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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 609–615
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Analysis of binding interaction between puerarin and bovine serum albumin by multi-spectroscopic method

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Received 10 June 2007; received in revised form 25 August 2007; accepted 30 August 2007
Available online 8 September 2007

Abstract

The interaction of puerarin and bovine serum albumin (BSA) was investigated by means of fluorescence spectroscopy, resonance light-scattering spectroscopy, infrared spectroscopy, and synchronous fluorescence spectra. The apparent binding constants (K_a) between puerarin and BSA were 1.13×10^4 (20 °C), and 1.54×10^4 l mol^{-1} (30 °C), and the binding sites values (n) were 0.95 ± 0.02. The experimental results showed that the puerarin could be inserted into the BSA, quenching the inner fluorescence by forming the puerarin–BSA complex. The addition of increasing puerarin to BSA solution leads to the gradual decrease in RLS intensity, exhibiting the formation of the aggregate in solution. It was found that both static quenching and non-radiation energy transfer were the main reasons for the fluorescence quenching. The positive entropy change and enthalpy change indicated that the interaction of puerarin and BSA was driven mainly by hydrophobic forces. The process of binding was a spontaneous process in which Gibbs free energy change was negative. The competing binding reaction with BSA between Fe^{3+}, Cu^{2+} and puerarin was investigated. The effect of Fe^{3+} and Cu^{2+} on the binding of puerarin with BSA is discussed.

Keywords: Bovine serum albumin; Fluorescence spectroscopy; Interaction; Puerarin

1. Introduction

The interaction between bio-macromolecules and drugs has attracted great interest among researchers since several decades [1–3]. Among bio-macromolecules, serum albumin is the major soluble protein constituent of circulatory system and has many physiological functions, e.g. it serves as a depot protein and a transport protein for many exogenous compounds [4,5]. The drug–protein interaction may result in the formation of a stable protein–drug complex, which has important effect on the distribution, free concentration and the metabolism of drugs in the blood stream. Thus, the drug–albumin complex may be considered as a model for gaining fundamental insights into drug–protein interactions. Therefore, studies on the binding of drug with protein will facilitate interpretation of the metabolism and transport process of drug, and will help to explain the relationship between structures and functions of proteins. In this regard, bovine serum albumin (BSA) has been studied extensively, partly because of its structural homology with human serum albumin (HSA) [6–10].

Puerarin, 4H-1-Benzopyran-4-one,8-β-D-glucopyranosyl-7-hydroxy-3-(4-hydroxy-phenyl) (Fig. 1), is one of the major isoflavonoid compounds isolated from *Puerarina lobata* and *P. mirifica* [11], widely used in China for various purposes. It has been shown to possess anticonvulsive actions [12]; suppresses alcohol intake [13], myocardial reperfusion injury [14], and arterial hypertension [15]; and has anti-arrhythmic actions [16].

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doi:10.1016/j.jpba.2007.08.032

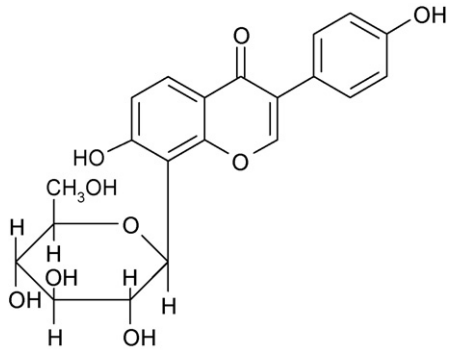

Fig. 1. Structure of puerarin.

Puerarin exerts its hypothermic and antipyretic effects by activating 5-HT_{1A} receptor and/or antagonizing 5-HT_{2A} receptors in the hypothalamus [17].

Fluorescence spectroscopy is an appropriate method to determine the interaction between the small molecule ligand and bio-macromolecules. By means of measurement and analysis of the emission peak, the transfer efficiency of energy, the lifetime, and fluorescence polarization, etc., much information may be obtained concerning the structural changes and the microenvironment surrounding the fluorophore in the macromolecule.

Resonance light-scattering (RLS), an elastic scattering, occurs when an incident beam is close to an absorption band. Pasternack et al. first established the RLS technique to study the bio-macromolecules on an ordinary fluorescence spectrometer [18,19]. RLS is a sensitive and selective technique for monitoring molecular assemblies. In recent years, RLS has been used to determine pharmaceuticals [20–22], bacteria [23], inorganic ions [24] and various bio-macromolecules [25,26]. To gain some insights into the medicinal action of puerarin, the interactions between puerarin and BSA were investigated by means of multi-spectroscopic method. Some important information such as the apparent binding constants and binding sites values were obtained.

