^{-1}) and HCl (0.1 mol L^{-1}), and the pH was adjusted to 7.40 by adding 0.5 mol L^{-1} NaOH. Solutions of common metal ions (2.0×10^{-2} mol L^{-1}) were prepared by $MgCl_2$, $AlCl_3$, $CoCl_2$, $ZnSO_4$ and $CuSO_4$, respectively, and their final concentration in fluorescence titration was 1.0×10^{-4} mol L^{-1}. Britton–Robinson buffer (pH 3.32). All reagents are of analytical grade unless specialized. Water is doubly distilled water.

Fluorescence spectra were measured using 2.5/2.5-nm slit widths and 700 V PMT. The excitation wavelength was 280 nm, and the emission was read at 290–420 nm. Fluorometric titration experiments: 2.0 mL 0.5 mol L^{-1} NaCl solution, 2.0 mL buffer and 2.0 mL BSA stock solution were pipetted into a 10 mL calibrated-flask, the filled with water to the scale. 1.0 mL of this solution was transferred to a 1-cm cell and was titrated by successive additions of GA or other chemical stock solution to give a solution of certain concentration. Titrations were done manually by using trace syringes, then mix with a vortex agitator and the fluorescence intensity was measured (excitation at 280 nm and emission at 340 nm). The temperature was controlled by air-conditioner and temperatures were kept in a certain value ($T \pm 0.5$ °C) throughout a certain experiment.

3. Results and discussion

3.1. Fluorescence quenching of BSA by GA

In BSA tryptophan and tysosine contribute to fluorescence spectra. This fluorescence decreases when some substance was added into the BSA solution. This phenomenon is called fluorescence quenching (FQ). FQ in serum albumin is widely used in measuring drug–protein binding affinity. The interaction of GA to BSA at a pH below or above the isoelectric point (p*I*) was evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of GA. Fig. 1 shows that addition of GA causes a dramatic change in the fluorescence emission spectra. Quenching of BSA was observed promptly in our experiments,

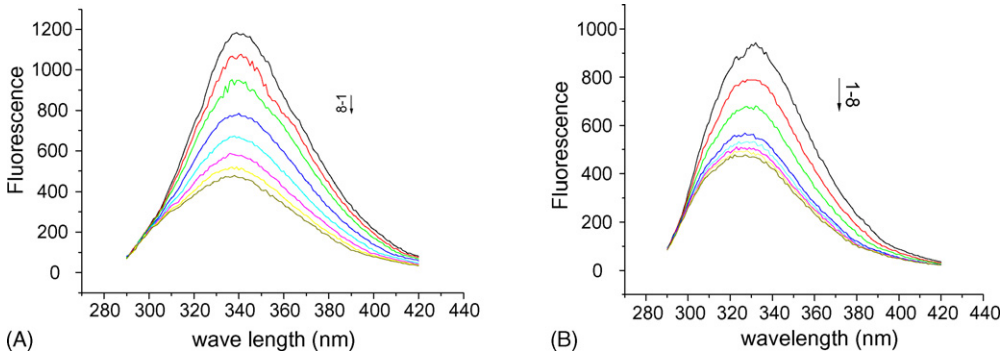

Fig. 1. Fluorescence spectra of BSA in the presence of GA. (A) pH 7.40, 297 K; (B) pH 3.32, 298 K. Curves 1–8 GA concentrations are 0, 5.3, 10.6, 21.3, 32, 42.6, 53.3, 63.9 ($\times 10^{-7}$ mol L^{-1}), respectively. BSA: 4×10^{-6} mol L^{-1}.

even at first addition of GA, suggesting [19] that a high affinity binding site to BSA is close to the single typtophan of this molecule. BSA has a strong fluorescence emission with a peak at 340 nm on excitation at 280 nm. With gradual increase in drug concentrations, we observe typical FQ behavior. The fluorescence intensity of protein decreases in the presence of GA and no distinct maximum emission wavelength shift was observed in the studied GA concentration at pH 7.40, but a slight blue shift at pH 3.32. Suggesting that the microenvironment around chromophore of BSA is changed after the addition of GA.

