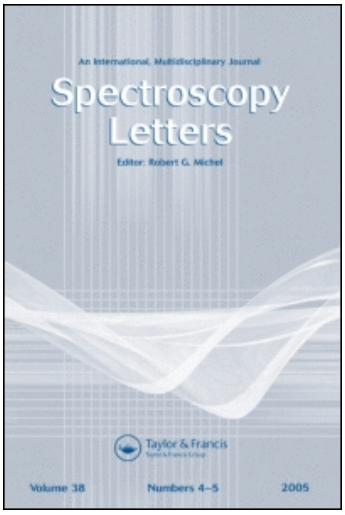

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Jun-Tao Wang,[1] Qiu-Hua Wu,[2] Chun Wang,[2] Jing-Jun Ma,[2] Xiao-Huan Zang,[2] Xiu-Min Yang,[2] and Na-Xin Qin[2]

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ABSTRACT The interaction of fangchinoline with human serum albumin (HSA) was studied by use of fluorescence quenching spectra, synchronous fluorescence spectra, and ultraviolet spectra. It was shown that fangchinoline has a strong ability to quench the fluorescence of HSA. The Stern-Volmer curves based on the quenching of the fluorescence of HSA by fangchinoline indicated that the quenching mechanism of fangchinoline on HSA was static quenching and non-radiation energy transfer. Based on the Förster theory of non-radiation energy transfer, the binding distances (r) and the binding constants (K_A) between fangchinoline and HSA were found. The thermodynamic parameters obtained revealed that the interaction between fangchinoline and HSA was mainly driven by hydrophobic force. The conformational changes of HSA were investigated by use of synchronous fluorescence. The result indicates that an ionic electrostatic interaction between fangchinoline and HSA could not be excluded.

KEYWORDS Fangchinoline, fluorescence quenching spectra, human serum albumin, synchronous fluorescence spectra, ultraviolet spectra

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in the systemic circulation. It is capable of binding an extraordinarily broad range of drugs. Because the overall distribution, metabolism, and efficacy of many drugs in the body are correlated with their affinities toward serum albumin, the information on the interaction of serum albumin with a particular drug can help people better understand the absorption and distribution of the drug. Therefore, the investigation of pharmaceuticals with respect to albumin–drug binding is imperative and of fundamental importance. Extensive investigations into the interaction between the serum albumin and internal compound or pharmaceutical molecule have been made,[1–4] but, thus far, only a very few studies

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of the interactions between serum albumin and the active component in Chinese herbal medicines have been reported.[5–7]

Traditional Chinese medicinal prescriptions have been used for more than 1000 years, and in recent years concentrated dosage forms have been widely adopted for clinical use. The traditional Chinese medicine Radix, *Stephaniae tetrandrae* (Chinese name: Fangji), that is, the root of *Stephaniae tetrandrae* S. Moore, has been demonstrated to have pain-relieving, blood pressure–depressing, antiphlogistic, antineoplastic, and antibiotic activities. It has been used clinically in China for centuries in the treatment of many diseases.[8] The main active constituents in Radix (*Stephaniae tetrandrae*) are two bisbenzylisoquinoline alkaloids: fangchinoline (FAN) and tetrandrine (TET). They have a similar chemical structure and pharmacological activities. However, a high dose of either TET or FAN is harmful. FAN (its chemical structure is shown in Fig. 1) has exhibited antioxidant, anti-inflammatory, and antihyperglycemic effects. It can also inhibit the release of histamine and promote phagocytosis activities.[9]

Fluorescence spectroscopy (FS) and ultraviolet spectroscopy (UV) have been widely used to study the interactions between small-molecule ligands and biomacromolecules.[1–7,10,11] From the measurements and analyses of the fluorescence emission spectra, fluorescence lifetime, fluorescence polarization, and so forth, a vast amount of information can be obtained about the structural fluctuations and the microenvironment changes surrounding the fluorophore in the macromolecule.[10,11] In this study, the interaction of FAN with HSA has been investigated by using FS and UV. The experimental results showed that FAN could quench the inner fluorescence of HSA and that both static quenching and non–radiation energy transfer were the main reasons leading to the fluorescence quenching. Moreover, the conformational changes of HSA were investigated by synchronous fluorescence.

