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Effect of Chinese medicine alpinetin on the structure of human serum albumin

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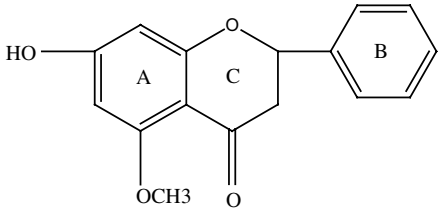
Abstract—Alpinetin (7-hydroxy-5-methoxyflavanone), one of the main constituents from the seeds of *Alpinia katsumadai* Hayata, belongs to flavonoids with its usefulness as antibacterial, anti-inflammatory and other important therapeutic activities of significant potency and low systemic toxicity. In this paper, the interaction of alpinetin to human serum albumin (HSA) has been studied for the first time by spectroscopic method including Fourier transform infrared (FT-IR) spectroscopy, circular dichroism (CD), and UV-absorption spectroscopy in combination with fluorescence quenching study under physiological conditions with drug concentrations of 3.3×10^{-6}–2.0×10^{-5} mol/L. The results of spectroscopic measurements and the thermodynamic parameters obtained (the enthalpy change ΔH^0 and the entropy change ΔS^0 were calculated to be -10.20 kJ/mol and 53.97 J/mol K^{-1} according to the Van't Hoff equation) suggest that hydrophobic interaction is the predominant intermolecular forces stabilizing the complex, which is also good agreement with the results of molecule modeling study. The alterations of protein secondary structure in the presence of alpinetin in aqueous solution were quantitatively estimated by the evidences from FT-IR and CD spectroscopy with reductions of α-helices about 24%, decreases of β-sheet structure about 2%, and increases of β-turn structure about 21%. The quenching mechanism and the number of binding site ($n\approx1$) were obtained by fluorescence titration data. Fluorescent displacement measurements confirmed that alpinetin bind HSA on site III. In addition, the effects of common ions on the constants of alpinetin–HSA complex were also discussed.

1. Introduction

Alternative medicines have become increasingly popular in the world. Flavonoids, comprising a vast family of polyphenolic secondary metabolites, exhibit a wide range of biological activities, such as antioxidant, antiviral, antibacterial, antiprotozoal and antifungal effects. They also play an important role in plant growth and defense mechanisms against infection and injury.[1] Alpinetin (Scheme 1) is a traditional Chinese medicine widely on the distribution in higher plants and prepared from their roots or seeds such as *Alpinia katsumadai* Hayata, *Amomum alnus*, *Populus*, *Polygonum* and *Scuiellaria* etc.[2] Like other flavonoid compounds, this drug is increasingly being recognized as possessing a broad spectrum of biological activities and important therapeutic applications,[3,4] including novel features, for example, as significant microbial resistance, anti-inflammatory and anti-hemostasis.[2] Another interesting aspect of flavomoids relates to its unusual fluorescence emission properties, such as dual fluorescence behavior; extraordinarily large Stokes shifts; and extreme sensitivity of the emission parameters to the surrounding medium (e.g., polarity, hydrogen bonding effects, pH, temperature, and related aspects).[5–7] In consideration of being one of the precious Chinese herbs and the possible pharmaceutical benefit led us to investigate the interaction of alpinetin with protein, so it can provide



Scheme 1. The chemical structure of alpinetin.

Keywords: Alpinetin; Human serum albumin; Fourier transform infrared (FT-IR) spectroscopy; Circular dichroism (CD); Secondary structure; Fluorescence spectroscopy.
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a molecular basis for elucidating the mechanism of the drug acting and predicting unfavorable drug interaction.

A number of biochemical and molecular biological investigations have revealed that proteins are frequently the 'targets' for therapeutically active flavonoids of both natural and synthetic origin.[8] However, very little is known about the mode of interactions of these compounds with their respective target proteins at the molecular level. Here, we have explored the interaction of alpinetin with human serum albumin (HSA). Human albumin (66.5 kDa) is the most abundant protein in blood serum with a concentration of ca. 0.63 mM. It is a single polypeptide chain of 585 amino acids with a largely-helical (≈67%) triple-domain structure that assemble to form a heart-shaped molecule. Other mammalian albumins are highly homologous with human albumin, all of which contain 17 disulfide bridges. HSA binds a number of the relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin, and bile acids and thus facilitates their transport throughout the circulation. HSA is also capable of binding a wide variety of drugs and xenobiotics.[9] 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.[10] Therefore, studying the interaction of active components in Chinese herbs with HSA has major biochemical importance and can be used as a model for elucidating the drug–protein complications.

In previous works[11] we reported optical spectroscopic studies to characterize the interaction of active components in Chinese herbs with HSA, yet the investigation on binding interaction of alpinetin and HSA has not been reported. This study is designed to examine the effect of alpinetin on the solution structure of HSA using UV–visible spectroscopic, circular dichroism (CD), Fourier transform infrared (FT-IR), and fluorescence spectroscopic methods at four temperatures under physiological conditions. Attempts were made to investigate the binding mechanism of alpinetin to HSA with respect to the binding constants, the binding sites, the thermodynamic functions, and the effect of alpinetin on the protein secondary structure. The partial binding parameters of the reaction were calculated through SGI FUEL workstations. These are the first spectroscopic results on alpinetin–HSA interactions and they can illustrate the nature of alpinetin–protein complications in vitro and in vivo.

