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Research Article

Interaction of Warfarin with Human Serum Albumin and Effect of Ferulic Acid on the Binding

Qiang Li,[1,2] Wen-yue Yang,[3] Ling-ling Qu,[3] Huan-Yang Qi,[4] Yun Huang,[3] and Zheng Zhang[1,2]

[1] *Heart Center, The First Hospital of Lanzhou University, Lanzhou, Gansu 730000, China*
[2] *The First Clinical Medical College of Lanzhou University, Lanzhou, Gansu 730000, China*
[3] *Pharmaceutical College, Hebei Medical University, Shijiazhuang 050017, China*
[4] *Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, Gansu 730000, China*

Correspondence should be addressed to Yun Huang; hy9317536@126.com and Zheng Zhang; zhangccu@163.com

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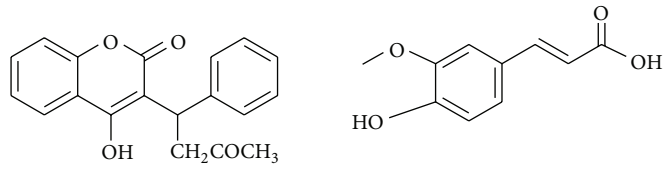
Angelica sinensis (Oliv.) Diels combined treatment with warfarin would increase the risk of bleeding. Ferulic acid is an abundant hydroxycinnamic acid in *A. sinensis* and warfarin is the most widely used oral anticoagulant. The studies on supermolecular interaction of warfarin with human serum albumin (HSA) and the influence of ferulic acid on the binding would contribute to the understanding of the metabolic processes of warfarin and the effect of ferulic acid. We focus on investigating the effect of warfarin on fluorescence spectrum of human serum albumin (HSA), fluorescence quenching mechanism, binding constant, Hill coefficient, binding mode, and the effect of different ferulic acid concentrations on the binding. Warfarin quenched the intrinsic fluorescence of HSA mainly by static quenching. Accession of ferulic acid reduced the binding of HSA-warfarin. By decreasing binding constant and the Hill coefficient of warfarin with HSA, ferulic acid could improve the plasma concentration of free warfarin, which would increase the risk of bleeding. Warfarin's free concentration increased at least 50% under the condition of simulated human body. The results indicated that *A. sinensis* combined treatment with warfarin would increase the risk of bleeding. And the results provide an important theoretical support for warfarin used as oral anticoagulant.

1. Introduction

Angelica sinensis (Oliv.) Diels, which belongs to the genus *Angelica* (family Apiaceae), was first documented in Shennong Bencao Jing (edited in Han Dynasty in China) and its root is known in Chinese as Danggui or Dongquai. *A. sinensis* is a popular traditional Chinese medicinal (TCM) herb which is easily obtained by the public [1–3]. Its roots as a Chinese herbal supplement are used for treatment of menstrual cramping, irregular menses, and menopausal symptoms. *A. sinensis* is used alone, or in combination with other herbs, as an antispasmodic, a "blood purifier," and a tonic to strengthen the heart, lung, and liver meridians. *A. sinensis* is also used as a health food product in Asia and as a dietary supplement in Europe and America for the treatment of a variety of gynecological problems [4].

Ferulic acid (Figure 1), the phenolic acid, which is believed to be one of the most biologically active components in *A. sinensis*, is used as a marker compound to evaluate the quality of *A. sinensis* in Chinese Pharmacopoeia [5–7]. In vivo and in vitro studies showed that ferulic acid from *A. sinensis* had antithrombotic activity. Ferulic acid interrupts platelet polymerization by inhibiting the release of serotonin and adenosine diphosphate from platelets and by reducing production of thromboxane A2 (TXA2) through inhibiting cyclooxygenase and thromboxane synthetase in arachidonic acid metabolism [8].