2. Experimental

2.1. Apparatus

Fluorescence and resonance light-scattering spectra were recorded on a JASCO FP-6500 spectrofluorometer equipped with a thermostated cell compartment using quartz cuvettes (1.0 cm) (Tokyo, Japan). The UV–vis spectra were recorded on a UV-2450 spectrophotometer using quartz cuvettes (1.0 cm) (Shimadzu, Japan). The pH measurements were carried out on a PHS-3C Exact Digital pH meter equipped with Phonix Ag–AgCl reference electrode (Cole-Paemer Instrument Co.), which was calibrated with standard pH buffer solutions.

2.2. Reagents

Puerarin was obtained commercially from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). A working solution of puerarin (1.0×10^{-4} M) was prepared by dissolving it in methanol–water solution (1:1, v/v). Bovine serum albumin (fraction V) was purchased from Sigma Co. (St. Louis, MO, USA). The working solution of BSA (1.0×10^{-5} M) was prepared with tris–HCl buffer and stored in refrigerator prior to use. Tris–HCl buffer (0.20 M, pH 7.4) containing 0.10 M NaCl was selected to keep the pH value and maintain the ionic strength of the solution. All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

2.3. Fluorescence spectra

Appropriate quantities of 1.0×10^{-3} M puerarin solution were transferred to a 10 ml flask, and then 0.5 ml of BSA solution was added and diluted to 10 ml with water. The resultant mixture was subsequently ultrasonicated for 5 min and incubated at 20 °C, or 30 °C for 2 hr. The solution was scanned on the fluorophotometer in the range of 300–550 nm. The fluorescence intensity at 342.8 nm was determined under the excitation at wavelength of 280 nm. The operations were carried out at fixed temperature (20 and 30 °C).

The UV spectra were scanned in the range of 220–400 nm at room temperature.

2.4. Resonance light-scattering spectra

An appropriate aliquot of BSA working solution was added to 1.0 ml puerarin working solution, and diluted to 10 ml with water. RLS spectra were obtained by synchronous scanning in the wavelength range of 250–750 nm on the spectrofluorophotometer at room temperature.

2.5. Fourier transform infrared spectroscopy

FI-IR measurements were carried out at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (USA) equipped with a Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the attenuated total reflection (ATR) method with a resolution of 4 cm^{-1} and using 60 scans. The spectra processing procedure involved collecting spectra of the buffer solution under the same conditions. Next, the absorbance of the buffer solution was subtracted from the spectra of the sample solution to obtain the FT-IR spectra of the proteins. The subtraction criterion was that the original spectrum of the protein solution between 2200 and 1800 cm^{-1} was featureless.

3. Results and discussions

3.1. Characteristics of the fluorescence spectra

The interaction of puerarin with BSA was evaluated by monitoring the intrinsic fluorescence intensity changes of BSA upon addition of puerarin. Fluorescence quenching spectra of BSA at

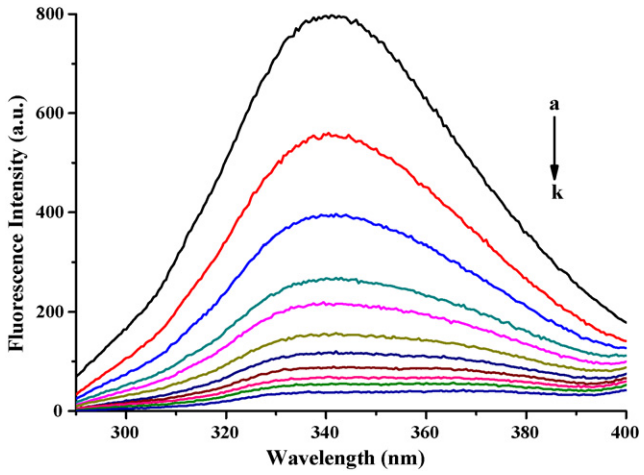

Fig. 2. The quenching effect of puerarin on BSA fluorescence intensity. λ_{ex} = 280 nm, (a–h) BSA, 1.00×10^{-6} M: 0.00, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00×10^{-5} M of puerarin.

the presence of various concentrations of puerarin are shown in Fig. 2.