2. Results and discussion

2.1. Binding studies using UV-absorption and CD spectrum

Figure 1 shows the UV-absorption spectra of HSA in the absence and presence of alpinetin. The absorption of HSA (about 210 nm) represents the content of α-helix structure of HSA.[12] As can be seen in Figure 1, HSA has strong absorbance with a peak at 209 nm and the absorbance of HSA increased with the addition of alpinetin; the chromophore of alpinetin–HSA gives a very specific pattern of the UV–vis spectrum with slightly dual absorbance spectra at higher concentration of alpinetin in the system, and the addition of alpinetin results in the distinct shift of alpinetin–HSA spectrum toward longer wavelength (from 278 to 287 nm). The two evidences clearly indicated the interaction between alpinetin and HSA.

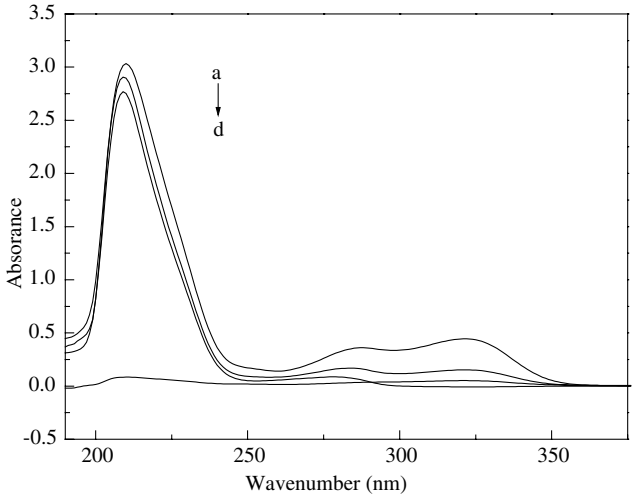

Figure 1. UV-absorption spectra of HSA in Tris buffer solution in presence of different alpinetin concentrations: (a) 3.0×10^{-5} mol/L; (b) 1.0×10^{-5} mol/L; (c) 0, [HSA] = 3.0×10^{-6} mol/L; (d) the spectra of alpinetin, 3.0×10^{-6} mol/L, 296 K.

To ascertain the possible influence of alpinetin binding on the secondary structure of HSA, we have performed CD studies in the presence of different concentrations of alpinetin. CD spectra of HSA exhibit two negative bands in the ultraviolet region at 209 and 222 nm characteristic for an α-helical structure of protein. Figure 2 is the CD spectra of HSA exhibited a characteristic of the

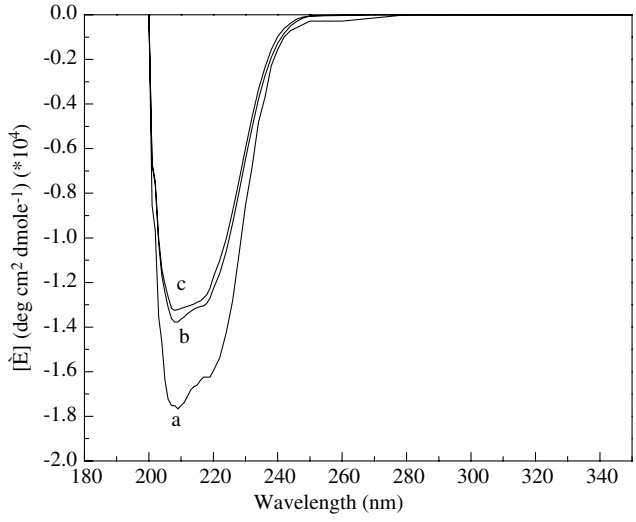

Figure 2. CD spectra of the HSA–alpinetin system: (a) 3.0×10^{-6} mol/L HSA; (b) 3.0×10^{-6} mol/L HSA + 3.0×10^{-6} mol/L alpinetin; (c) 3.0×10^{-6} mol/L HSA + 6.0×10^{-6} mol/L alpinetin.

typical (α + β)-helix structure of the free HSA and its alpinetin complex with negative bands at 208 and 217 nm. The binding of alpinetin to HSA caused a decrease in band intensity at all wavelength of the far-UV CD without any significant shift of the peaks, indicating the decrease of the α-helical content in protein. However, the CD spectra of HSA in presence and absence of alpinetin are similar in shape, indicating that the structure of HSA is also predominantly α-helical. From the above results, it is apparent that the effect of alpinetin on HSA causes a conformational change of the protein, with the loss of α-helical stability. The calculating results exhibited a reduction of α-helix structures from 47.12% to 33.85% and 32.85% at molar ratio alpinetin/HSA of 1:1 and 2:1.

2.2. FT-IR spectroscopy

Additional evidence regarding the alpinetin–HSA complications comes from FT-IR spectroscopy results obtained for drug–protein complexes. Since infrared spectra of proteins exhibit a number of the so-called amide bands, which represent different vibrations of the peptide moiety. Of all the amide modes of the peptide group, the single most widely used one in studies of protein secondary structure is amide I. 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}.[13] Figure 3 is the FT-IR spectra of the alpinetin-free and alpinetin-bound form of HSA with its difference absorption spectrum. From Figure 3, we concluded that the secondary structure of HSA is changed because the peak position of amide I band (1645.4 cm^{-1}) and amide II band (1544.1 cm^{-1}) in the HSA infrared spectrum has evident shifts and their peak shapes are also changed. Figures 4 and 5 showed a quantitative analysis of the protein secondary structure of HSA before and after the interaction with alpinetin in Tris buffer. The free protein contained major amounts of α-helices (55%), β-sheets (22%), β-turn structures (11%), and β-antiparallels (12%).[14] The data obtained from Figure 5 suggested that upon alpinetin–HSA complexes, the α-helix structures were reduced from 54.4% to 30.3%, β-sheet decreased slightly from 39.2% to 36.9%, and β-turn structures increased from 6.4% to 26.7%.