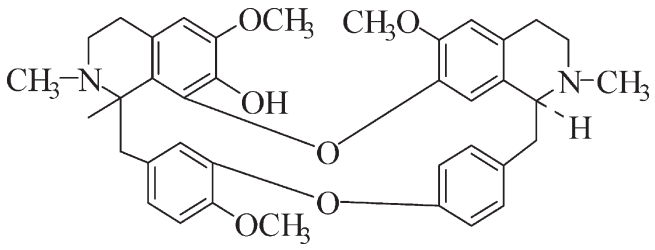

FIGURE 1 Molecular Structure of FAN

In addition, the binding constants and the thermodynamic parameters of the process are also proposed here. To our knowledge, this is the first report on the interactions between FAN and HSA.

MATERIALS AND METHODS

Materials

FAN and TET were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), HSA was from Sigma (St. Louis, MO, USA), and Tris (no less than 99.5% pure) was from Serva (New York, NY, USA). Tris-HCl buffer solution 0.05 mol/L was used to keep the pH of the solution at 7.4, and NaCl solution (0.1 mol/L) was used to keep the ionic strength. HSA solution (1.0×10^{-5} mol/L) was prepared in pH 7.4 Tris-HCl buffer solution. FAN and TET solutions at 1.0 mg/mL were obtained by dissolving an appropriate amount of the compound in 95% ethanol, respectively. $CuCl_2$, $FeCl_3$, and $ZnCl_2$ 1.0×10^{-2} mol/L solutions were prepared in water, respectively. All other chemicals were of analytical reagent grade, and all solutions were prepared with doubly distilled water.

Apparatus

A UV-250 recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and a Hitachi F-4500 fluorescence spectrophotometer equipped with a 1.0-cm quartz cell and a thermostat bath (Hitachi, Ltd. Tokyo, Japan) were employed throughout this study.

Spectroscopic Measurements

Appropriate quantities of FAN (1.0 mg/mL) ethanol solution were transferred to a 10-mL flask and evaporated to dryness under a gentle stream of nitrogen gas; to this, 5.0 mL 1.0×10^{-5} mol/L HSA solution was added. The resultant mixture was subsequently ultrasonicated for 5 min and incubated at 27°C, 37°C, or 47°C for 2 h. A blank system containing 5.0 mL $1.0 \times 1\,0^{-5}$ mol/L HSA solution alone was prepared similarly. Fluorescence quenching spectra were obtained by recording the fluorescence emission from 300 to 500 nm at the excitation wavelength of 283 nm, with a slit width of 5.0 nm. The synchronous fluorescence spectroscopy was scanned at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$), respectively.

The absorption spectra curves were obtained by scanning the 1.0×10^{-5} mol/L FAN solution prepared in a pH 7.4 Tris-HCl buffer solution from 300 to 500 nm at 27°C, 37°C and 47°C, respectively.

