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Spectroscopic studies on binding of shikonin to human serum albumin

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Abstract

The binding properties on shikonin to human serum albumin (HSA) have been studied for the first time using fluorescence spectroscopy in combination with UV-visible absorbance spectroscopy, Fourier transform infrared (FT-IR) spectroscopy and circular dichroism (CD) spectroscopy. The results of spectroscopic measurements suggested that the hydrophobic interaction is the predominant intermolecular force stabilizing the complex, which is in good agreement with the results of molecule modeling study. And the enthalpy change ΔH° and the entropy change ΔS° were calculated to be $-13.86\,\mathrm{kJ\,mol^{-1}}$ and $51.16\,\mathrm{J\,mol^{-1}\,K^{-1}}$ according to the Vant'Hoff equation. The fluorescence quenching mechanism and the number of binding site ($n \approx 1$) were also obtained from fluorescence titration data. The efficiency of Förster energy transfer provided a distance of 2.12 nm between tryophan and shikonin binding site. The alterations of protein secondary structure in the presence of shikonin in aqueous solution were quantitatively calculated from FT-IR and CD spectroscopy with reductions of α helices content about 2.8–5.4% and with increases of β structures about 2.4%. In addition, the effect of common ions on the binding constants of shikonin–HSA complexes was also discussed.

Keywords: Shikonin; Human serum albumin; Fluorescence quenching; Fourier transform IR; Circular dichroism spectroscopy; Molecular modeling

1. Introduction

A full understanding of the modes of drugs' action requires the study of their interactions with all possible chemical and biological targets, including amino acids, hormones, peptides and proteins. Studies based on drugs–protein interactions improve the armory of molecular tools for detecting and manipulating the biological roles of biomacromolecules. Such studies also crucially determine the bioavailability and toxicology of any injected drug. Human serum albumin (HSA), serving as the major soluble protein constituents of the circulatory system, is the most abundant protein in the blood plasma and has many physiological functions. It contributes significantly to colloidal osmotic blood pressure and many transport and regulatory processes. Crystal structure analysis has revealed that HSA is a globular protein composed of a single polypeptide chain of 585 amino acid residues. It consists of three homologous domains. Each domain can be divided into two subdomains A and B, which are formed from six and four α-helical, respectively. HSA is characterized by high α-helical content and a large number of disulfide bonds. Despite very high stability, HSA is a flexible protein with the 3D structure susceptible to environmental factors such as temperature, pH, etc. [1]. It is the typical site of coordination for a wide variety of endogenous and exogenous substances such as amino acid, fatty acids, hormones and foreign molecules such as drugs. The majority of these drug-binding studies involving HSA have shown that the distribution, free concentration and the metabolism of various drugs can be significantly altered as a function of their binding constants to HSA [2,3]. Nowadays, many researches on the binding of drugs to HSA have been carried out, but seldom report on the interaction of protein with the main component of Chinese herb medicine. Shikonin (Fig. 1, 1,4-naphthalenedioe, 5,8-dihydroxy-2(1-hydroxy-4-methy3-pentenyl)), one of the active components isolated

Abbreviations: HSA, human serum albumin; UV, ultraviolet visible absorbance spectroscopy; FT-IR, Fourier transform infrared spectroscopy; CD, circular dichroism

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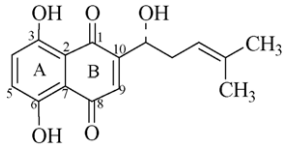

Fig. 1. The chemical structure of shikonin.

from the root of Arnebia euchroma (Royle) Johnst, has been used in traditional Chinese medicine for over 1000 years[4]. Multiple pharmacological actions have been attributed to shikonin, e.g. anti-inflammatory [5], antigonadotropic [6]. And inhibition of thyroid and pituitary hormone effects has been measured [7,8]. It also displays anti-HIV-1 activity [9]. The various reported mechanisms of action for shikonin led us to investigate its interaction with HSA, so it can provide a molecular basis for elucidating the mechanism of drug acting and predicting unfavorable drug–protein interaction.

In previous works [10], we reported optical spectroscopic studies on the interaction of active components in Chinese herbs with HSA. This study is designed to examine the influence of shikonin on the solution structure of HSA at different temperatures under physiological pH conditions. Attempts were made to investigate the binding mechanism between shikonin and HSA regarding the binding constants, the binding sites, the thermodynamic functions and the effect of on the protein secondary structure. The partial binding parameters of the reaction were calculated using SGI FUEL workstations. These are the first spectroscopic results on shikonin–HSA interactions, which illustrate the nature of shikonin–protein complications.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA, fatty acid free <0.05%) was purchased from Sigma Chemical Company. All HSA solutions were prepared in pH 7.40 buffer solution, and HSA stock solution was kept in the dark at 4 °C. Shikonin (analytical grade) was obtained from the National Institute for Control of Pharmaceutical and Bioproducts, China. The stock solution was prepared in methanol. NaCl (analytical grade, 1.0 mol L^{-1}) solution was used to maintain the ion strength at 0.1. Buffer (pH 7.40) consists of tris (0.2 mol L^{-1}) and HCl (0.1 mol L^{-1}), and the pH was adjusted to 7.40 by 0.5 mol L^{-1} NaOH when the experiment temperature was higher than 296 K. The pH was checked with a suitably standardized pH meter. The common ions were prepared at a concentration of 20 μg/ml. All reagents were of analytical reagent grade and distilled water was used throughout the experiment.