Assumed the procedure to be dynamic quenching. By Stern–Volmer quenching equation:

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

where K_q, K_{sv}, τ_0 and $[Q]$ are the quenching rate constant of the biomolecule, the dynamic quenching constant, the average lifetime of the molecule without quencher and concentration of quencher, respectively. Because fluorescence lifetime of biopolymer is 10^{-8} s [30], quenching constant (K_q: L mol^{-1} s^{-1}) at pH 7.40 can be obtained by the slope: 297 K, $K_q = 2.47 \times 10^{13}$, $r = 0.9991$; 287 K, $K_q = 2.11 \times 10^{13}$, $r = 0.9988$. 298 K, $K_q = 1.20 \times 10^{13}$, $r = 0.9910$ and 292 K, $K_q = 1.38 \times 10^{13}$, $r = 0.9907$ at pH 3.32, respectively. Fig. 2 shows that curves have fine linear relationships, but changes of slopes with the increase of temperature show different result below and above p*I*. This indicates there are distinct quenching mechanism below and above p*I* between GA and HSA.

However, maximum scatter collision quenching constant of various kinds of quenchers to biopolymer is 2.0×10^{10} L mol^{-1} s^{-1} [31]. Obviously, the rate constant of protein quenching procedure initiated by GA is greater than K_q of scatter procedure. This shows that above quenching is not initiated by dynamic collision but forms compound. This phenomenon might be the result of the radiationless energy transfer between GA and BSA.

3.2. Binding constant and number of binding sites

Assumed 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 [32–34]:

$$\log\left(\frac{F_0 - F}{F}\right) = \log K + n\ \log[Q] \quad (2)$$

where in this work, K is the binding constant to a site, and n the number of binding sites per BSA. According to the Eq. (2), the binding constant K and the number of binding sites n at pH 7.40 can be obtained as $K = 8.28 \times 10^5$ L mol^{-1}, $n = 1.1$ ($r = 0.9985$) at 297 K and $K = 1.60 \times 10^5$ L mol^{-1}, $n = 0.986$ ($r = 0.9981$) at 287 K, respectively. While at pH 3.32, $K = 9.63 \times 10^4$ L mol^{-1}, $n = 0.962$ ($r = 0.9947$) at 292 K and $K = 2.21 \times 10^4$ L mol^{-1}, $n = 0.821$ ($r = 0.9956$) at 298 K, respectively. The binding parameters of GL to BSA are $K = 4.65 \times 10^4$ L mol^{-1}, $n = 1.03$ ($r = 0.9976$) at 297 K and pH 7.40.

3.3. The binding force between GA and BSA

The acting forces between pharmaceutical and biomolecule include hydrogen bonds, van der Waals forces, electrostatic forces and hydrophobic interaction forces, etc. If the temperature changes little, the reaction enthalpy change is regarded as a constant. By van't Hoff and thermodynamic equation:

$$\ln K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R};$$
$$\Delta G^\circ = RT\ \ln K = \Delta H^\circ - T\Delta S^\circ \quad (3)$$

Here ΔH°, ΔG°, ΔS° are enthalpy change, free energy change and entropy change, respectively, and can be obtained. ΔH° and ΔS° are 117 and 0.507 kJ at pH 7.40, while are −177 and −0.511 kJ at pH 3.32, respectively, in the binding reaction between GA and BSA. So it can be deduced that the acting force is mainly hydrophobic interaction force above p*I* (pH 7.40) and hydrogen bonds, van der Waals forces below p*I* (pH 3.32) [35].

3.4. Energy transfer between GA and BSA

Förster nonradiative energy transfer contains lot of useful information [36,37] concerning the molecular details of donor–acceptor, for instance, it can evaluate the distance between the ligand and the tryptophan residues in the protein.