As seen in Fig. 2, the addition of puerarin led to a concentration-dependent quenching of BSA intrinsic fluorescence intensity along with a slight red shift of maximum emission wavelength, implying that the binding of puerarin to BSA occurs and the microenvironment around the chromophore of BSA is changed upon addition of puerarin. With the increasing concentration of puerarin, a new fluorescence peak (λ_{max} = 477 nm) was observed. It is the same as the fluorescence emission spectra of puerarin.

Fluorescence quenching could proceed via different mechanisms, usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature. Higher temperatures will result in faster diffusion and hence larger amounts of collisional quenching and higher temperatures will typically result in the dissociation of weekly bound complexes and hence smaller amounts of static quenching. For the dynamic quenching, the mechanism can be described by the Stern–Volmer equation [7,27]:

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

where F_0 and F represent the fluorescence intensities in the absence and in the presence of quencher, K_q is the quenching rate constant of the biomolecule, K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime of the molecule without quencher and $[Q]$ is the concentration of the quencher.

To clarify the fluorescence quenching mechanism of BSA by puerarin, it was first assumed that the interaction proceeds via a dynamic way. The temperature-dependent fluorescence quenching of BSA by puerarin was then carried out. From the experimental data, the corresponding dynamic quenching constants for the interaction between puerarin and BSA were $K_{SV} = 2.52 \times 10^4\,l\,mol^{-1}$ (20 °C, $F_0/F = 1.009 + 25180\,[Q]$; $R = 0.9966$), and $K_{SV} = 2.14 \times 10^4\,l\,mol^{-1}$ (30 °C, $F_0/F = 1.002 + 21350\,[Q]$; $R = 0.9989$), respectively. Because the fluorescence life time of the biopolymer is 10^{-8} s [28–30], the quenching constants K_q at 20 and 30 °C were calculated to be 2.52×10^{12}, and $2.14 \times 10^{12}\,l\,mol^{-1}\,s^{-1}$, respectively.

According to the literature [30,31], for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is $2.0 \times 10^{10}\,l\,mol^{-1}\,s^{-1}$, and the K_{SV} increases with increasing temperature. Considering that in our experiment the rate constant of the BSA quenching procedure initiated by puerarin is much greater than $2.0 \times 10^{10}\,l\,mol^{-1}\,s^{-1}$ and that the K_{SV} decreased with increasing temperature, it can be concluded that the nature of quenching is not dynamic but probably static, resulting from the formation of puerarin–BSA complex.

3.2. Characteristics of the RLS spectra

The RLS spectra of puerarin-BSA in Tris–HCl buffer solution (0.020 M) are shown in Fig. 5. Upon addition of trace amount of puerarin to BSA solution, a remarkably decreased RLS with a maximum peak at 559.1 nm and a secondary one at 469.9 nm was observed (Fig. 3 b and a).

The production of RLS is correlated with the formation of an aggregate and the RLS intensity is dominated primarily by the particle dimension of the formed aggregate. Bearing these points in mind, it is inferred from the results that the added BSA may interact with puerarin in solution, forming a new puerarin–BSA complex that could be expected to be an aggregate. The newly formed puerarin–BSA complex may be ascribed to the higher electrostatic attraction between puerarin and BSA. The size of puerarin–BSA particles may be smaller than that of BSA, and thus the decreased light-scattering signal occurred under the given conditions. Moreover, the dimension of the resultant puerarin–BSA particles may be much less than the incident wavelength, and thus the enhanced light-scattering signal occurs under the given conditions.

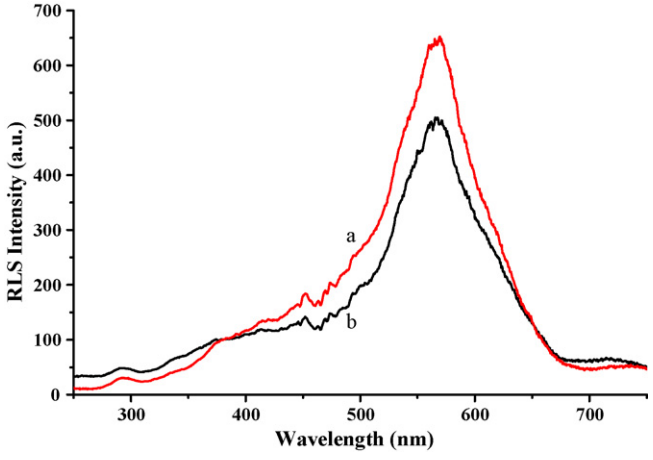

Fig. 3. RLS spectra of 1.0×10^{-6} M BSA (a), and 3.12×10^{-5} M puerarin and 1.0×10^{-6} M BSA (b).