2.2. Apparatus and methods

Fluorescence spectra were measured with a RF-5301PC spectrofluorophotometer (Shimadzu), using 5 nm × 5 nm slit widths. The emission spectra were recorded between 300 and 500 nm (excitation wavelength 280 nm). Fluorescence titration experiments: 3.0 ml solution containing appropriate concentration of HSA was titrated manually by successive addition of a 1.0×10^{-3} mol L^{-1} methanol stock solution of shikonin (to give a final concentration of 3.3×10^{-6} to 3.0×10^{-5} mol L^{-1}) with trace syringes, and the fluorescence intensity was measured (excitation at 280 nm and emission at 341 nm). All experiments were measured at three temperatures (296, 303, and 310 K). The temperature of sample was kept by recycle water throughout experiment. The data thus obtained were analyzed by using the Scatchard and Stern–Volmer equations to calculate the binding constants.

The common ions were prepared according to the handbook on the preparations of standard ions. The methods of fluorescence titration experiments were used to determine the binding constants according to the modified Stern–Volmer equation (excitation at 280 nm and emission at 341 nm, 296 K, tris–HCl buffer, pH 7.40). Then, collected the data of binding constant obtained from modified Stern–Volmer equation without addition of common ions, it is used to compare the values of binding constant with addition of common ions between the protein and shikonin.

The absorbance spectra of the shikonin–HSA with concentrations of shikonin from 0 to 2.0×10^{-5} mol L^{-1} were recorded on a Cintra-10 e UV–vis spectrometer (GBC, Australia). The range of wavelength is from 190 to 500 nm.

FT-IR measurements were made at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (America) equipped with a Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the ATR method with resolution of 4 cm^{-1} and 60 scans. Spectra processing procedures: spectra of buffer solution were collected at the same condition. Then, subtract the absorbance of buffer solution from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectrum of protein solution between 2200 and 1800 cm^{-1} was featureless [11]. In this study Fourier self-deconvolution and secondary derivative were applied to these two ranges respectively to estimate the number, position and width of component bands in the region of 1500–1725 cm^{-1}. Based on these parameters curve-fitting process was carried out by Galactic Peaksolve software (Version 1.0) to get the best Gaussian-shaped curves that fit the original protein spectrum.

Circular dichroism (CD) measurements were carried out on a Jasco-20 automatic recording spectropolarimeter (Japan) in a cell of path length 2 mm at room temperature. The induced ellipticity was obtained by the ellipticity of the drug–HSA mixture subtracting the ellipticity of drug at the same wavelength and is expressed in degrees. The results are expressed as mean residue ellipticity (MRE) in deg cm^2/dmol, which is defined as [MRE θ_{obs} (m deg)/10*nl* Cp]. The θ_{obs} represents the CD in millidegree, *n* is the number of amino acid residues (585), *l* is the path length of the cell and Cp is the mole fraction. The

a-helical content of HSA was calculated from the MRE value at 208 nm using the equation α% helix = [(MRE208-4000)/33000-4000] × 100 [12].

3. Results and discussion

3.1. Fluorescence quenching studies of HSA by shikonin

The effect of shikonin on HSA and the conformation changes of HSA were evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of shikonin. Fig. 2 shows the fluorescence emission spectra of HSA with the addition of different concentrations of shikonin. HSA shows a strong fluorescence emission with a peak at 341 nm at λ_{ex} 280 nm due to its single tryptophan residue (Trp-214), while shikonin was almost non-fluorescent under the present experiment conditions. It can be seen that addition of shikonin to HSA leads to a significant reduction in the fluorescence intensity with a slight shift of emission to a shorter wavelength from 341 to 333 nm, indicating that the binding of shikonin to HSA quenches the intrinsic fluorescence of the single tryptophan in HSA (Trp-214). It also implied that the conformational changes are induced in HSA by shikonin under the conditions.

The fluorescence quenching data are analyzed by the Stern–Volmer equation [13]:

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

where F_0 and F are the fluorescence intensities in the absence and presence of shikonin, respectively. [Q] is the concentration of the quencher, and K_{SV} is the Stern–Volmer dynamic quenching constant. The possible quenching mechanism can be interpreted by the Stern–Volmer curves. Fig. 3 shows the

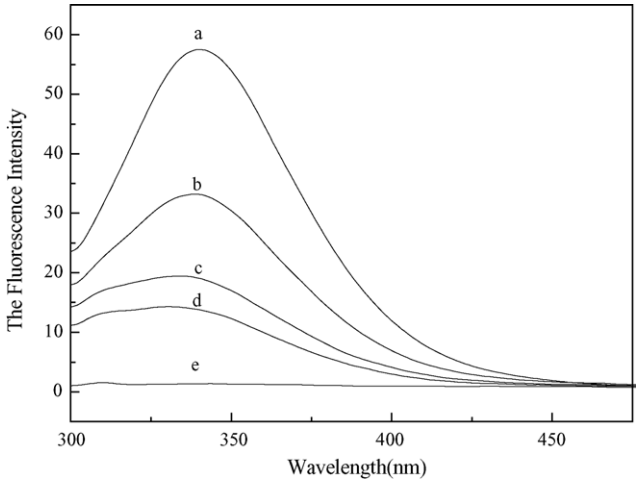

Fig. 2. The fluorescence spectra of shikonin–HSA system. The concentration of HSA was 3.0×10^{-6} mol L^{-1} while the shikonin concentration corresponding to 0, 3.0, 6.67, 13.33×10^{-6} mol L^{-1} from the (a) to (d); (e) [shikonin] = 3.0×10^{-6} mol L^{-1}; λ_{ex} = 280 nm, T = 296 K; pH 7.40; tris buffer.