RESULTS AND DISCUSSION

Fluorescence Quenching Spectra and Quenching Mechanisms

The maximum fluorescence intensity of the tryptophan and tryosine residues of proteins excited at 283 nm is about 340 nm and 300 nm, respectively.[2] Under the experimental conditions, the maximum fluorescence intensity of HSA was at 340 nm. The fluorescence of serum albumin at 340 nm is due to the tryptophan and tryosine residues present in the protein, which can act as intrinsic probes. The main contributions to the fluorescence intensity of HSA came from tryptophan residue, as the fluorescence emission wavelength maximum of HSA was closer to that of tryptophan residue. If the drug can quench the fluorescence of the tryptophan residues, the tryptophan residues are possibly located in or near the binding position. In this experiment, the concentrations of HSA solution were stabilized at 1.0×10^{-5} mol/L to ensure sensitivity. The effect of FAN on HSA fluorescence intensity was investigated by changing its concentration from 0 to 0.5×10^{-5}, 1.0×10^{-5}, 1.5×10^{-5}, 2.0×10^{-5}, 3.0×10^{-5}, 4.0×10^{-5}, and 5.0×10^{-5} mol/L, respectively. The results are shown in Figure 2. We observed that the fluorescence intensity of HSA decreased regularly with increased concentration of FAN and that there was no significant λ_{em} shift with the addition of FAN, which indicated that FAN could quench the intrinsic fluorescence of HSA and that the interaction between FAN and HSA indeed existed. Crystal structure analyses have revealed that the main drug-binding regions are located in subdomains IIA and IIIA of HSA.[1] There is only one tryptophan residue (Trp 214) in the HSA molecule, which was located in subdomain IIA. The large degree of quenching of HSA fluorescence by FAN at 340 nm indicated that the trytophanyl group in subdomain IIA is the main binding site of FAN with HSA.

Quenching can occur by different mechanisms, usually classified as dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their different dependencies on temperature and excited-state lifetime. Dynamic quenching depends upon diffusion. Because high temperatures will result in large diffusion coefficients, the bimolecular quenching constants are expected to increase with increased temperatures. If the bimolecular quenching constants decreased with increased temperatures, it could be concluded that the quenching process is not a dynamic quenching but a static quenching.[4–6] For dynamic quenching, the mechanism can be described by the Stern–Volmer equation:

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

Here, F_0 represents the fluorescence intensities in the absence of quencher, F represents the corrected fluorescence intensities in the presence of quencher (the correction method is the same as that early employed by us[5]), K_q is the quenching rate constant of the bimolecule, K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime of the molecule without quencher

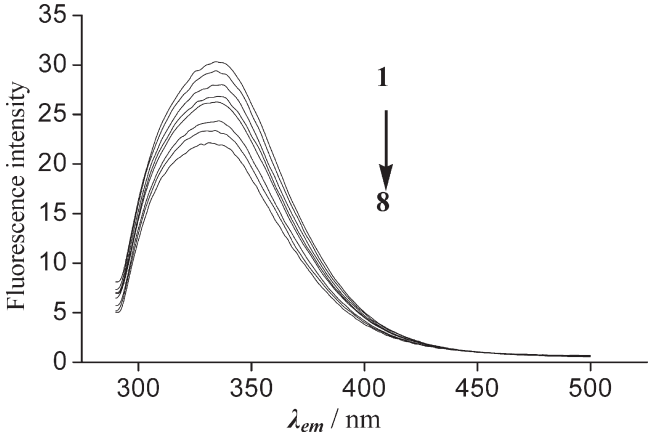

FIGURE 2 The Fluorescence Quenching Spectra of FAN–HSA. $C_{HSA} = 1.0 \times 10^{-5}$ mol/L; $C_{FAN}/10^{-5}$ mol/L, from 1 to 8: 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0.

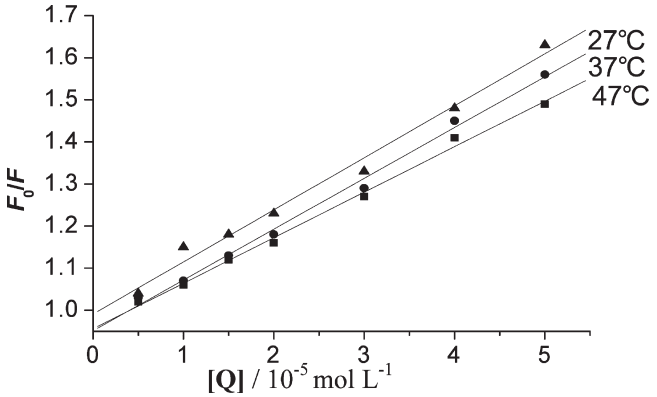

FIGURE 3 Stern–Volmer Curves of Fluorescence Quenching of HSA by FAN.