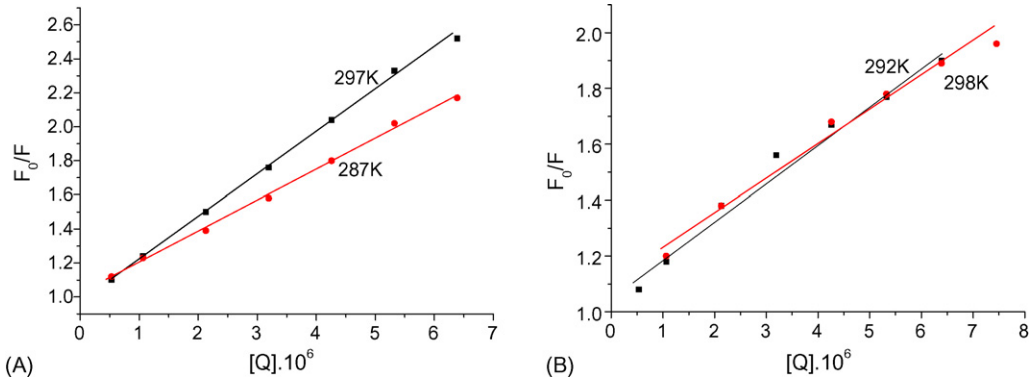

Fig. 2. The Stern–Volmer curves for quenching of GA to BSA. (A) pH 7.40; (B) pH 3.32 ($\lambda_{ex} = 280$ nm; $\lambda_{em} = 340$ nm; GA: from 5.3 to 64 × 10^{-7} mol L^{-1}; BSA: 4 × 10^{-6} mol L^{-1}).

While proper overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor exists, nonradiative energy transfer can be detected. The rate of energy transfer depends upon the extent of overlap, the relative orientation of the donor and acceptor transition dipoles, and the distance between these molecules. According to the Förster nonradiative energy transfer theory [36,38], the efficiency of energy transfer (E) is related to the distance R between donor and acceptor by

$$E = \frac{R_0^6}{R_0^6 + r^6} \tag{4}$$

where R_0 is a characteristic distance, called the Förster distance or critical distance, at which the efficiency of transfer is 50%, and r is the distance between the donor and acceptor. R_0 can be denoted by Eq. (5).

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J. \tag{5}$$

In Eq. (5), K^2 is the spatial orientation factor of the dipole of the donor and acceptor, N the refractive index of the medium, F the fluorescence quantum yield of the donor in the absence of acceptor and the overlap integral J expresses the degree of spectral overlap between the donor emission and the acceptor absorption. J can be given by:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{6}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. The efficiency of transfer (E) could be obtained by the Eq. (7). Where F and F_0 are the relative fluorescence intensity in the presence and absence of acceptor.

$$E = \frac{1 - F}{F_0} \tag{7}$$

The overlap spectra of the absorption spectra of GA and the fluorescence emission spectra of BSA are showed in Fig. 3. So J can be evaluated by integrating the spectra in Fig. 3 for $\lambda = 280 - 420$ nm and is 8.926×10^{-15} cm^3 L mol^{-1}. Under these experimental conditions, we found $R_0 = 2.47$ nm from Eq. (5) using $K^2 = 2/3$, $N = 1.36$, $\Phi = 0.15$ [39], the energy transfer effect, $E = 0.514$ from Eq. (7), and the distance between GA and amino acid residue in BAS $r = 2.45$ nm. The data of R_0 and r are also in the academic range, which prove that nonradiative energy transfer occurs between GA and BSA. The average distance $r < 8$ nm [40], and $0.5R_0 < r < 1.5R_0$ [41] indicate that the energy transfer from BSA to GA occurs with high probability.

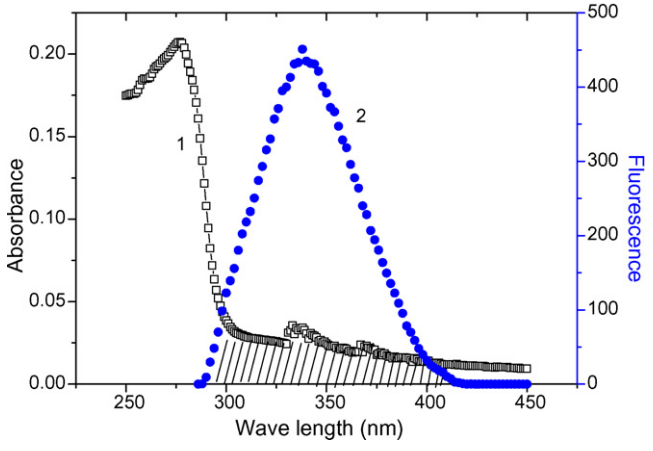

Fig. 3. The overlap of fluorescence emission spectra (1) and the absorption spectra (2) when the molar ratio of GA and BSA is 1:1. BSA: 1.0×10^{-6} mol L; GA: 1.0×10^{-6} mol L.