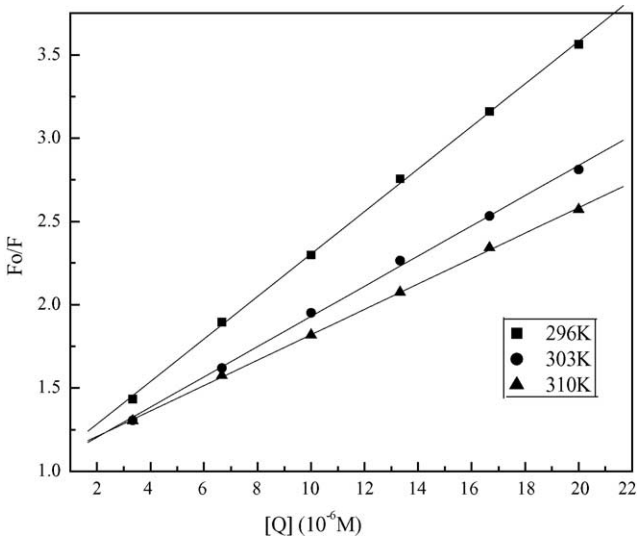

Fig. 3. The Stern–Volmer plot of HSA quenched by shikonin. [HSA] = 3.0 μM, λ_{ex} = 280 nm, λ_{em} = 341 nm, pH 7.40, tris buffer.

Stern–Volmer quenching plots of shikonin with HSA at different temperatures (296, 303, and 310 K). There is a linear dependence between F_0/F and Q and the slopes decrease with increasing temperature, which is indicative of the homogeneity of static quenching. Static quenching arises from the formation of a dark complex between the fluorophore and quenching agent. Therefore, the quenching of HSA fluorescence by shikonin depends on the formation of the new complex of shikonin and HSA.

3.2. CD spectra, UV absorption spectra and FT-IR spectra

To ascertain the possible influence of shikonin binding on the secondary structure of HSA, we have performed CD studies in the presence of same concentrations of Shikonin. Fig. 4 shows the CD spectra of HSA, which exhibited characteristic features of the typical (α + β) helix structure of the free HSA and its shikonin complex with negative bands at 208 and 220 nm. The binding of shikonin to HSA caused a decrease in band intensity without any significant shift of the peaks, indicating a decrease of the α-helical content in protein. From the above results, it is apparent that the effect of shikonin on HSA causes a conformational change of the protein, with the loss of α-helical stability. The calculated results exhibited a reduction of α-helix structures from 47.12 to 41.70% at molar ratio of shikonin/HSA of 2:1.

Fig. 5 shows the UV absorption spectra of HSA in the absence and presence of shikonin. The absorption of HSA (about 210 nm) represents the content of α-helix structure of HSA [14]. As can be seen in Fig. 5, HSA has strong absorbance with a peak at 209 nm and the peak intensity increased with the addition of shikonin. Meanwhile, the formation of chromophore of shikonin–HSA results in the distinct shift of shikonin–HSA spectrum towards longer

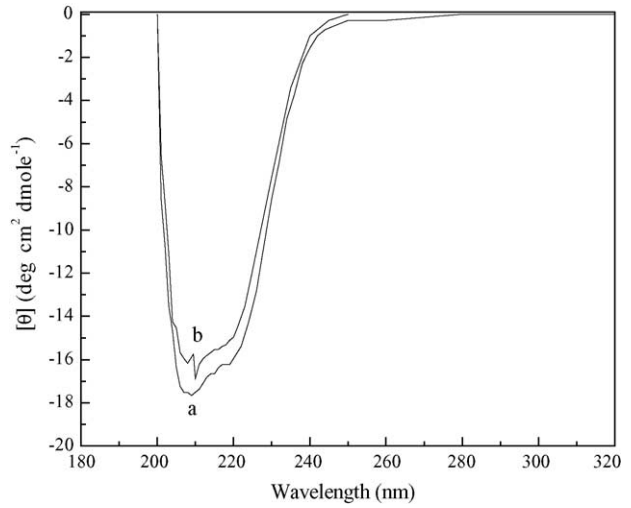

Fig. 4. CD spectra of the HSA–shikonin system. (a) 3.0×10^{-6} mol L^{-1} HSA; (b) 3.0×10^{-6} mol L^{-1} HSA + 6.0×10^{-6} mol L^{-1} shikonin. T = 296 K, tris buffer, pH 7.40.

wavelength. The above two evidences clearly indicated the interaction between shikonin and HSA, inducing the change of α-helix structure of protein.