TABLE 1 Stern–Volmer Quenching Constants of the System of FAN and HSA

T(°C)	K_{SV} (L/mol)	K_q (L/[mol · s])	R
27	1.24×10^4	1.24×10^{12}	0.994
37	1.21×10^4	1.21×10^{12}	0.997
47	1.08×10^4	1.08×10^{12}	0.998

(for the biopolymer, τ_0 is often taken as 10^{-8} s[12,13]) and [Q] is the concentration of the quencher. In order to confirm the quenching mechanism, the process of fluorescence quenching was first assumed to be dynamic quenching. Figure 3 displays the Stern–Volmer plots of the quenching of HSA by FAN at 27°C, 37°C, and 47°C, which were much below the denaturing temperature of HSA according to the literature[14] (the denaturing temperature of HSA ranging from 60°C to 100°C).

Based on the experimental data from Figure 3, the corresponding dynamic quenching constants for the interaction between FAN and HSA were calculated according to Eq. (1) and are shown in Table 1.

According to the literature,[15,16] for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is about 2.0×10^{10} L/(mol · s). Considering that in this experiment, the rate constant of the protein quenching procedure initiated by FAN is much greater than 2.0×10^{10} L/(mol · s) and that the K_{SV} decreased with increased temperatures, it can be concluded that the quenching was not initiated by dynamic quenching but probably by static quenching resulting from the formation of FAN–HSA complexes. Then, the process should be handled by the static quenching model as follows.

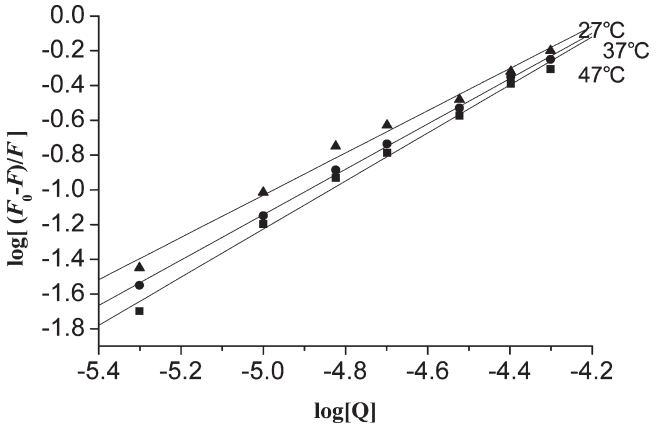

FIGURE 4 Double-log Plot of Fangchinoline Quenching effect on HSA Fluorescence at Different Temperatures.

TABLE 2 The Binding Constants (K_A), Binding Numbers (n), Correlation Coefficients (R), and Binding Distance (r) Between FAN and HSA

T (°C)	K_A	n	R	r (nm)
27	5.04×10^5	1.2	0.995	2.42
37	5.38×10^5	1.3	0.999	2.71
47	5.67×10^5	1.4	0.997	2.80

Binding Constants and Binding Sites

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the following formula:[17,18]

$$\log(F_0 - F)/F = \log K + n \log[Q], \qquad (2)$$

where K is the binding constant, and n is the number of binding sites per HSA. After the fluorescence quenching intensities on HSA by FAN were measured at 336 nm, the double-logarithm algorithm was assessed by Eq. (2). The resulting double-logarithm curves are shown in Figure 4, and the corresponding calculated results are given in Table 2. The correlation coefficients for the curves were larger than 0.995, indicating that the interaction between FAN and HSA agrees well with the site-binding model underlying Eq. (2). The result illustrates that there is a strong binding force between FAN and HSA, and that one binding site would be formed.