2.3. Analysis of fluorescence quenching of HSA by alpinetin

Fluorescence quenching of the single tryptophan residue in HSA was used to measure drug-binding affinity. Tryptophan fluorescence is the most frequently examined among the most frequently examined among the three intrinsic aromatic fluorophores in HSA molecules to obtain information about conformational changes. The interaction of alpinetin to HSA and the conformation changes in HSA were evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of alpinetin. Figure 6 shows that addition of alpinetin causes a dramatic change in the fluorescence

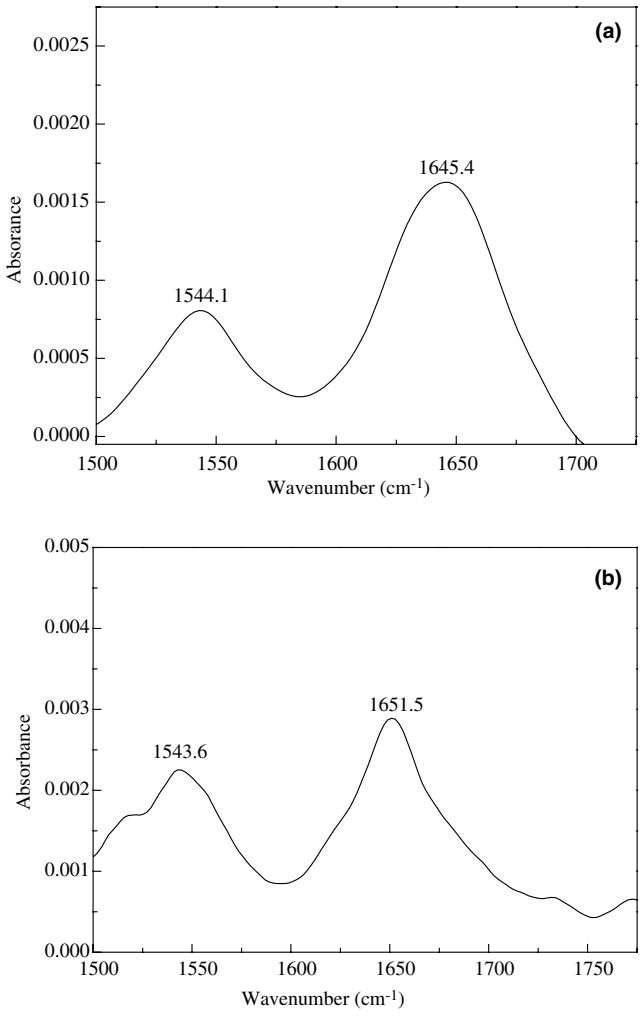

Figure 3. FT-IR spectra and different spectra of HSA in aqueous solution: (a) FT-IR spectrum of HSA; (b) FT-IR difference spectrum of HSA obtained by subtracting the spectrum of the alpinetin-free form from that of the alpinetin-bound form in the region of 1775–1500 cm^{-1} at physiological pH ([HSA]: 3.0 μM; [alpinetin]: 3.0 μM).

emission spectra. HSA has a strong fluorescence emission with a peak at 337 nm on excitation at 280 nm. With gradual increase in drug concentrations, we observe typical dual fluorescence behavior. The fluorescence intensity of protein decreases in the presence of alpinetin and the maximum emission wavelength were shifted from 337 to 348 nm, suggesting that the microenvironment around HSA is changed after the addition of alpinetin. For alpinetin, it caused a concomitant increase in the fluorescence emission at 431 nm, which is the characteristic wavelength of the bound HSA. This phenomenon might be the result of the radiationless energy transfer between alpinetin and HSA. Moreover, the occurrence of an isoactinic point at 378 nm might also indicate the existence of bound and free alpinetin in equilibrium.

It is well known that the fluorescence of HSA comes from the tyrosine, tryptophan, and phenylalanine residues. According to Miller,[15] with large $\Delta\lambda$ values such as 60 nm, the synchronous fluorescence of HSA is