Additional evidence regarding the shikonin–HSA complications comes from the FT-IR spectroscopy of the drug–protein complexes. Since infrared spectra of proteins exhibit a number of so-called amide bands, which represent different vibrations of the peptide moieties. Of all the amide modes of the peptide group, the amide I is most widely used one in studies of protein secondary structure. This vibration mode originates from the C=O stretching vibration of the amide group (coupled to the in-phase bending of the N–H bond and the stretching of the C–N bond) and gives rise to infrared bands in the region between approximately 1600 and 1700 cm^{-1} [15]. Fig. 6 shows the FT-IR spectra of the shikonin-free and shikonin-bound form of HSA. From Fig. 6, we concluded that the secondary structure of HSA is changed because the peak positions of amide I band (1645.5 cm^{-1}) and amide II band (1543.6 cm^{-1}) in the HSA infrared spectrum has evident shifts and their peak shapes are also changed. Fig. 7 showed a quantitative analysis of the protein secondary structure of HSA before and after the interaction with shikonin in tris buffer. The free protein contained major amounts of α-helices (55%), β-sheets (22%), β-turn structures (11%), and β-antiparallels (12%) [16]. The data obtained from Fig. 7 suggested that upon shikonin–HSA complexes, the α-helix structures were reduced from 54.4 to 51.6%, β structures increased from 44.1 to 46.6%.

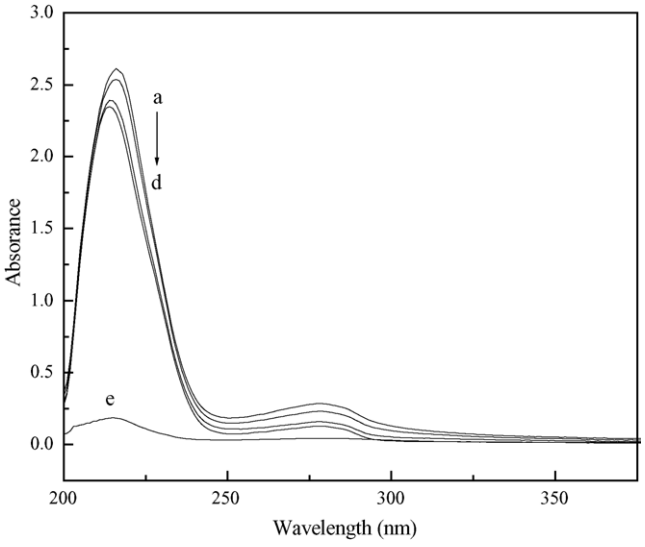

Fig. 5. UV absorption spectra of HSA in tris buffer solution (pH 7.40, 296 K) in presence of different shikonin concentrations from (a) to (d). (a) 1.33×10^{-5} mol L^{-1}, (b) 6.67×10^{-6} mol L^{-1}, (c) 3.33×10^{-6} mol L^{-1}, (d) 0. [HSA] = 3.0×10^{-6} mol L^{-1}; (e) the spectra of shikonin, [shikonin] = 3.0×10^{-6} mol L^{-1}.

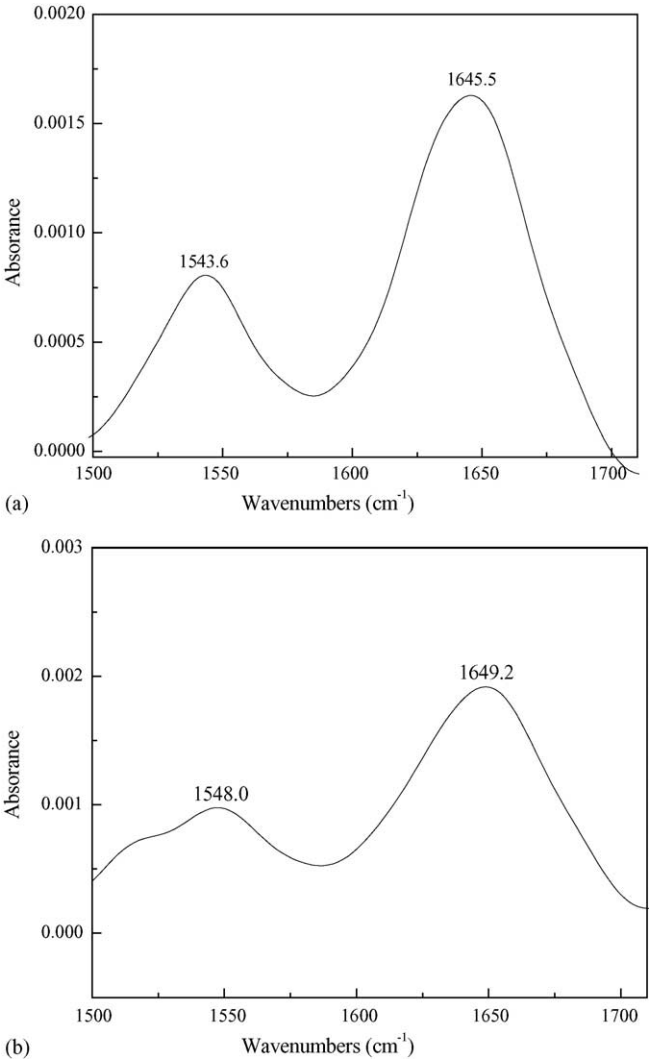

Fig. 6. FT-IR spectra and different spectra of HSA in aqueous solution: (a) FT-IR spectrum of HSA; (b) FT-IR difference spectrum of HSA obtained by subtracting the spectrum of the shikonin-free form from that of the shikonin-bound form in the region of 1725–1500 cm^{-1} at 296 K tris buffer (pH 7.40) ([HSA] = 3.0×10^{-6} mol L^{-1}; [shikonin] = 6.0×10^{-6} mol L^{-1}).