3.5. The effect of metal ions and other bioactive compound on the binding constants

The effects of some common metal ions and other bioactive compound (GL) on the binding are investigated at 297 K. The fluorescence intensity was changed before and after the addition of common ion at $\lambda_{em} = 340$ nm. The results are listed in Table 1. The competition between the common metal ions and GA led to GA–BSA binding constants dropped 10–70% as compared to the binding constant without ions. The presence of GL makes the binding constant of GA–BSA reduce 80%, while the presence of GA lets the binding constant of GL–BSA decrease almost 100%. The results show the presence of common metal ions reduces the GA–BSA binding, causing GA to be quickly cleared from the blood, which may lead to the need for more doses of GA to achieve the desired medicinal effect. Moreover, remarkable effects on respective binding exist among bioactive components. High binding constant of GA to serum albumin may be an explanation of the wide use of *Glycyrrhiza Uralensis Fisch* in many TCM formulations.

3.6. Identification of binding sites on BSA

Similar to HSA, BSA consist of amino acids chains forming a single polypeptide which contain three homologous α-helices domains (I–III). Each domain is divided into antiparallel six-helix and four sub-domains (A and B). The adherence of two sub-domains, with their grooves towards each other forms a domain, and three of such domains make up an albumin molecule [17]. The binding sites of BSA for endogenous and exogenous ligands may be in these domains and the principal regions of ligand binding sites of albumin are located in hydrophobic cavities in sub-domains IIA and IIIA. Many ligands bind specifically

Table 1
The binding constant between BSA and GA at 297 K in the presence of common ions and bioactive compound

Ions (bioactive compound)	$K'/10^5$	R^2	K'/K
Without	8.28	0.9971	–
Al^{3+}	1.64	0.9981	0.198
Co^{2+}	2.70	0.9979	0.326
Cu^{2+}	2.95	0.9986	0.356
Mg^{2+}	3.05	0.9981	0.368
Zn^{2+}	1.00	0.9924	0.121
GL^{+}	1.49	0.9979	0.180
GA^{+}	0.003*	0.9941	0.001

(*) is the binding constant between BSA and GL in the presence of GA. (+) Addition of GL or GA is 4 μM.

to serum albumin, for example warfarin and phenylbutazone for site I, flufenamic acid (FA) and ibuprofen for site II [42] and digitoxin [43] for site III. In order to identify the location of the GA-binding site on BSA, site marker competitive experiments were carried out, using drugs mentioned above. According to the Stern–Volmer equation (Eq. (1)), the fluorescence data were used to obtain the values of quenching constant K_{sv} (2.30×10^5, $r = 0.9990$; 2.70×10^5 mol/L, $r = 0.9993$, 2.42×10^5, $r = 0.9990$, for PB, FA, and IB, respectively, at 296 K). While in term of Eq. (2), the binding constant K are 1.72×10^5, $r = 0.9996$; 9.04×10^5, $r = 0.9992$; 9.48×10^5, $r = 0.9948$, respectively. Considering K_{sv} and K is 2.47×10^5 and 8.28×10^5 without probe in the same condition, the results calculated indicated that the bound of GA to HSA was affected by adding FA in contrast with the addition of PB or IB to the same complex, which has shown remarkable effect. So, we suggest the binding site of GA to BSA is site II.

3.7. Effects of GA on the conformation of BSA by UV-visual and synchro-fluorescence spectra

It is proverbial that the fluorescence of BSA comes from the tyrosine, tryptophan, and phenylalanine residues. The spectrum of BSA was sensitive to the micro-environment of these chromophores and it allows non-intrusive measurements of protein in low concentration under physiological conditions. So spectroscopic methods were usually applied to the study of conformation of serum protein. In synchronous fluorescence spectroscopy, according to Miller [44], distinction of the difference between excitation wavelength and emission wavelength ($\Delta\lambda$) reflects the spectra of disparate chromophores, with large $\Delta\lambda$ values such as 60 nm, the synchronous fluorescence of HSA is characteristic of tryptophan residue and with small $\Delta\lambda$ values such as 15 nm is characteristic of tyrosine. The maximum emission wavelength (λ_{max}) is also very useful in estimating the hydrophobicity of the trytophan resides. λ_{max} at 330–332 indicated that trytophan resides are located in the nonpolar region, that is, they are buried in a hydrophobic cavity in BSA; λ_{max} at 350–352 nm suggests that tryptophan residues are exposed to water, says, the hydrophobic cavity in BSA is disagglomerated and the structure of HAS is looser.