Warfarin (Figure 1), the 4-hydroxycoumarin compound, is the most widely used oral anticoagulant. Onset and duration of the action are rapid and predictable, and its bioavailability is excellent. Warfarin is highly water soluble and rapidly absorbed from the stomach and the upper



Warfarin Ferulic acid

Figure 1: The tentative structure of warfarin and ferulic acid.

gastrointestinal tract. Its plasma concentrations peak occurs 60 to 90 minutes after oral administration. Warfarin binding to the enzyme vitamin K 2,3-epoxide reductase in liver microsomes stops the cycle of vitamin K and reduces gamma-carboxylation of the precursors of vitamin D-dependent pro- and anticoagulant factors [9].

In 2000, a review "*Herb-drug interactions*" in Lancet indicated that Chinese traditional herb *A. sinensis* combined treatment with warfarin would increase the risk of bleeding [10]. In 2002 and 2010, there were two reviews in JACC commented the drug interactions between *A. sinensis* and warfarin. Case reports were also suggestive of interaction between warfarin and *A. sinensis* [11, 12]. A patient stabilized on warfarin experienced an increase in prothrombin time (PT) and international normalized ratio (INR) after taking *A. sinensis* for perimenopausal symptoms [8].

For the clinical studies, several cases published previously showed gross over-anticoagulation and bleeding complications on patients received chronic warfarin therapy in combination with *A. sinensis*. However, until now, the mechanism of interactions of *A. sinensis* and warfarin was not clear and little attention has been paid to the effect of ferulic acid on warfarin binding with human serum albumin (HSA). HSA is the most abundant protein constituent of blood plasma and serves as a protein storage component. As one of the most extensively studied model proteins, HSA has high affinity to many endogenous and exogenous compounds, serving in transporting drugs or other organic molecules to their targets [13, 14]. Therefore, investigating the interaction of active components in Chinese herbs with HSA can provide useful information of drug actions and can be used as a model for elucidating the drug-protein complex [15].

In order to further explore whether the interactions between warfarin and HSA occur and the influence of ferulic acid on them, we primarily focus on investigating the effect of warfarin on fluorescence spectrum of HSA, fluorescence quenching mechanism, binding constant, Hill coefficient, binding mode, and the effect of different ferulic acid concentrations on the binding, which are expected to provide more information about the interaction between warfarin and HSA. The studies on supermolecular interaction of warfarin with HSA and the influence of ferulic acid on the binding would contribute to the understanding of the metabolic processes of warfarin. And the results may provide an important theoretical support for warfarin used as oral anticoagulant.

2. Materials and Methods

2.1. Materials. HSA purchased from Sigma (New York, USA) was 98.0-99.0% pure (agarose gel electrophoresis) and Tris (no less than 99.5% pure) was purchased from Sigma. Ferulic acid and warfarin (no less than 98.0% pure) were bought from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals were from Beijing Chemical Reagents Company and were of analytical reagent grade. Double distilled water was used throughout the experiment.

2.2. Apparatus. Fluorescence measurements were performed on a Perkin-Elmer spectrofluorimeter Model LS-55 equipped with a 150 W Xenon lamp and a thermostat bath. The widths of both the excitation slit and emission slit were set at 5.0 nm. The absorption spectra were measured on a Shimadzu UV-2450 UV-Vis spectrophotometer. The pH measurements were carried out on a PHS-3C digital pH meter (Shanghai Leici Instrument Factory). Titrations were done manually by using trace syringes. A quartz cell of 1.00 cm pathlength was used for the measurements.

2.3. Procedures. HSA (5×10^{-4} M), warfarin (1×10^{-3} M), and ferulic acid (1×10^{-3} M) were diluted with Tris-HCl buffer (0.05 M, pH 7.40, containing 0.10 M NaCl) to some lower concentration for actual use. A 5×10^{-4} M HSA stock solution was diluted to some lower concentrations with Tris-HCl buffer solution. The final concentrations of HSA varied from 0 to 5×10^{-6} M. 3 mL of HSA (4×10^{-6} M) was titrated to give 0, 6, 12, 18, 24, 30, 36, 42, or 48 μL of warfarin (1×10^{-3} M), respectively, and a certain amount of Tris-HCl buffer solution to give the final volume of 3.05 mL. Another solution containing HSA in the absence and presence of ferulic acid (6×10^{-6} M) was prepared similarly. The final concentrations of warfarin varied from 0 to 16×10^{-6} M at an increment of 2×10^{-6} M. The resultant mixture was subsequently ultrasonicated for 1 min and incubated for 5 min at 298 or 310 K.