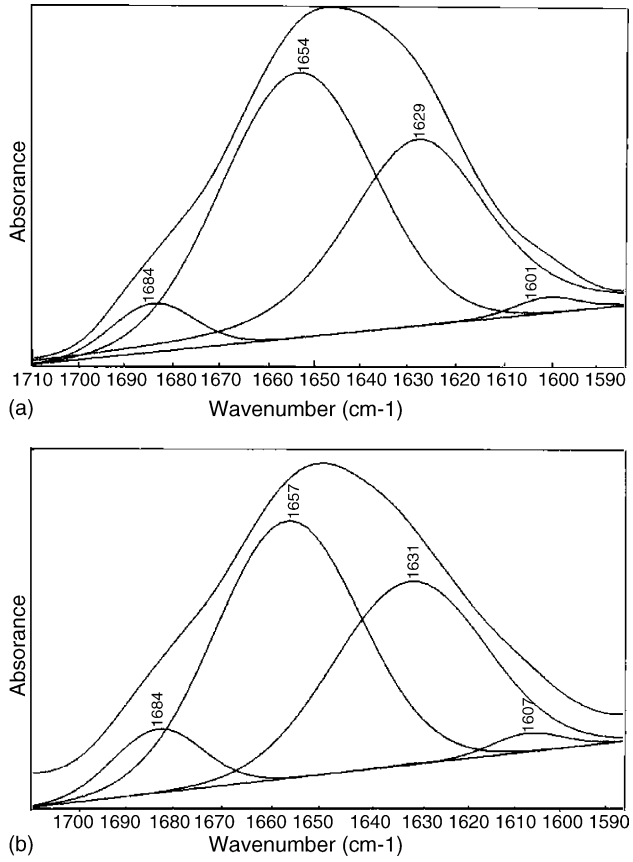

Fig. 7. Curve-fitted amide I (1700–1600) regions of free HSA (a) and shikonin–HSA (b) in buffer solution with [shikonin]/[HSA] = 2:1. T = 296 K, tris buffer, pH 7.40.

3.3. Binding constant and number of binding sites

In drug–protein binding studies, several equations have been used for binding constant calculation. One equation frequently used is the Scatchard equation [17]:

$$\frac{r}{D_f} = nK - rK \tag{2}$$

where r is the number of mol of bound drug per mol of protein, D_f is the concentration of unbound drug, K and n are the binding constant and number of binding sites, respectively.

Quenching data were also analyzed according to the modified Stern–Volmer equation [18]:

$$\frac{RF_0}{\Delta RF} = \frac{1}{[Q]}\frac{1}{fK} + \frac{1}{f} \tag{3}$$

where RF_0 and ΔRF are the relative fluorescence intensities of protein in the absence and presence of quencher, respectively. f is the fractional maximum fluorescence intensity of protein summed up and K is a constant. The dependence of $RF_0/\Delta RF$ on the reciprocal value of the quencher concentration $1/[Q]$ is linear with the slope equal to the value of $(fK)^{-1}$. The value $1/f$ is fixed on the ordinate. Association constant K is a quotient of an ordinate $1/f$ and slope $(fK)^{-1}$.

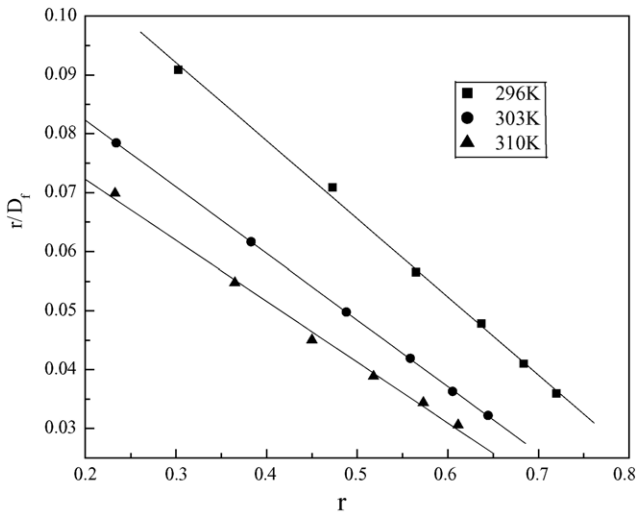

Fig. 8. The Scatchard plot for the shikonin–HSA at tris buffer (pH 7.40). [HSA] = 3.0×10^{-6} mol L^{-1}, [shikonin] = 3.33×10^{-6} to 2.0×10^{-5} mol L^{-1}, λ_{ex} = 280 nm, λ_{em} = 341 nm.

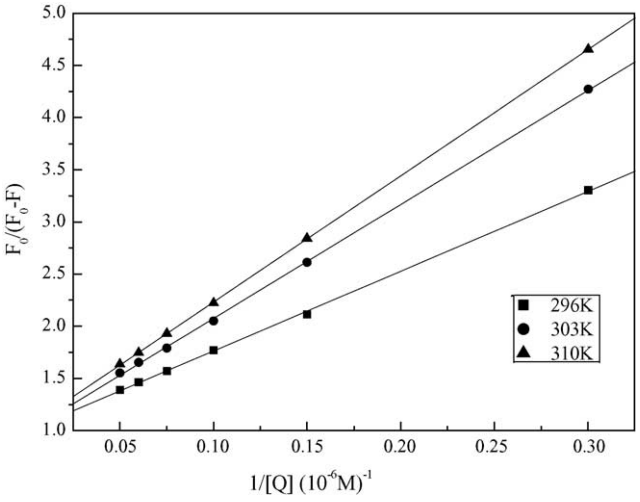

Fig. 9. The modified Stern–Volmer for the shikonin–HSA at tris buffer (pH 7.40). [HSA] = 3.0×10^{-6} mol L^{-1}, [shikonin] = 3.33×10^{-6} to 2.0×10^{-5} mol L^{-1}, λ_{ex} = 280 nm, λ_{em} = 341 nm.