Table 1
The binding parameters for the puerarin–BSA system

Temperature (°C)	K_a ($l\,mol^{-1}$)	n	R
20	1.13×10^4	0.93	0.9991
30	1.54×10^4	0.97	0.9991

3.3. Binding constant and binding sites

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the binding constant formula [31]:

$$lg\frac{F_0 - F}{F} = lgK_a + nlg\,[Q] \quad (2)$$

where K_a is the binding constant, and n is the number of binding sites per BSA. After the fluorescence quenching intensities on BSA at 340 nm were measured, the double-logarithm algorithm was assessed by Eq. (2).

Table 1 presents the corresponding calculated results. The apparent binding constants (K_a) between puerarin and BSA were $1.13 \times 10^4\,l\,mol^{-1}$ (20 °C, $\lg(F_0 - F)/F = 4.05225 + 0.92761 \lg[Q]$; $R = 0.9991$), and $1.54 \times 10^4\,l\,mol^{-1}$ (30 °C, $\lg(F_0 - F)/F = 4.18618 + 0.97268 \lg[Q]$; $R = 0.9991$), and the number of binding sites per BSA (n) was 0.95 ± 0.02. The data clearly showed that the number of binding sites per BSA was independent of temperature from 20 to 30 °C. The results illustrated that there is a strong binding force between puerarin and BSA. The correlation coefficients are larger than 0.9990, indicating that the interaction between puerarin and BSA agrees well with the site binding model underlying the Eq. (2). From the temperature dependence of the binding equilibrium constants, it is possible to calculate values for the thermodynamic functions involved in the binding and dissociation process. The increase in the binding constant with increase in temperature, suggested the involvement of non-ionic interactions in the binding of puerarin to BSA. The temperature may affect the diffusion coefficient and stability of the puerarin–BSA system. The increasing temperature may result in the increasing diffusion coefficient, and also it leads to the lower stability of the puerarin–BSA system. The competition of the diffusion coefficient and stability of the puerarin–BSA system with increased temperature may induce the above results.

The obtained value $n = 0.95$ corresponds to the high affinity binding sites; however the existence of the low affinity sites was not studied in this work. Recently Berezhkovskiy illustrated that the calculated number of binding sites increased with the increase of compound concentration using the measured values of unbound drug fraction [32]. When simple binding to the receptor with n sites of the same reaction is considered, and $K_d = 1/K_a$ is the dissociation binding constant (affinity of the binding site), it is necessary to have the ligand concentration roughly equal to $10 \times K_d$ to occupy 90% of the binding sites. The low affinity site (K_d about or greater than 1000 μM) was not practically occupied (compared to the binding to high affinity site) and thus was not detected at low concentration of puerarin used in the experiments. Though if the number of low affinity sites is significant (for instance 10 with $K_d = 1000$ μM), binding to them will be comparable to binding to a single high affinity site. This is because the increase of the quantity of sites leads to the increase of ligand bound to them.

3.4. Thermodynamic parameters and nature of the binding forces

The interaction between drug and biomolecule may involve hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, etc. According to the data of enthalpy change (ΔH) and entropy change (ΔS), the model of interaction between drug and biomolecule can be concluded [33]: (1) $\Delta H > 0$ and $\Delta S > 0$, hydrophobic forces; (2) $\Delta H < 0$ and $\Delta S < 0$, van der Waals interactions and hydrogen bonds; (3) $\Delta H < 0$ and $\Delta S > 0$, electrostatic interactions. In order to elucidate the interaction of puerarin with BSA, we calculated the thermodynamic parameters from Eqs. (3)–(5). If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta G = -RT \ln K \quad (3)$$

where R is the gas constant, T is the experimental temperature, and K is the binding constant at the corresponding T. Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from the Eqs. (4) and (5):

$$\ln\frac{K_2}{K_1} = \left[\frac{1}{T_1} - \frac{1}{T_2}\right]\frac{\Delta H}{R} \quad (4)$$

where K_1 and K_2 are the binding constant at the experiment temperatures T_1 and T_2, respectively.