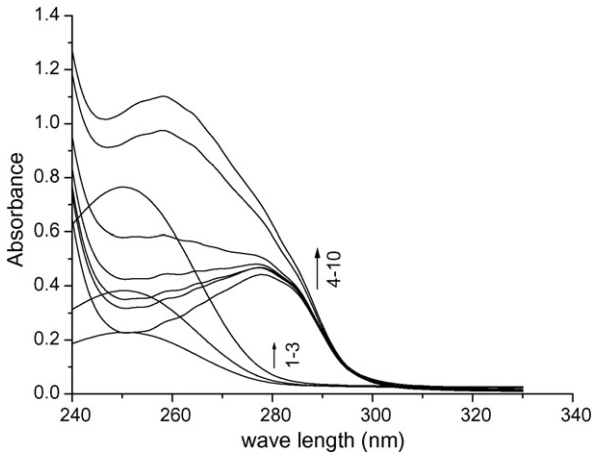

Fig. 5. UV absorption spectra of the GA–BSA system obtained at 297 K and pH 7.40. (1–3) GA (μM): 6.75, 20.25, 54; (4–10) GA (μM): 0, 6.75, 10.8, 20.25, 33.75, 47.25, 67.5 and BSA keep in 10 μM. pH 7.40, 297 K.

Fig. 4 shows the effect of addition of GA on the synchronous fluorescence spectrum of BSA. Addition of the drug results in the strong fluorescence quenching of tryptophan with the maximum emission wavelength at 281 nm, no distinct shift of wavelength was observed; while fluorescence strength of tyrosine change little at 286 nm. Moreover, considering the fine linearity of the Stern–Volmer quenching in the same condition and the binding site number in the same condition, we can conclude that GA bind to a specific site near one of the two tryptophans in BSA. Since tryptophan 212 of BSA is located in sub domain IIA [19], we can conclude that the binding site is in the vicinity of tryptophan 212. λ_{max} of emission spectra is 340 nm shows this tryptophan partially locates in the hydrophobic medium. All these indicate that the interaction with GA does not affect the conformation of BSA in the studying condition. On the other hand, λ_{max} of emission spectra is 332 nm and a blue shift of wavelength from 332 to 325 nm was observed at pH 3.32 suggest tryptophan is buried in a hydrophobic cavity in BSA and the conformation of BSA has been changed.

The UV–vis spectra of BSA, GA and GA–BSA were shown in Fig. 5. It is clear from the figure that in the invisible region, the absorption peaks of GA and BSA is in 250 and 278 nm,

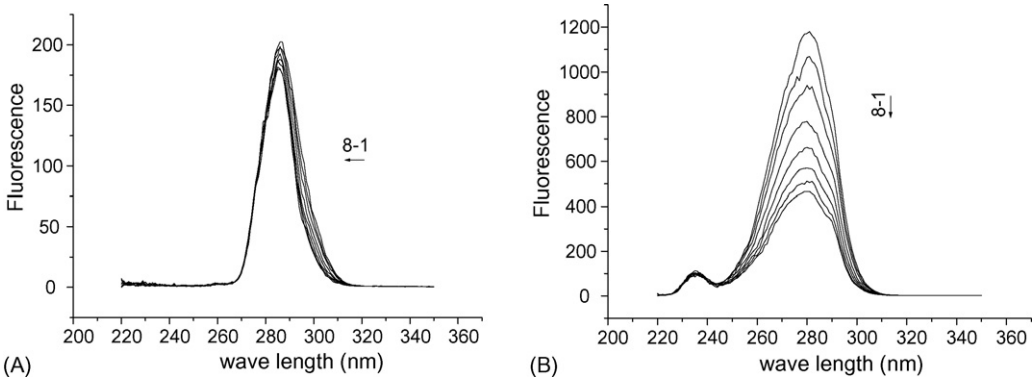

Fig. 4. Synchronous fluorescence spectra of BSA (4.0 μM) with $\Delta\lambda = 15$ nm (A) and $\Delta\lambda = 60$ nm (B) in the absence and presence of GA (1–8, μM): 0, 0.53, 1.06, 2.13, 3.20, 4.26, 5.33, 6.39. pH 7.40, 297 K.

respectively. A new peak was formed with the increasing addition of GA which λ_{max} is 258 nm. This indicates new complex is formed. The conclusion is consistent with the result from fluorescence quenching investigation.

4. Conclusions

In this paper, the interaction between GA and BSA has been studied by the fluorescence method, and UV-visual spectroscopy. The results presented have clearly indicated that GA is a strong quencher. Meanwhile, from the thermodynamic parameter calculation, it can be shown that the acting force was mainly the hydrophobic. Displacement experiments show that GA has one reactive site in BSA, that is, high affinity site (site IIA).

Acknowledgements

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