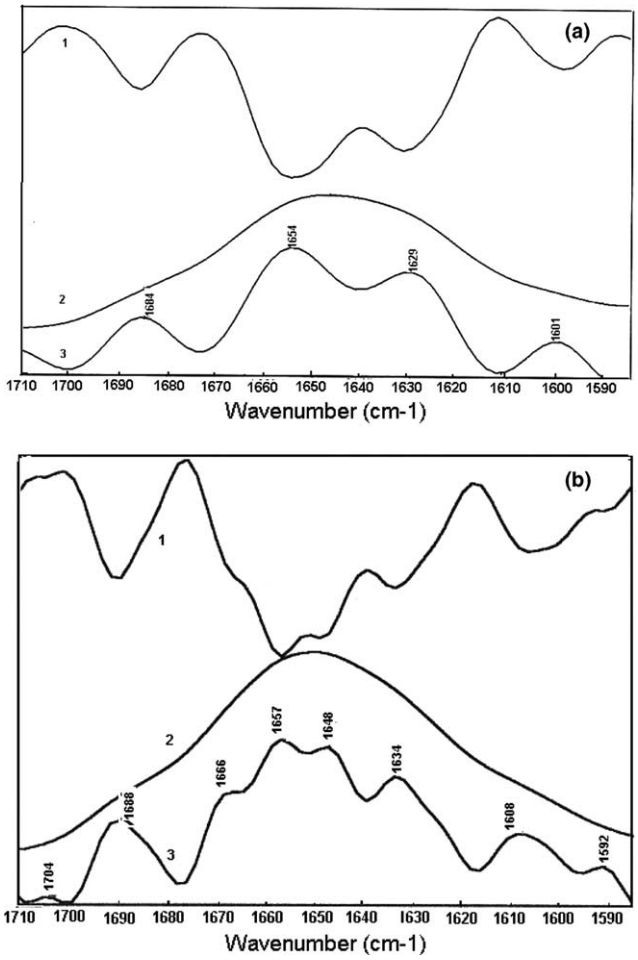

Figure 4. The comparisons of second-derivative resolution enhancement (1) and FT-IR spectrum of Fourier self-deconvolution (3) in buffer solution: (a) the spectra of HSA; (b) the spectrum of HSA + alpinetin system with C_{drug}/C_{HSA} = 1:1. The middle trace (2) represent a synthetic band contour obtained from different Lorentzian curves.

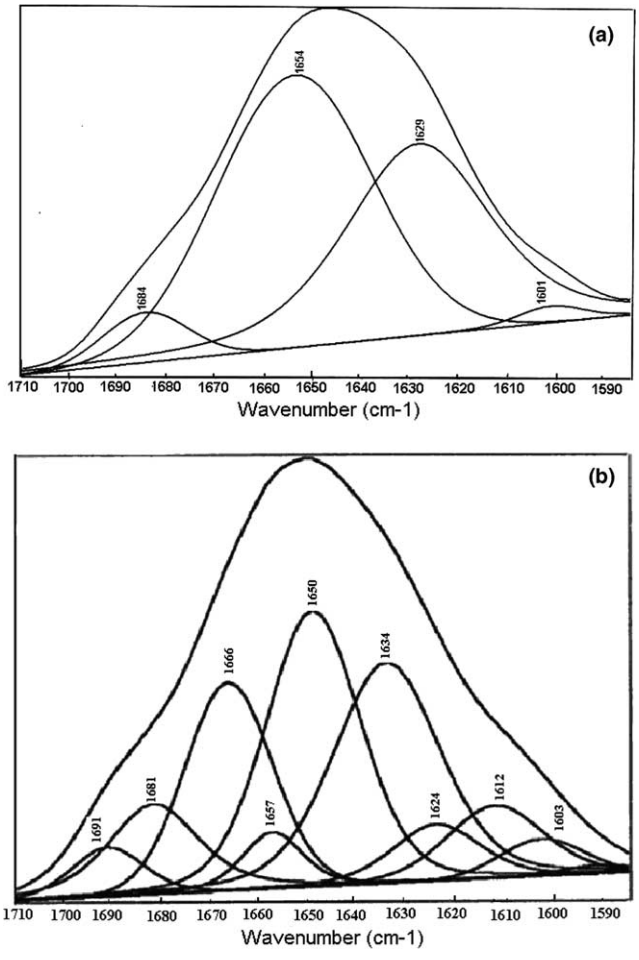

Figure 5. Curve-fitted amide I (1700–1600) regions of free HSA (curve a) and alpinetin–HSA (curve b) in buffer solution with C_{drug}/C_{HSA} = 1:1.

characteristic of tryptophan residue. Figure 7 shows the effect of addition of alpinetin on the synchronous fluorescence spectrum of HSA when $\Delta\lambda$ = 60 nm. The addition of the drug results in the strong fluorescence quenching of tryptophan with the maximum emission wavelength at 338 nm. It is reported that the maximum emission wavelength (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 HSA; λ_{max} at 350–352 nm shows that tryptophan residues are exposed to water, that is, the hydrophobic cavity in HSA is disagglomerated and the structure of HSA is looser. Figure 7 suggests that alpinetin mainly bound to the hydrophobic cavity of HSA, which is in accordance with the results from binding mode.

2.4. Binding constant and number of binding sites

In drug–protein binding studies, several equations have been for binding constant calculation. Another one frequently used is Scatchard equation[16]

$$r/D_f = nK - rK, \quad (1)$$

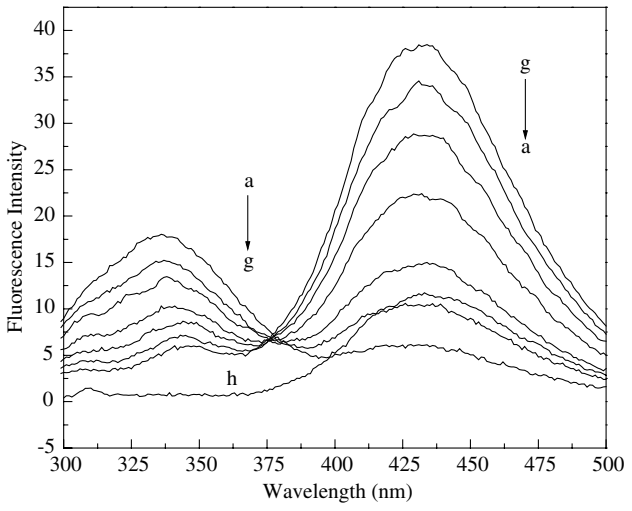

Figure 6. The fluorescence spectra of alpinetin–HSA system. The concentration of HSA was 3.0×10^{-6} mol/L while the alpinetin concentration corresponding to 0, 0.67, 1.33, 2.0, 2.67, 3.33, 4.0×10^{-5} mol/L from a to g; (h) [alpinetin] = 0.67×10^{-5} mol/L; T = 296 K; pH 7.40; λ_{ex} = 280 nm, λ_{em} = 337 nm.