Fluorescence spectra were recorded at 298 and 310 K in the range from 300 to 450 nm at excitation wavelength 287 nm. The synchronous fluorescence spectra were recorded when $\Delta\lambda = 15$ nm, $\Delta\lambda = 60$ nm, and $\Delta\lambda = 90$ nm. The three-dimensional fluorescence spectra were performed under the following conditions: emission wavelengths over a range of 280–450 nm, excitation wavelengths over a range of 220–320 nm with an increment of 2 nm. The UV-Vis absorption

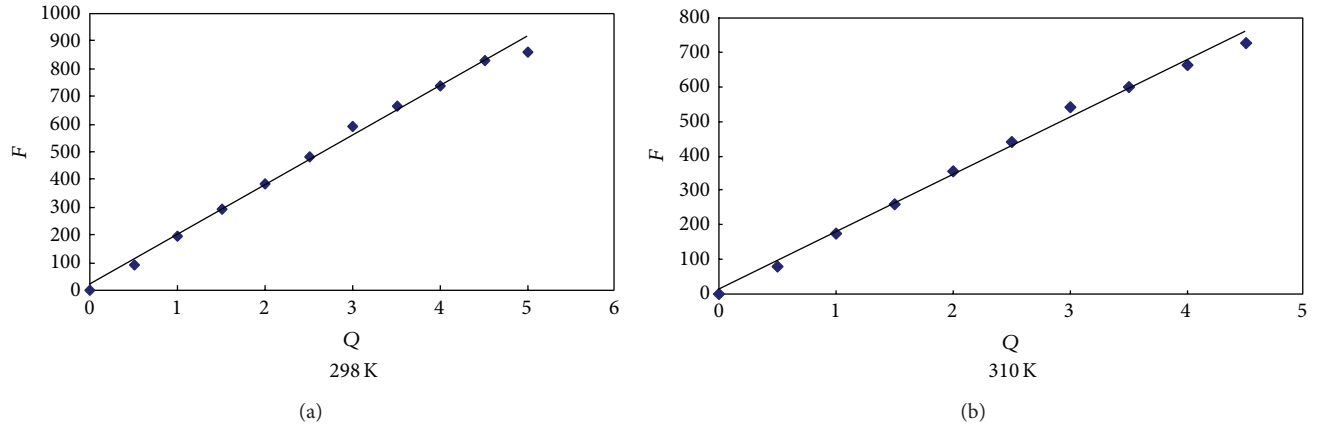


Figure 2: Standard curve of HSA at 298 and 310 K when $\Delta\lambda = 60$ nm, $C_{(HSA)} = 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 \times 10^{-6}$ M.

spectrum of warfarin (HSA or warfarin-ferulic acid) was recorded in the range from 280 to 450 nm using UV-2450.

3. Results and Discussion

3.1. Determination of Free HSA Concentration. Synchronous fluorescence spectrum shows the fluorescence of Trp residues when $\Delta\lambda = 60$ nm. The intrinsic fluorescence is mainly manifested by emission of Trp residues in molecule [16]. In this research, the standard curve of HSA was got from synchronous fluorescence spectrum of Trp residues. So the free concentration of HSA can be derived from the standard curve of HSA. The standard curves of HSA were shown in Figure 2 and Table 1.

3.2. Interaction of HSA with Warfarin

3.2.1. Effect of Warfarin on Fluorescence Spectrum of HSA. Figure 3 shows the fluorescence quenching spectra of HSA with different concentrations of warfarin, suggesting that warfarin could combine with HSA. The fluorescence intensity of HSA at 344 nm decreased regularly with the increase of warfarin concentration, suggesting that the complex of warfarin-HSA was formed, which is responsible for quenching HSA fluorescence.