Figs. 8 and 9 show the Scatchard plot and the Stern–Volmer plot for the shikonin–HSA system at different temperatures, respectively. In Table 1 the binding constants obtained for the different methods are listed for shikonin associated with HSA. The Scatchard plots obtained from

Table 1
Binding parameters for shikonin–HSA system (296 K, tris buffer, pH 7.40)

Temperature (K)	Binding parameters		
	Modified Stern–Volmer method	Scatchard method	
	K ($\times 10^5$)	K ($\times 10^5$)	n
296	1.307	1.326	0.994
303	1.136	1.130	0.928
313	1.067	1.034	0.899

this procedure showed a straight line for different temperatures tested. The linearity of Scatchard indicates that shikonin binds to one class of sites on HSA, which was in full agreement with the number of binding site n; and the binding constants K decreased with the increasing temperature. It is shown that the binding between shikonin and HSA is very strong, and the temperature has an effect on it.

3.4. Binding mode

The molecular forces contributing to protein interactions with small molecular substrates may be a Van' der Waals interaction, hydrogen bonds, ionic, electrostatic and hydrophobic interactions, etc. The signs and magnitudes of thermodynamic parameters for protein reactions can be accounted for the main forces contributing to protein stability. Because the temperature effect is very small, the reaction enthalpy change can be regard as a constant if the temperature range is not too wide. Therefore, from the Vant'Hoff equation:

$$\ln K = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (4)$$

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

where K is the Stern–Volmer dynamic quenching constant at corresponding temperature and R is the gas constant, the enthalpy change (ΔH°) is calculated from the slope. The free energy change ΔG° is estimated from Eq. (5). According to the binding constants K_{SV} at the three temperatures, the thermodynamic parameters were determined from linear Vant'Hoff plot (Fig. 10) and are presented in Table 2. As shown in Table 2, ΔH° and ΔS° for the binding reaction between shikonin and HSA are found to be $-13.86\,\mathrm{kJ\,mol^{-1}}$ and $51.16\,\mathrm{J\,mol^{-1}\,K}$. Thus, the formation of shikonin–HSA coordination compound is an exothermic reaction accompanied

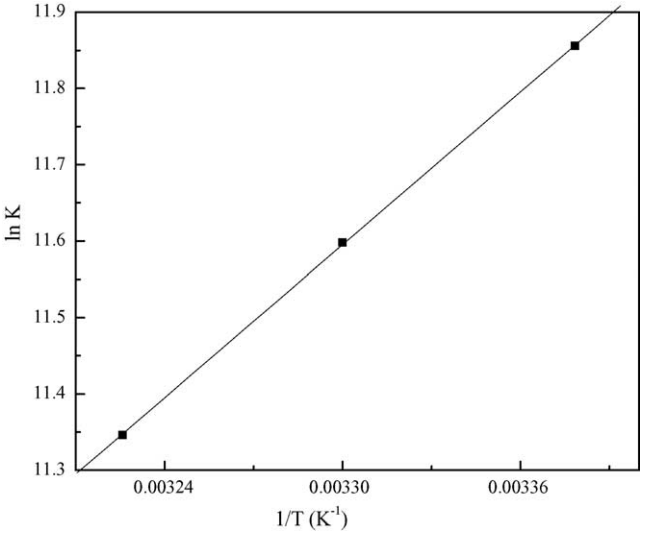

Fig. 10. Vant'Hoff plot for the interaction of HSA and shikonin in tris buffer, pH = 7.40.

Table 2
Thermodynamic parameters of shikonin–HSA interaction at pH 7.4 (tris buffer)

Temperature (K)	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K)
296	−29.00	−13.86	51.16
303	−29.36		
310	−29.72		

by positive ΔS° value. Ross and Subramanian [19] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes. From the point of view of water structure, a positive ΔS° value is frequently taken as evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS° and a negative ΔH° value. Accordingly, it is not possible to account for the thermodynamic parameters of shikonin–HSA coordination compound on the basis of a single intermolecular force model. It is more likely that hydrophobic, electrostatic interactions are involved in its binding process. For the shikonin–HSA system, the shikonin is unionized under the experimental conditions (pH 7.40, pK_a value is 9.15). Hence, electrostatic interactions can be precluded from the binding process. Thus shikonin-bound to HSA is mainly based on the hydrophobic interaction.