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

The thermodynamic parameters for the interaction of puerarin with BSA are shown in Table 2. The negative sign for ΔG means that the interaction process is spontaneous. The free energy of binding of drugs to bovine serum albumin consists of two main contributions: (a) from electrostatic interactions and (b) from hydrophobic forces. The positive ΔH and ΔS values indicated that hydrophobic forces may play a major role in the binding between puerarin and BSA [34].

The characteristic of albumin to allow a variety of ligands to bind is amazing. Albumin is the principal carrier of drugs that are otherwise insoluble in the circulating blood. Several authors have demonstrated a correlation between the hydrophobic character and protein binding of low molecular

Table 2
The thermodynamic parameters of puerarin–BSA binding procedure

Temperature (°C)	ΔH ($kJ\,mol^{-1}$)	ΔG ($kJ\,mol^{-1}$)	ΔS ($J\,mol^{-1}\,K^{-1}$)
20	22.87	−22.75	155.62
30	–	−24.30	155.60

weight substances. Shaikh et al. found that hydrophobic interactions play a role in the binding of bromopyrogallol red and isothipendyl hydrochloride to BSA [1,3]. Cui et al. found that *N*-(*p*-chlorophenyl)-*N*′-(1-naphthyl) thiourea had a strong ability to quench the intrinsic fluorescence of both bovine serum albumin and human serum albumin through static quenching procedure, and the hydrophobic interaction was the predominant intermolecular force stabilizing the complex [5]. These results suggested that hydrophobic interactions played an important role in protein binding with organic compounds. Several authors have found that the emission maxima shifted with the addition of drugs to serum albumin. The blue shift of daidzein bound protein fluorescence is indicative of the role of hydrophobic interactions in the binding of this aglycone to HAS with the emission maxima shifting from 465 to 457 nm [28]. The binding of daidzein to a hydrophobic pocket in HSA may be a cause for this phenomenon.

In this study, the quenching of BSA fluorescence indicated the increase of polarity of the fluorophore environment, probably due to the hydrogen bonds between puerarin and NH_2, OH and SH groups in the protein. The native protein contains hydrophobic groups in the interior of the tertiary structure while polar groups are at the surface. The hydrophobic forces may take place by binding puerarin to a hydrophobic pocket in BSA. The hydrophobic forces also played a major role in binding phenthoate and 3,4, 5-trimethoxybenzoic acid to BSA [29,30].

3.5. Analysis of BSA conformation after puerarin binding

We had ascertained that it is the binding of puerarin to BSA to cause the fluorescence quenching of BSA, but it is still a puzzle about whether the binding affects the conformation and/or microenvironment of BSA. To further verify the binding of puerarin to BSA and investigate BSA structure after puerarin binding, the method of synchronous fluorescence and FTIR were utilized.

Synchronous fluorescence is a kind of simple and effective means to measure the fluorescence quenching and the possible shift of the maximum emission wavelength λ_{max}, relative to the alteration of the polarity around the chromophore microenvironment. $\Delta\lambda$, representing the value of difference between excitation and emission wavelengths, is an important operating parameter. As $\Delta\lambda$ is 15 nm, synchronous fluorescence offers characteristics of tyrosine residues, while when $\Delta\lambda$ is 60 nm, it provides the characteristic information of tryptophan residues. When $\Delta\lambda$ is set at 15 or 60 nm the shift of the λ_{max} and the fluorescence quenching of BSA imply the alteration of polarity microenvironment around Tyr or Trp residues and the state of puerarin binding to BSA [35]. The synchronous fluorescence spectra of interaction between puerarin and BSA are presented in Fig. 4. A weak blue shift (~1.0 nm) can be observed, and at the same time, the fluorescence intensity of BSA weakened regularly along with the adding of puerarin, implying that puerarin bonded to BSA and located in close proximity to the Tyr residues, the polarity near the tyrosine residues was decreased and so the hydrophobicity was increased obviously. When the hydrophobic part of puerarin molecule is close enough to the phenyl moiety of Tyr, hydrophobicity near the tyrosine residues