where r is the number of mol of bound drug per mol of protein, D_f is the concentration of unbound drug, K and

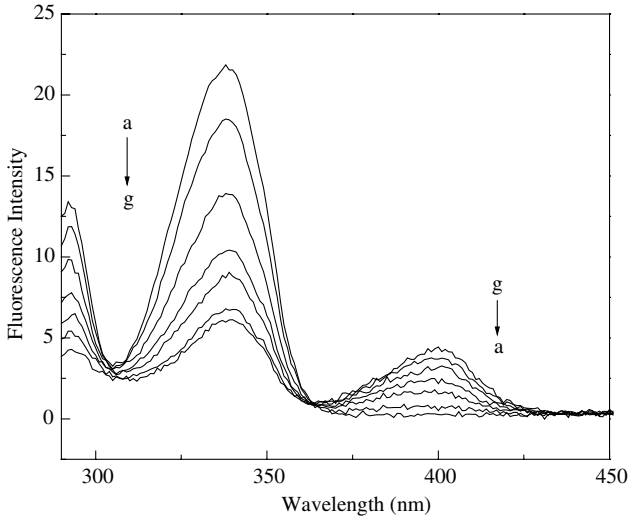

Figure 7. Synchronous fluorescence spectra of HSA (3.0 μM) with $\Delta\lambda = 60$ nm in the absence and presence of increasing amount of alpinetin (μM): (a) 0; (b) 6.67; (c) 13.33; (d) 20; (e) 26.67; (f) 33.33; (g) 40. Tris buffer, pH 7.4, 296 K.

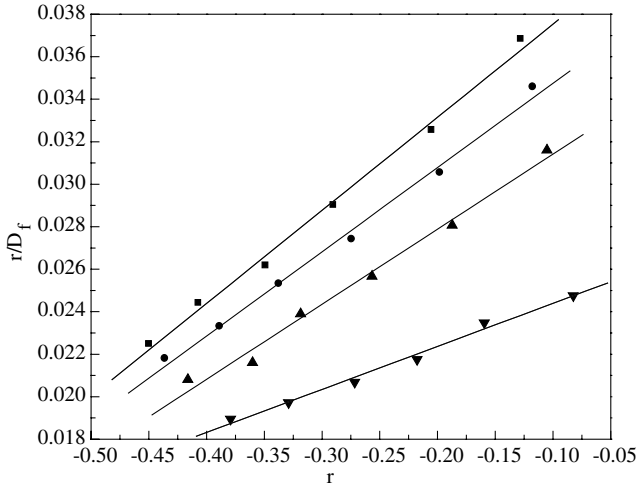

Figure 8. Scatchard plot for the alpinetin–HSA at pH 7.40. HSA concentration: 3.0×10^{-6} mol/L; pH 7.40; ▼ 318 K, ▲ 310 K; ● 303 K, ■ 296 K; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 337$ nm.

n are the binding constant and number of binding sites, respectively.

Quenching data were also analyzed according to the modified Stern–Volmer equation[17]

$$\frac{RF_0}{\Delta RF} = \frac{1}{[Q]}\frac{1}{fK} + \frac{1}{f}, \quad (2)$$

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 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 $(fK)^{-1}$.

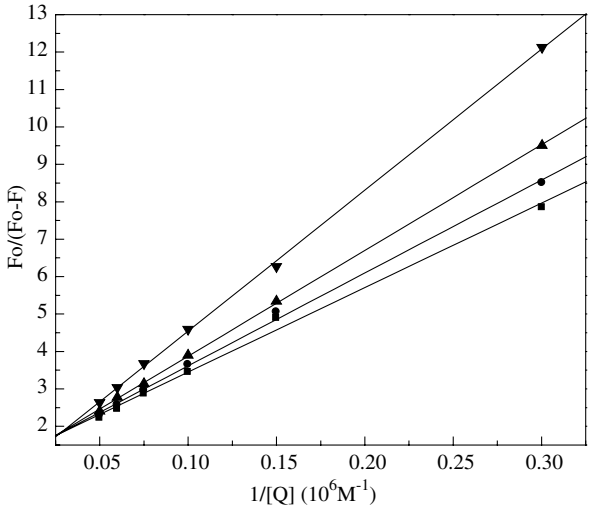

Figure 9. The modified Stern–Volmer for the alpinetin–HSA at pH 7.40. HSA concentration: 3.0×10^{-6} mol/L; pH 7.40; ▼ 318 K; ▲ 310 K; ● 303 K; ■ 296 K; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 337$ nm.

Figures 8 and 9 show the Scatchard plot and the Stern–Volmer plot for the alpinetin–HSA system at different temperatures, respectively. In Table 1 the binding constants obtained for the different methods are listed for alpinetin associated with HSA. The Scatchard plots obtained from this procedure showed a straight line for different temperatures tested. The linearity of Scatchard indicates that alpinetin binds to one class of sites on HSA, which was 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 alpinetin and HSA is very strong, and the effect of temperature is very small. Thus, alpinetin can be removed by the proteins in the body.