3.2.2. Binding Rate of Warfarin with HSA. According to the literature, the proportion of HSA, warfarin, and ferulic acid in human body is 100 : 0.5 : 1. And 99.9% of warfarin combines with HSA in blood [17, 18]. So the concentrations of warfarin and ferulic acid are 2×10^{-8} M and 3×10^{-8} M, respectively, when the concentration of HSA is 4×10^{-6} M. The binding rate of HSA with warfarin is very high. And the amount of HSA is much more than warfarin in this research. So it can be concluded that almost all of warfarin was combined with HSA.

Figure 4 shows that synchronous fluorescence intensity was declined after warfarin was added. The fluorescence intensity was 759 and 754 when warfarin was absent and present. The free concentration of HSA was 3.98×10^{-6} M when warfarin was added according to the equations of Table 1. The free concentration of HSA was reduced by 2×10^{-8} M and the concentration of warfarin was 2×10^{-8} M. So it can be speculated that the binding site of warfarin with HSA was 1 : 1. And then ferulic acid was added and the fluorescence intensity was 755 nm. At this point, the free concentration of HSA was 3.99×10^{-6} M according to the equations of Table 1. It did not only decrease, but also increased to 1×10^{-8} M. So it can be speculated that the accession of ferulic acid reduced the binding of HSA-warfarin. Under the condition of simulated human body, the free concentration of warfarin increased at least 50%.

Table 1: Equation of standard curve of HSA when $\Delta\lambda = 60$ nm.

T/K	Equation	R^2
298 K	$F = 178.44[Q] + 20.636$	0.9941
310 K	$F = 165.35[Q] + 11.473$	0.9953

3.2.3. Fluorescence Quenching Mechanism and Quenching Constant. The quenching mechanism can be interpreted by the fluorescence quenching spectra of the protein and the Stern-Volmer plots of warfarin with HSA as shown in Figure 3. The procedure of the fluorescence quenching is assumed to be dynamic quenching in order to confirm the view. The Stern-Volmer equation is described as

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

where F_0 and F are the relative fluorescence intensities in the absence and presence of quencher, K_q is the quenching rate constant of biomolecule, τ_0 is the average lifetime of the biomolecule (10^{-8} s) without quencher, and $[Q]$ is the free concentration of the quencher. K_{SV} is the dynamic quenching constant [19]. Dynamic and static quenching can be distinguished by their different dependence on temperature. The K_{SV} values decrease with an increase in temperature for static quenching, and the reverse effect would be observed for dynamic quenching [20]. The Stern-Volmer plots from our