Binding Distance Between the Drug and the Amino Acid Residues of HSA

According to the Förster non–radiation energy transfer theory,[19,20] the energy-transfer effect is related not only to the distance between the acceptor and donor but also to the critical energy-transfer distance (R_0).

$$E = R_0^6/(R_0^6 + r^6) \qquad (3)$$

$$E = 1 - (F/F_0), \qquad (4)$$

where E is the energy transfer efficiency, R_0 is the critical distance when the transfer efficiency is 50%, and r is the binding distance between donor and acceptor

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J, \qquad (5)$$

where K^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of the

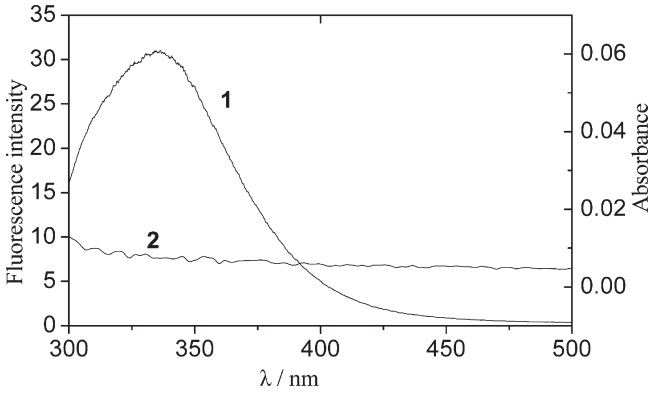

FIGURE 5 Overlap Between Fluorescence Emission Spectrum of HSA (1) and UV Absorption Spectrum of FAN (2).

donor and the absorption spectrum of the acceptor. Therefore,

$$J = \frac{\left(\sum I_P(\lambda)\varepsilon_D(\lambda)\lambda^4\Delta\lambda\right)}{\left(\sum I_P(\lambda)\Delta\lambda\right)}. \quad (6)$$

Here, $I_P(\lambda)$ is the fluorescence intensity of the fluorescence donor at wavelength λ, and $\varepsilon_D(\lambda)$ is the molar absorptivity of the acceptor at wavelength λ.

The overlap of the absorption spectrum of FAN and the fluorescence emission spectrum of HSA is shown in Figure 5. The overlap integral was calculated to be 1.10×10^{-15} cm^3 L/mol for FAN-HSA system at 27°C by integrating the spectra from 300 to 500 nm in Figure 5. The critical distance, R_0, corresponding with 50% energy transfer from HSA to FAN, was calculated to be 1.76 nm from Eq. (5) when $K^2 = 2/3$, $N = 1.336$, and $\Phi = 0.15$.[21,22] The binding distance, r, between FAN and the amino acid residue in HSA was calculated to be 2.42 (27°C), 2.71 (37°C), and 2.80 (47°C) (shown in Table 2), which are much smaller than 7 nm, a criterion value for energy transfer phenomenon to occur,[21,22] suggesting that the energy transfer from HSA to FAN may occur with high possibility.

Thermodynamic Parameters and Nature of the Binding Forces

The interaction forces between drug and biomolecule may involve hydrophobic force, electrostatic interactions, van der Waals interactions, hydrogen bonds, and so forth. In order to elucidate the interaction of FAN with HSA, we calculated the thermodynamic parameters from the Eqs. (7–9). 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:

$$\Delta G = -RT \ln K, \quad (7)$$

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 following equations:

$$\ln K_2/K_1 = [1/T - 1/T_2]\Delta H/R \quad (8)$$

$$\Delta G = \Delta H - T\Delta S. \quad (9)$$

TABLE 3 The Thermodynamic Parameters of FAN–HSA Binding Procedure

T (°C)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/[mol · s])
27	5.05	−32.75	126.01
37	5.05	−34.01	125.99
47	5.05	−35.24	125.91