Table 1. Binding parameters for alpinetin–HSA system

Temperature (K)	Binding parameters		
	Stern–Volmer method	Scatchard method	
	K (10^4)	K (10^4)	n
296	5.267	4.389	0.957
303	4.562	3.967	0.976
313	3.716	3.534	0.989
318	2.064	2.026	1.304

2.5. 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 account for the main forces contributing to protein stability. If the enthalpies change (ΔH^0) does not vary significantly over the temperature range

studied, then its value and that of ΔS^0 can be determined from the Van't Hoff equation

$$\ln K = -\Delta H^0/RT + \Delta S^0/R, \quad (3)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0. \quad (4)$$

In Eq. 3, K is the binding constant at corresponding temperature and R is the gas constant. The enthalpy change (ΔH^0) is calculated from the slope of the Van't Hoff relationship. The free energy change ΔG^0 is estimated from the Eq. 4. Figure 10 is the Stern–Volmer quenching plots of alpinetin with HSA at different temperatures (296, 303, 310, 313 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. According to the binding constants of alpinetin to HSA obtained at the four temperatures above, the thermodynamic parameters were determined from linear Van't Hoff plot (Fig. 11) and presented in Table 2. As shown in Table 2, ΔH^0 and ΔS^0 for the binding reaction between alpinetin and HSA are found to be −10.197 kJ/mol and 53.972 J/mol K. Thus, the formation of alpinetin–HSA coordination compound is an exothermic reaction accompanied by positive ΔS^0 value. Ross and Subramanian[18] 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, as described below. From the point of view of water structure, a positive ΔS^0 value is frequently taken as a typical evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS^0 and a negative ΔH^0 value, while negative entropy and enthalpy changes arise from van der Waals forces and hydrogen formation in low dielectric media. Accordingly, it is not possible to account for the thermodynamic parameters of alpinetin–HSA coordination compound on the basis of a single intermolecular force model. Therefore, the binding of alpinetin to HSA might involve hydrophobic interaction strongly as evidenced by the positive values of ΔS^0 and the electrostatic interaction can also not be excluded. Meanwhile, it is found that the major contribution to ΔG^0 arises from the ΔH^0 term rather than from ΔS^0, so the binding process is enthalpy driven, and the increase of entropy might be based on the destruction of the iceberg structure induced by the hydrophobic interaction. For the alpinetin–HSA system, alpinetin is unionized under the experimental conditions (pH 7.40) in view of its pK_a value of 8.40, so electrostatic interactions can be precluded from the binding process. The additional evidences come from the synchronous fluorescence and molecular modeling, which is in good agreement with the above results. Thus the hydrophobic interaction might play a major role in the binding alpinetin to HSA.

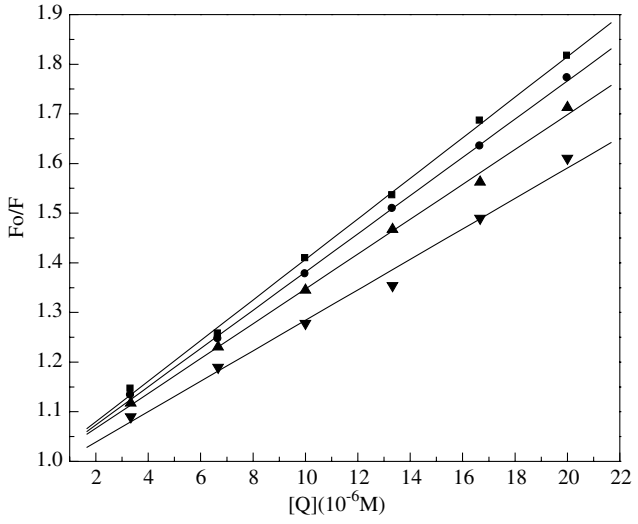

Figure 10. The Stern–Volmer plot of HSA quenched by alpinetin: ■ 296 K; ● 303 K; ▲ 310 K; ▼ 318 K. [HSA] = 3.0 μM, λ_{ex} = 280 nm, λ_{em} = 337 nm, pH = 7.40.

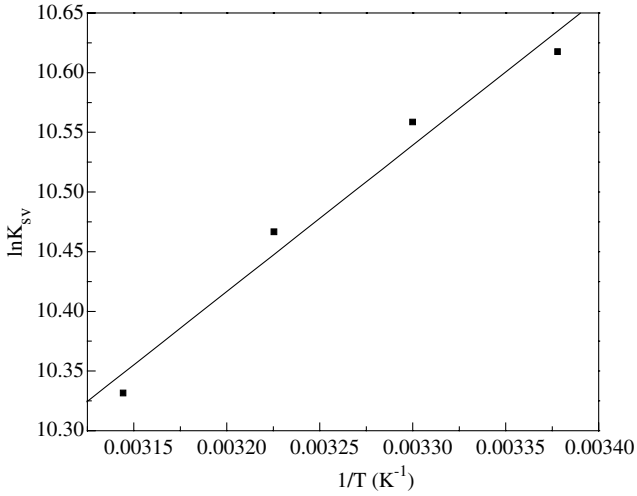

Figure 11. Van't Hoff plot for the interaction of HSA and alpinetin in Tris buffer, pH = 7.40.