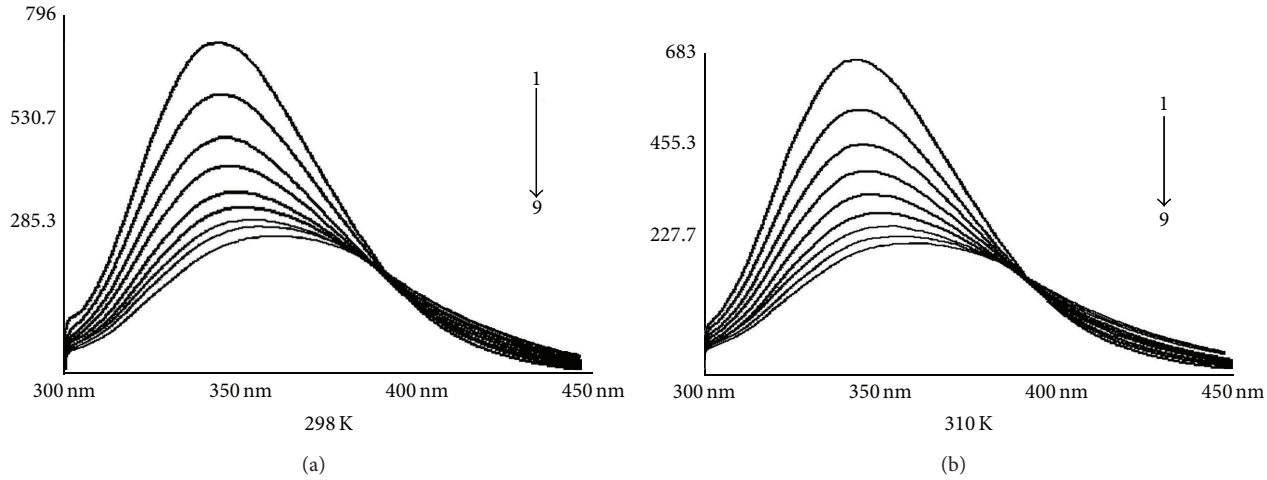


Figure 3: Effect of warfarin on fluorescence spectrum of HSA; form curve 1 → 9, $C_{(HSA)} = 4 \times 10^{-6}$ M, and $C_{(warfarin)} = 0, 2, 4, 6, 8, 10, 12, 14, 16 \times 10^{-6}$ M, respectively.

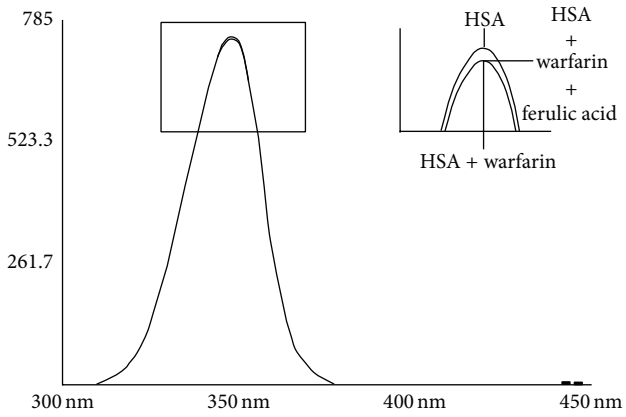


Figure 4: Synchronous fluorescence of effect of absence and presence of ferulic acid on HSA-warfarin, $C_{(HSA)} = 4 \times 10^{-6}$ M, $C_{(warfarin)} = 2 \times 10^{-8}$ M, and $C_{(ferulic\ acid)} = 3 \times 10^{-8}$ M.

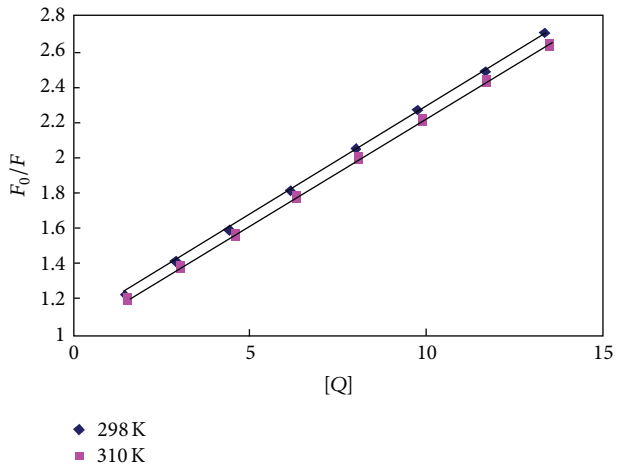


Figure 5: Stern-Volmer plots of warfarin-HSA at different temperatures.

data (Table 2 and Figure 5) had good linearity and the slope declined (K_{SV} from 1.22×10^5 to 1.18×10^5) with increasing temperature. The result suggests that warfarin effectively quenched the intrinsic fluorescence of HSA mainly by static quenching. The static quenching constant K_{LB} (Table 2) was developed by the Lineweaver-Burk equation

$$(F_0 - F)^{-1} = F_0^{-1} + K_{LB}^{-1} F_0^{-1} [Q]^{-1}. \quad (2)$$

The UV-Vis absorption spectra of HSA with warfarin were shown in Figure 6. We found that the maximum absorption wavelengths of HSA were located at 278 nm when warfarin was absent and present. The UV absorbance intensity of HSA was increased with the addition of warfarin, which indicates the formation of a complex between HSA with warfarin and change in protein conformation [21]. This result once again confirmed that warfarin effectively quenched the intrinsic fluorescence of HSA mainly by static quenching.