As a result, the resulting thermodynamic parameter ΔG, ΔH, and ΔS values for the interaction of FAN with HSA are $\Delta G = 5.05$ kJ/mol $\Delta H = -32.75$ kJ/mol, and $\Delta S = 126.01$ J/(mol · K) (T = 27°C); $\Delta G = 5.05$ kJ/mol, $\Delta H = -34.01$ kJ/mol, and $\Delta S = 125.99$ J/(mol · K) (T = 37°C); $\Delta G = 5.05$ kJ/mol, $\Delta H = -35.24$ kJ/mol, and $\Delta S = 125.91$ J/(mol · K) (T = 47°C). They are also given in Table 3. The negative sign for ΔG means that the interaction process is spontaneous. The positive ΔH and ΔS values indicate that the binding between FAN and HSA is mainly ΔS-driven, with little contribution from the enthalpy factor, and that hydrophobic force may play a major role in the reaction.[23]

Conformational Studies

The synchronous fluorescence spectra can provide information about the molecular environment in the vicinity of the chromophore molecules. The conformational changes of HSA caused by FAN were evaluated by measuring the synchronous fluorescence intensity of HSA before and after the addition of FAN. In this work, the synchronous fluorescence characteristics of HSA were studied at different scanning intervals $\Delta\lambda$ ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$). When $\Delta\lambda$ was stabilized at 15 nm or 60 nm, the synchronous fluorescence gave the characteristic information of tyrosine

or tryptophan residues in HSA.[24] A useful method of studying the environment of amino acid residues was to measure the possible shift in fluorescence emission wavelength maximum λ_{max}. The shift in the position of emission maximum corresponds with the changes of the polarity around the chromophore molecule.[25–27] According to the literature,[28] a blue shift of λ_{max} suggests that the amino acid residues are located in a more hydrophobic environment and are less exposed to the solvent, and a red shift of λ_{max} implies that the amino acid residues are in a more polar environment and are more exposed to the solvent. Thus, the conformational changes of HSA can be evaluated by measuring the changes of λ_{max}.

With the HSA concentration being unchanged and the concentration of FAN being increased, the synchronous fluorescence spectra for the system were obtained by scanning at $\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm, respectively (shown in Figure 6).