3.5. Molecular modeling

The complementary application of molecular modeling by computer methods has been employed to improve the understanding of the interaction of shikonin and HSA. Descriptions of 3D structure of crystalline albumin have revealed that HSA comprises of three homologous domains (I–III): I (residues 1–195), II (196–383), III (384–585), each domain is a product of subdomains that posses common structural motifs. The principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA, which are consistent with sites I and II, respectively. One tryptophan residue (Trp-214) of HSA is in subdomain IIA [1]. The crystal structure of HSA was taken from the Brookhaven Protein Data Bank (entry codes 1h9z). The potential of the 3D structure of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structure of all the molecules was generated by molecular modeling software Sybyl 6.9. The geometries of these compounds were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. FlexX program was applied to calculate the possible conformation of the ligands that binds to the protein. The conformer with RMS (root-means-square) was used for further analysis. Based on this kind of approach, a computational model of the target receptor has been built, by which partial binding parameters of the shikonin–HSA system were calculated through SGI FUEL workstations. The best energy ranked results is shown in Fig. 11, on which the naphthalenedioe moiety is located within the binding pocket

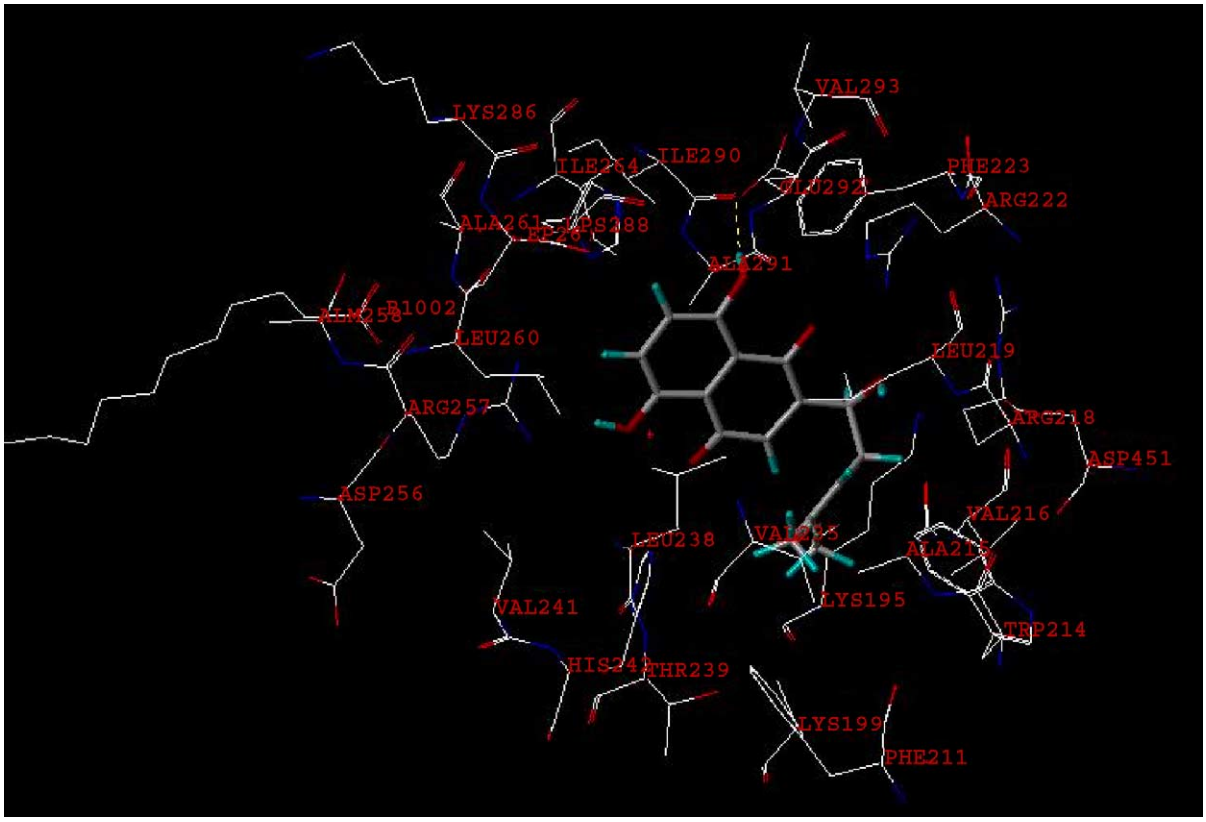

Fig. 11. The binding mode between shikonin and HSA. The residues of HSA are represented using line and the shikonin structure is represented using ball and stick model. The hydrogen bond between shikonin and HSA is represented using dashed line.

and both rings are practically coplanar. It is important to note that the trptophan residue (Trp214) and the lysine residue (Lys195) of HSA are in close proximity to the pentenyl moiety of shikonin suggesting the existence of hydrophobic interaction between them. Further, this finding provides a good structural basis to explain the efficient fluorescence quenching of HSA emission in the presence of shikonin. There is also a hydrogen bond between the drug and the polar amino acid residues; 3-OH substituent of shikonin is donating hydrogen to carbonyl of the residues Ile 290 of HSA. On the other hand, the amino acid residues with benzene ring can match that of the structure of shikonin in space in order to firm the conformation of the complex. The ligand binding regions of HSA located in hydrophobic cavities in subdomains IIA are large to accommodate the shikonin. The calculated binding Gibbs free energy (ΔG°) is $-21.34\,\mathrm{kJ\,mol^{-1}}$, which is not close to the experimental data ($-29.00\,\mathrm{kJ\,mol^{-1}}$) in some degree. However, the results obtained from modeling indicated that the interaction between shikonin and HSA is dominated by hydrophobic force.