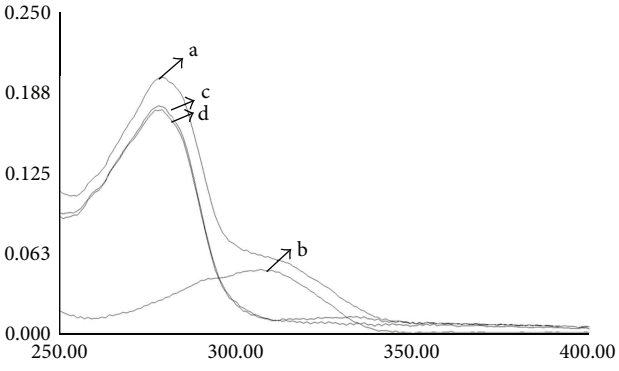


Figure 6: The UV absorption spectra of HSA-warfarin (a), warfarin (b), (HSA-warfarin)-warfarin (c), and HSA (d) $C_{(HSA)} = C_{(warfarin)} = 4 \times 10^{-6}$ M.

Table 2: The binding parameters of warfarin-HSA at different temperatures.

T/K	Stern-Volmer	Lineweaver-Burk	Double logarithm		
	K_{sv} (M)	K_{LB}	K_a	n_H	R^2
298 K	1.22×10^5	1.30×10^5	8.42×10^4	0.9643	0.9969
310 K	1.18×10^5	1.39×10^5	6.17×10^4	0.9428	0.9990

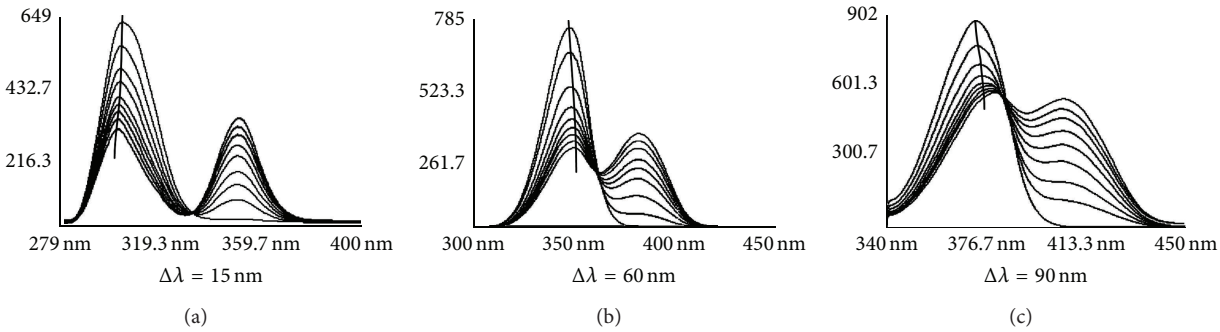


Figure 7: Synchronous fluorescence spectra of interaction between HSA and warfarin.

3.2.4. The Binding Constant, Hill Coefficient, and Binding Distance. For the static quenching interaction, if it is assumed that there are similar and independent binding sites in the biomacromolecule, binding constant (K_a), and the Hill coefficient (n_H) can be got from the double logarithm regression curve equation

$$\lg \frac{(F_0 - F)}{F} = \lg K_a + n_H \lg [Q]. \quad (3)$$

Table 2 gives the values of K_a and n_H at 298 and 310 K.

3.2.5. Conformation Investigation. Synchronous fluorescence spectrum can simplify the spectrum, narrow band, and reduce the spectral overlap. It shows the fluorescence of Tyr residues when $\Delta\lambda = 15$ nm, Trp residues when $\Delta\lambda = 60$ nm, and Phe residues when $\Delta\lambda = 90$ nm. The change of the position of synchronous fluorescence reflects the change of surrounding microenvironment polarity of amino acid residues [22–24]. If the λ_{em} exhibits blueshift, it indicates that the microenvironment polarity of amino acid residues was decreased and hydrophobicity was increased. And redshift, on the other hand, indicates that the microenvironment polarity of amino acid residues was increased and hydrophobicity was decreased. Three synchronous fluorescence spetra were shown in Figure 7. It can be learned that the maximum emission wavelength of Trp and Phe residues had a significant redshift and Tyr residues had a buleshift. It indicated that the conformation of HSA was changed, the polarity around the Trp and Phe residues was increased, and the hydrophobicity was decreased. And the Tyr residues, on the other hand, were decreased, and the hydrophobicity was increased.