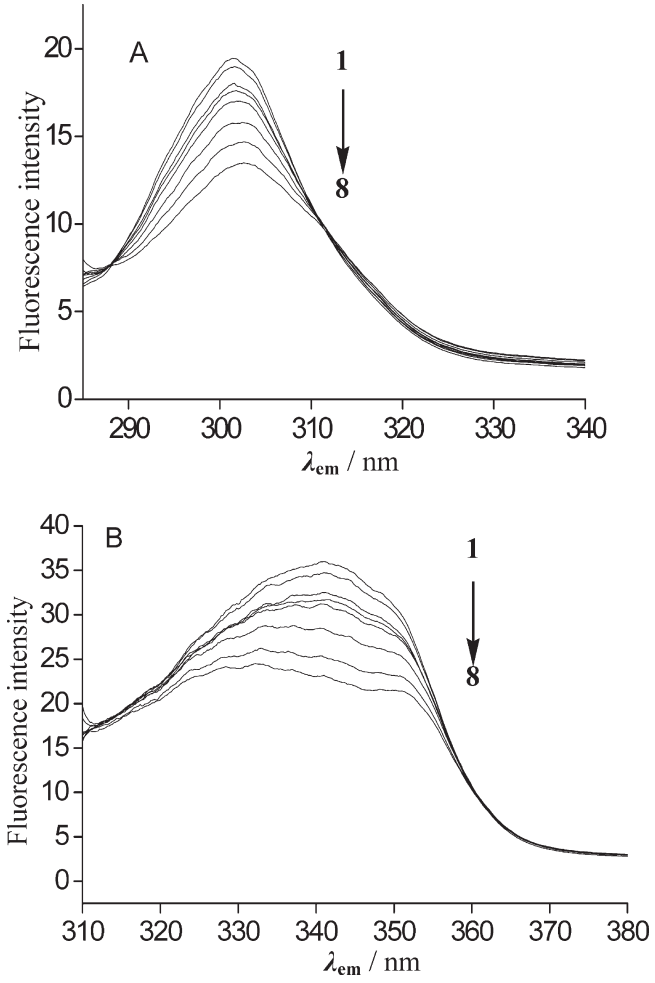

FIGURE 6 Synchronous Fluorescence Spectra of HSA–FAN. (A) $\Delta\lambda = 15$ nm, (B) $\Delta\lambda = 60$ nm; $C_{FAN}/10^{-5}$ mol/L, from 1 to 8: 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0.

The effects of FAN on the fluorescence intensity of the tyrosine (Figure 6A) and tryptophan residues (Figure 6B) of HSA indicated that the main contributions to the fluorescence intensity of HSA came from tryptophan residues, as the fluorescence emission wavelength maximum of tryptophan residues was closer to that of HSA, and the fluorescence intensity of tryptophan residues was much bigger than that of tyrosine residues. Nevertheless, the interaction of tyrosine with FAN could not be excluded, as both the fluorescence of tryptophan and tyrosine residues were quenched by FAN.

It is also observed from Figure 6 that, upon the addition of the drug, there is a small red-shift of the maximum emission wavelength for tyrosine residues (Fig. 6A), and there are great changes of the peak shape and strong blue-shifts of the maximum emission wavelength for tryptophan residues (Fig. 6B), suggesting that the interaction of HSA with FAN leads to a more polar environment for tyrosine residues and a more hydrophobic environment for tryptophan residues, with the latter to a larger degree. The above result indicates that FAN could insert in HSA molecules and result in molecular conformational changes of HSA, the interaction forces between FAN and HSA may involve hydrophobic force and ionic electrostatic interactions, and the hydrophobic force may play a major role in the reaction.

Effect of the Metal Ions and Competing Drug TET on the Binding Constants Between FAN and HSA

The metal ions could affect the interaction between the pharmaceutical molecule and the serum albumin.[29] In this work, the effect of Cu^{2+}, Fe^{3+}, and Zn^{2+} on the binding reaction between FAN and HSA was investigated each at a concentration of 5.0×10^{-5} mol/L at 27°C. The results shown in Table 4 reveal that the binding constants of FAN and

TABLE 4 The Effect of Metal Ions and TET on the Binding Constants (27°C)

Compound	K'_A	r	K'_A/K_A
Fe^{3+}	3.56×10^5	0.993	0.706
Zn^{2+}	4.98×10^5	0.996	0.988
Cu^{2+}	3.75×10^5	0.990	0.745
TET	4.77×10^5	0.991	0.946

HSA in the presence of these ions were 70.6% to 98.8% of the binding constant obtained with no metal ions. This result implied that there was a competition between FAN and metal ions for the interaction with HSA when both of them existed in the HSA solution.

TET has a similar chemical structure and pharmacological activities with FAN. The influence of TET on the FAN-HSA interaction was also investigated. We can observe from Table 4 that the binding constant decreased in the presence of TET, suggesting that TET could compete with FAN when they existed in the HSA solution at the same time.

CONCLUSIONS

In this paper, the nature and magnitude of the interaction of HSA with FAN was investigated by fluorescence spectra and UV spectra. The results showed that the Stern–Volmer quenching constant K_{SV} was inversely correlated with temperature, which indicated that the probable quenching mechanism of the HSA–FAN system was initiated by static quenching. The thermodynamic parameters showed that the interaction between FAN and HSA was spontaneous, and that the hydrophobic force was a major factor in the interaction. A blue shift of the maximum emission wavelength of HSA from synchronous fluorescence spectra suggested that FAN could result in conformational changes of HSA, causing a more hydrophobic environment for the tryptophan residues and a more polar environment for the tyrosine residues in HSA, with the former to a larger degree. This suggests that the ionic electrostatic interaction between FAN and HSA could not be excluded.

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