Table 2. Thermodynamic parameters of alpinetin–HSA interaction at pH 7.4

Temperature (K)	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (J/mol K)
296	−26.189	−10.197	53.972
303	−26.928		
310	−27.090		
318	−27.360		

2.6. Energy transfer between alpinetin and HSA

Förster's theory of dipole–dipole energy transfer was used to determine the distances between the protein residue (donor) to the bound drug (acceptor) in HSA. By Förster's theory,[19] the efficiency of energy transfer (E) is related to the distance R (Å) between donor and acceptor by

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r_0^6), \quad (5)$$

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

$$R_0^6 = 8.8 \times 10^{25} \kappa^2 \Phi_d J \eta^{-4}, \quad (6)$$

where η is the refractive index of the medium, κ^2 is the orientation factor, and Φ_d 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:

$$J(\lambda) = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda / \sum F(\lambda) d\lambda, \quad (7)$$

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 of r, also can be calculated.

Figure 12 is the overlap of the fluorescence spectra of HSA and the absorption spectra of alpinetin. Under these experimental conditions, it has been reported for HSA that, $\kappa^2 = 2/3$, $\Phi_d = 0.118$, $\eta = 1.336$.[20] So the value of the overlap integral calculated from Figure 12 is 16.506×10^{-14} cm^3 L/mol, and the value of R_0 is 3.60 nm and the r is 4.96 nm, respectively. Obviously, they are lower than 7 nm after interaction between alpinetin and HSA. This accord with conditions of Förster's non-radiative energy transfer theory indicated again the static quenching interaction between alpinetin and HSA.

2.7. Identification of binding sites on HSA

HSA has a limited number of binding sites for endogenous and exogenous ligands that are typically bound reversibly and have binding constants in the range 10^4–10^8 M^{-1}.[21] The principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry. The binding locations have been determined crystallographically for several ligands. The IIIA subdomain is the most active in accommodating of many ligands, for example, digitoxin, ibuprofen, and tryptophan.[22] In order to identify the location of the alpinetin-binding site on HSA, site marker competitive experiments were carried out, using drugs, which specifically bind to a known site or region on HSA. The competitors used included PB (phenylbutazone) being a characteristic marker for site I,[23] FA (flufenamic) for site II,[24] and Dig (digitoxin) for site III.[25] According to the modified Stern–Volmer equation (Eq. 2), the fluorescence data were used to obtain the values of binding constant K (6.017×10^4, $r = 0.991$; 5.728×10^4, $r = 0.991$; 2.685×10^4 mol/L, $r = 0.999$ for PB, FA, and Dig, respectively, at 296 K). The results calculated indicated that the bound-alpinetin to HSA was affected by adding Dig, in contrast with the addition of PB or FA to the same complex, which has shown notable effect. Another important evidence comes from the results of the linear Stern–Volmer plots, which indicated the existence of a binding site for alpinetin in the proximity of the trytophan.[26] So in agreement with the above considerations, these evidences suggests that one of the primary binding site of alpinetin is at least the same as that of Dig to HSA.

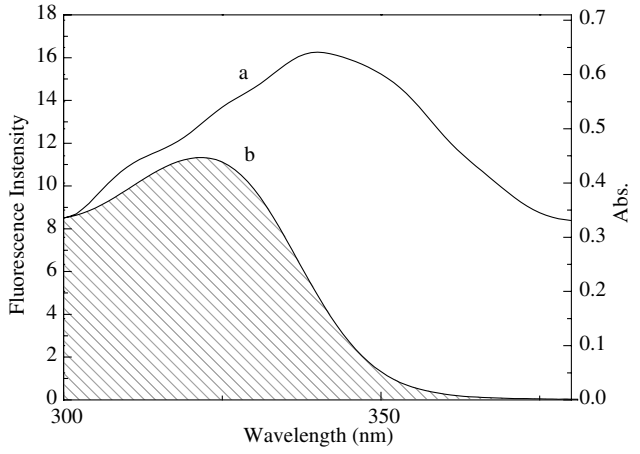

Figure 12. Overlapping between the fluorescence emission spectrum of HSA (a) (λ_{ex} = 280 nm) and absorption UV spectrum of alpinetin (b). C_{drug}/C_{HSA} = 1:1; T = 296 K.

2.8. Molecule modeling

The complementary applications of molecule modeling have been employed by computer methods to improve the understanding of the interaction of alpinetin and HSA. Descriptions of 3-D 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. There is a large hydrophobic cavity in subdomain IIA that many drugs can bind.[22] The crystal structure of HSA in complex with Dig (digitoxin) was taken from the Brookhaven Protein Data Bank (entry codes 1h9z). The potential of the 3-D structure of HSA was assigned according the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were 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, partial binding parameters of the alpinetin–HSA system were calculated through SGI FUEL workstations. The best energy ranked results is shown in Figure 13. As shown in Figure 13, the flavonoids moiety is located within the binding pocket and the A- and C-rings are practically coplanar. It is important to note that the tryptophan residue of HSA (Trp214) is in close proximity to the B-ring of alpinetin 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 alpinetin. On the other hand, the amino acid residues with benzene ring can match that of the alpinetin in space in order to firm the conformation of the complex. The calculated binding Gibbs free energy (ΔG) is -18.26 kJ/mol, binding constant $K = 4.09 \times 10^4$, which is close to the