3.6. Distance measurement between tryptophan and shikonin binding site

Förster' theory of dipole–dipole energy transfer was used to determined the distances between the protein residue (donor) to the bound drug (acceptor) in HSA. By Förster' theory [20], the efficiency of energy transfer (E) is related to the distance R (Å) between donor and acceptor by

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{6}$$

R_0 is the distance (Å) at which the transfer efficiency equals to 50%, is given by the following equation:

$$R_0^6 = 8.8 \times 10^{-25} K^2 n^{-4} \Phi J \tag{7}$$

where n is the refractive index of the medium, K^2 is the orientation factor, and Φ is the quantum yield of the donor. The spectral overlap integral (J) between the donor emission spectrum and the acceptor absorbance spectrum was approximated by the following summation:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda} \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescence reagent when the wavelength is λ, $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor at the wavelength of λ. From these relationships J, E and R_0 can be calculated; so the value r, also can be calculated.

Fig. 12 shows the overlap of the fluorescence spectra of HSA and the absorption spectra of shikonin. Under

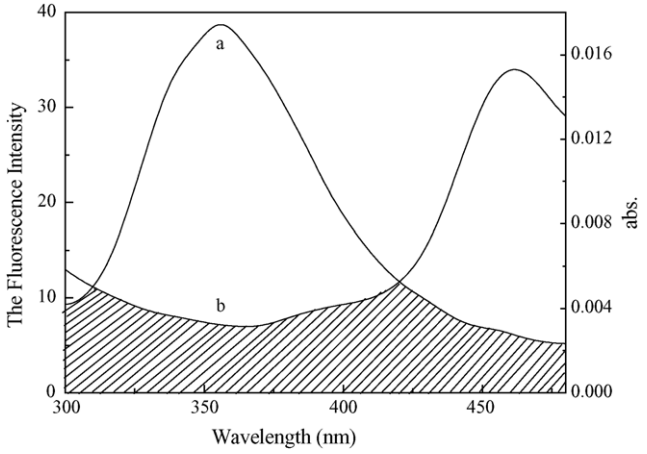

Fig. 12. Overlapping between the fluorescence emission spectrum of HSA + shikonin (a) (λ_{ex} = 280 nm) and absorption UV spectrum of the system (b). [HSA]/[shikonin] = 1:1. T = 296 K, tris buffer, pH 7.40.

these experimental conditions, it has been reported for HSA that, $K^2 = 2/3$, $\Phi = 0.118$, $n = 1.336$ [21]. So the value of the overlap integral calculated from Fig. 11 is 3.7634×10^{-14} cm^3 L mol^{-1}, and R_0 is 2.08 nm and the r is 2.12 nm, respectively. Obviously, they are lower than 7 nm after interaction between shikonin and HSA. This accords with conditions of Foster's non-radioactive energy transfer theory indicated again a static quenching interaction between shikonin and HSA.

3.7. Effect of co-ions on binding of shikonin to HSA

The multiple binding sites underlie the exceptional ability of HSA to interact with many organic and inorganic molecules and make this protein an important regulator of intercellular fluxes and the pharmacokinetic behavior of many drugs [22]. The previous studies indicated that HSA has a high-affinity metal-binding site at different regions: the Cu(II)–Ni(II) sites at N-terminus side of the protein; the Cd(II)–Zn(II) site, involving three histidines (His105, His146 and His247) and one aspartate (Asp249) residues; the single free thiol group of the Cys34 residue responsible for the binding of metal ions such as Hg(II), Au(I) and Ag(I); Mn(II) and Bi(III) ions bind to human serum albumin through strong or weaker binding sites [23,24]. In this work, we are interested in examining the effect of inorganic cations and anions on the solution system of shikonin–HSA by analyzing their different binding constants (according to the modified Stern–Volmer equation), which can only be used as a model for investigating the interaction of shikonin to HSA. Table 3 shows the results of the effect of common ions on the binding constants at 296 K. In contrast to the ions including Zn^{2+}, F^- and $SO_2{}^{4-}$, the competition between the other ions and pharmaceutical belittled the binding constant between protein and pharmaceutical, constantly decreased from 16 to 39%. As a result, the binding force between serum albumin protein and pharmaceutical also decreased, shortened the stored time of pharmaceutical in blood plasma and improved maximum reaction intensity of pharmaceutical.

Table 3
The binding constants K' (L mol^{-1}) between shikonin and HSA at 296 K (tris buffer, pH 7.40) in the presence of common ions ($K = 1.307 \times 10^5$)

Ions	K' ($\times 10^5$)	R	K'/K
Mg^{2+}	1.012	0.9994	0.763
Al^{3+}	1.111	0.9991	0.838
Ca^{2+}	0.948	0.9996	0.714
Cu^{2+}	0.799	0.9991	0.602
Fe^{2+}	0.801	0.9993	0.604
Zn^{2+}	1.408	0.9992	1.062
F^-	1.852	0.9996	1.397
Cl^-	1.101	0.9998	0.830
Br^-	0.870	0.9994	0.656
$SO_4{}^{2-}$	1.437	0.9998	1.084
$CO_3{}^{2-}$	1.109	0.999	0.836

4. Conclusions

The results of fluorescence quenching measurements and molecular modeling study suggested that shikonin could bind to HSA through the hydrophobic interaction and hydrogen bond with high affinity. The spectroscopic evidences (CD, FT-IR and UV) also showed that the secondary structure of HSA changed after shikonin-bound to HSA. The number of binding site and the binding distance between the drug and HSA was obtained based on the fluorescence and UV experiment results. It is noteworthy that the spectroscopic research described herein signifies a promising approach, exploiting the new use of Chinese medicine shikonin for probing their interactions with relevant target proteins.

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