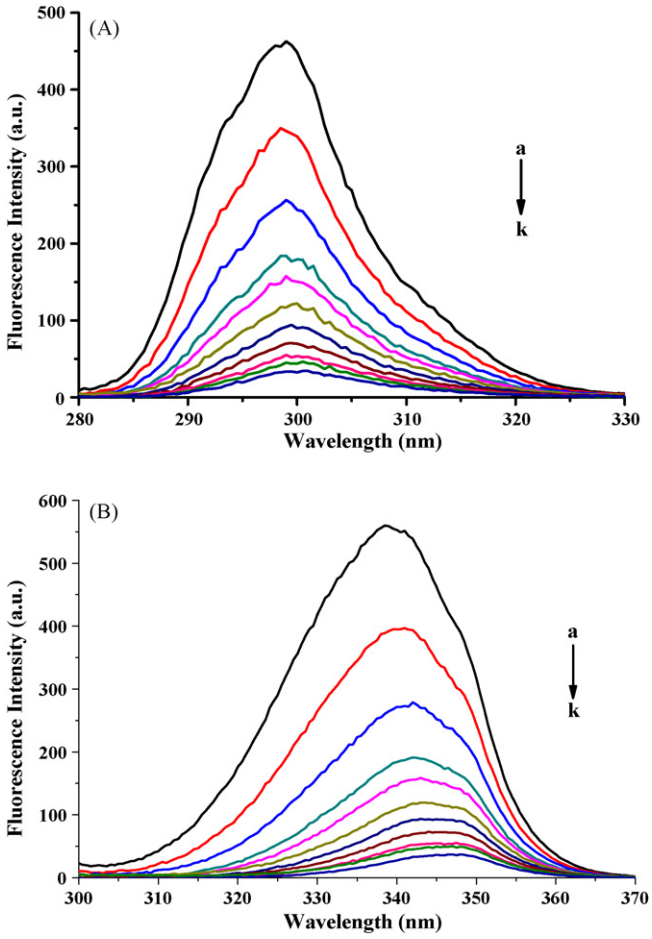

Fig. 4. Synchronous fluorescence spectra of interaction between BSA and puerarin at $\Delta\lambda$ =15 nm (A) and at $\Delta\lambda$ =60 nm (B). Concentrations of BSA was 1.00×10^{-6} M while concentrations of puerarin were 0.00, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00×10^{-5} M from the (a) to (k), (pH = 7.4, 30 °C).

increases involuntarily. An obvious shift of the λ_{max} (9.5 nm) with a gradual quenching is shown in Fig. 4B indicating that puerarin was also closed to Trp residue and had effect on the microenvironment around the Trp residue.

Fig. 5 shows the FT-IR spectra of the puerarin-free and puerarin-bound forms of BSA with their difference absorption spectrum. The spectrum in Fig. 5a was obtained by subtracting the absorption of Tris buffer from the spectrum of the protein solution. The difference spectrum in this paper (Fig. 5b) was obtained by subtraction of the spectrum of the puerarin-free form from that of the puerarin-bound form. As shown in Fig. 5, many peaks in the BSA infrared spectrum (from 1600 to 1700 cm^{-1}) were not found after puerarin binding, which also indicates that the secondary structure of the protein has been altered after puerarin binding.

3.6. Influences of common ions on binding constant

Metal ions, especially those of bivalent type, are vital to human body and playing an essentially structural role on many proteins based on coordinate bonds. The presence of metal ions