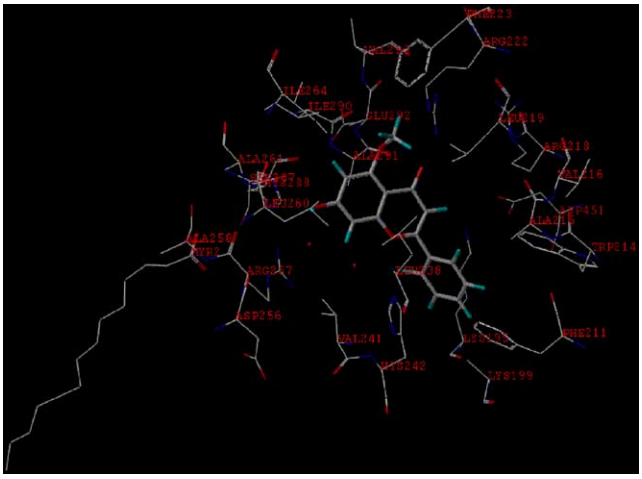

Figure 13. The binding mode between alpinetin and HSA, only residues around 8 Å of alpinetin is displayed. The residues of HSA are represented using line and the alpinetin structure is represented using ball and stick model.

experimental data (−26.19 kJ/mol, binding constant $K = 4.19 \times 10^4$) in some degree. Therefore, the results obtained from modeling indicated that the interaction between alpinetin and HSA is dominated by hydrophobic force.

2.9. Effect of co-ions on binding of alpinetin to HSA

The previous studies indicated that HSA has a high-affinity metal-binding site at N-terminus. 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.[27] The common ions chosen are widely distributed in human and animals. Therefore, we are interested in examining the effect of inorganic cations and anions on the solution system of alpinetin–HSA that can be used as a model for investigating the interaction of alpinetin to HSA. Table 3 shows the results of the effect of common ions on the binding constants at 296 K. It is shown that the binding constants between the protein and alpinetin have changed in the presence of common ions, constantly decreased from 5.5% to 89.8%. As a result, the binding force between 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 alpinetin and HSA at 23 °C in the presence of common ions

Ions	K' ($\times 10^4$)	R	K'/K
Mg^{2+}	3.410	0.9998	0.647
Al^{3+}	1.645	0.9968	0.312
Ca^{2+}	1.242	0.9978	0.236
Cu^{2+}	4.354	0.9999	0.827
Fe^{2+}	4.978	0.9996	0.945
Zn^{2+}	4.765	0.9991	0.905
F^-	5.101	0.9996	0.968
Cl^-	4.562	0.9998	0.866
Br^-	4.689	0.9994	0.890
SO_4^{2-}	0.538	0.9957	0.102
CO_3^{2-}	4.151	0.9992	0.788

3. Conclusions

The interaction between alpinetin and HSA has been investigated by fluorescence spectroscopy combined with UV–vis, CD, and FT-IR spectrophotometric techniques under simulative physiological conditions for the first time. This study showed that the intrinsic fluorescence of HSA was quenched through static quenching mechanism. Experimental results also showed that the binding of alpinetin to HSA induced a conformational change of HSA, which was further proved by the quantitative analysis data of CD and FT-IR spectrum. It is noteworthy that the spectroscopic research described herein signifies a promising approach, exploiting the new use of Chinese medicine alpinetin for probing their interactions with relevant target proteins. Accurate measurements of alpinetin's albumin binding properties and knowledge of its binding site locations are important to prevent adverse drug reactions leading to life-threatening hypoglycemia, and provide a basis for systematically predicting, which drugs have the potential to displace alpinetin from albumin and potentate its hypoglycemic effect. Therefore, this is also expected to open the door to new avenues in the screening and design of appropriate flavonoid-based drugs that will be of important in modern medical research.

4. Materials and methods

4.1. Materials

Alpinetin was of analytical grade, and purchased from the National Institute for Control of Pharmaceutical and Bioproducts, China, and the stock solution was prepared in ethanol. Human serum albumin (HSA, fatty acid free <0.05%) purchased from Sigma Chemical Co. and used without further purification; the molecular weight of 66,500 was used to calculate the molar concentrations. All HSA solution was prepared in the pH 7.40 buffer solution, and HSA stock solution was kept in the dark at 4 °C. NaCl (analytical grade, 1.0 mol/L) solution was used to maintain the ion strength at 0.1. Buffer (pH 7.40) consists of Tris (0.2 mol/L) and HCl (0.1 mol/L), and the pH was adjusted to 7.40 by 0.5 mol/L 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 the concentration of 20 μg/mL. All reagents were of analytical reagent grade and double water was used throughout the experiments.

4.2. Apparatus and methods

The absorbance spectra of the alpinetin–HSA with concentrations of alpinetin from 0 to 2.0×10^{-5} mol/L were recorded on a Cintra-10 and 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 (AIR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the attenuated total reflection (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.[28] 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 1700–1600 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 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)/10nlC_p]. 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 C_p is the mol fraction. The α-helical content of HSA was calculated according to the equation.[29]

Fluorescence spectra were measured with a RF-5301PC spectrofluorophotometer (Shimadzu), using 5 nm/5 nm slit widths. The emission spectra were recorded from 300 to 500 nm (excitation wavelength 280 nm). Synchronous fluorescence spectra of HSA in the absence and presence of increasing amount of alpinetin (0–2.0×10^{-5} mol/L) were recorded λ_{ex}: 290–500 nm. All experiments were measured at four temperatures (296, 303, 310, 313 K). The temperature of the sample was maintained by recycling water throughout experiment. The data thus obtained were analyzed by using the Scatchard equation to calculate the binding constants.

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