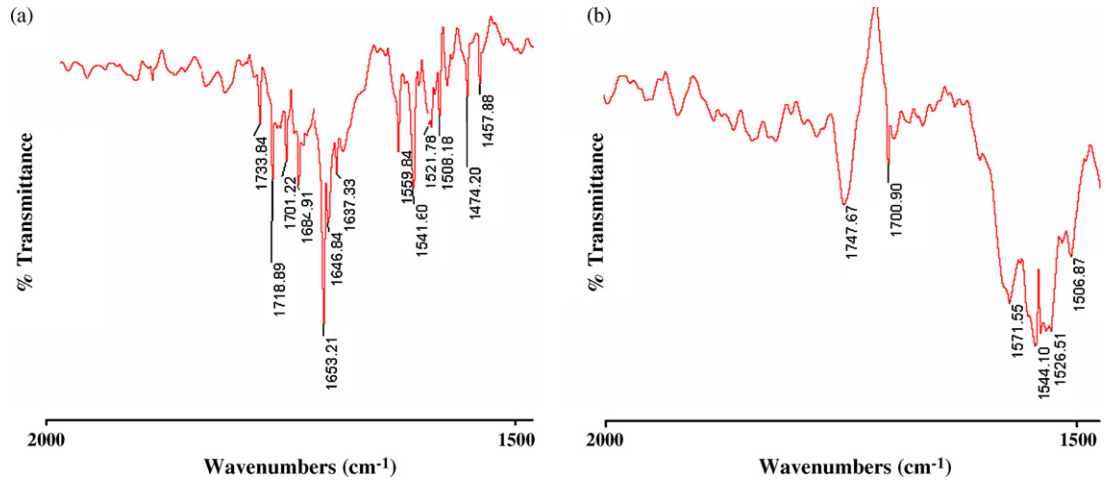

Fig. 5. FT-IR spectra and difference spectra of BSA in aqueous solution. (a) FT-IR spectrum of BSA; (b) FT-IR difference spectrum of BSA obtained by subtracting the spectrum of the puerarin-free form from that of the puerarin-bound form in the region of 1780–1380 cm^{-1} at physiological pH (BSA: 1.0×10^{-6} M; puerarin: 1.56×10^{-5} M).

Table 3
Binding constants K_a and the number of binding sites per BSA, n, in the presence of various metal ions at 30 °C

	K_a ($l\,mol^{-1}$)	n	R^2
Puerarin–BSA	$1.54 \times 10^4 \pm 5.11$	0.95	0.9991
Puerarin–BSA–Cu^{2+}	$6.44 \times 10^5 \pm 1.96$	1.30	1
Puerarin–BSA–Fe^{3+}	$2.44 \times 10^5 \pm 2.67$	1.24	1

$C_{BSA} = C_{Cu^{2+}} = C_{Fe^{3+}} = 1.0 \times 10^{-6}$ M.

in plasma may affect interaction of drugs with BSA. Effects of common bivalent metal ions (e.g. Cu^{2+} and Fe^{3+}) on binding constants of puerarin–BSA complex were investigated at 30 °C. Cu^{2+} and Fe^{3+} (1.0×10^{-6} M) decrease the fluorescence intensity of puerarin–BSA systems, but these two metal ions do not affect the fluorescence intensity of puerarin. This possibly resulted from the formation of metal ion–BSA complexes, which also quench BSA fluorescence. The values of binding constant K_a and the number of binding sites per BSA acquired in the present of metal ions are listed in Table 3.

It can be seen from Table 3 that the presence of Cu^{2+} and Fe^{3+} ions increased the binding constants and binding sites of puerarin–BSA complex. The higher binding constant possibly results from the formation of metal ion–puerarin complexes via metal ion bridge. The larger stability of puerarin–BSA complex is due to the larger number of binding sites per BSA (n). This may prolong storage period of puerarin in blood plasma and enhance its maximum effects.

4. Conclusion

Albumin represents 52–60% of the total plasmatic protein content and plays an important role in transport drugs mostly through the formation of non-covalent complexes at specific binding sites, actuating in the regulation of their plasmatic concentrations. Most drugs bind reversibly to a number of binding sites on albumin, and there is evidence of conformational changes in protein induced by its interaction with low molecular weight drugs. These changes appear to affect the secondary and tertiary structure of albumin.

Qi et al. studied the non-covalent binding between puerarin and HSA and α_1-acid glycoprotein (AAG) by measuring the molecular weight of puerarin, HSA, AAG and their complexes by ESI-MS [36]. The binding constant between puerarin and HSA was $7.59 \times 10^4\,l\,mol^{-1}$ according to the Scatchard equation. This data is similar to our report $1.54 \times 10^4\,l\,mol^{-1}$. Qi et al. calculated the thermodynamic parameters at very high temperature (170–190 °C). However, puerarin decomposes at 187 °C. So the obtained thermodynamic parameters did not reflect the binding nature.

The binding interactions of puerarin with BSA in dilute aqueous solution were studied using fluorescence spectra, resonance light-scattering spectra, infrared spectroscopy, and synchronous fluorescence spectra. The apparent binding constants (K_a) between puerarin and BSA were $1.13 \times 10^4\,l\,mol^{-1}$ (20 °C), and $1.54 \times 10^4\,l\,mol^{-1}$ (30 °C), and the number of binding sites per BSA (n) were 0.95 ± 0.02. The positive entropy change and the enthalpy change indicated that the interaction of puerarin and BSA was driven mainly by hydrophobic forces. The process of binding was a spontaneous process in which Gibbs free energy change was negative.

Acknowledgments

The authors are grateful for financial support from the Natural Science Research Project of Nantong University (grant no. 06Z021) and Natural Science Basic Research Project of Jiangsu Province University (grant no. 05KJD430163 and BK2006545).

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