3.2.6. Binding Forces. The interaction forces between small organic molecular and biomacromolecule mainly involve hydrogen bonds, Vander Waals forces, electrostatic interactions, hydrophobic forces, and so forth. According to the views of Ross and Subramanian, $\Delta H > 0$ and $\Delta S > 0$ imply a hydrophobic interaction, $\Delta H < 0$ and $\Delta S < 0$ reflect hydrogen bonding and van der Waals interaction, and $\Delta H < 0$ and $\Delta S > 0$ imply electrostatic force. Consider thermodynamic equations [25]

Table 3: Thermodynamic parameter of interaction between HSA and warfarin.

T/K	ΔH (KJ·mol^{-1})	ΔS (J·k^{-1})	ΔG (KJ·mol^{-1})
298	4.28	112.26	−29.17
310		112.26	−30.52

$$\ln\left(\frac{K_{LB2}}{K_{LB1}}\right) = \left(\frac{1}{T1} - \frac{1}{T2}\right)\frac{\Delta H}{R},$$
$$\Delta G = \Delta H - T\Delta S, \quad (4)$$
$$\Delta G = -RT \ln K_{LB}.$$

The values of ΔH, ΔS, and free energy change (ΔG) for warfarin binding to HSA are listed in Table 3. Consequently, the positive ΔH and positive ΔS values suggested that hydrophobic interaction played major roles in warfarin binding to HSA. The negative value of ΔG reveals that the interaction process was spontaneous.

3.3. Effect of Ferulic Acid on Binding Constant and Hill Coefficient. Figure 8 shows the fluorescence spectra of warfarin-HSA in the presence of ferulic acid. According to (3), the K_a and n_H can be got as $K_a = 6.53 \times 10^4$ and $n = 0.9505$. It can be learned that the K_a and n_H were decreased after ferulic acid was added.

Figure 9 presents the contour spectra of (a) HSA, (b) HSA-warfarin, and (c) HSA-warfarin-ferulic acid, respectively. Peak 1 is the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$), and peak 2 is the spectral behavior of Trp residues. The stoke shifts

Table 4: Contour spectra characteristics of HSA, HSA-warfarin, and HSA-warfarin-ferulic acid systems.

System	Peak 2 ($\lambda_{ex}/\lambda_{em}$)	$\Delta\lambda$ (nm)	Intensity
HSA	284/336	52	462
HSA-warfarin	284/338	54	397
HSA-warfarin-ferulic acid	284/339	55	415

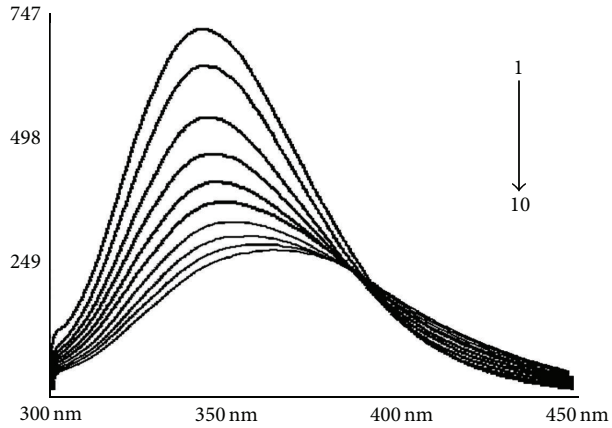


Figure 8: Fluorescence spectra of the effect of ferulic acid on warfarin-HSA, form curve 1 → 10, $C_{(HSA)} = 4\times10^{-6}$ M, $C_{(ferulic\ acid)} = 6\times10^{-6}$ M, and $C_{(warfarin)} = 0, 2, 4, 6, 8, 10, 12, 14, 16 \times 10^{-6}$ M, respectively.

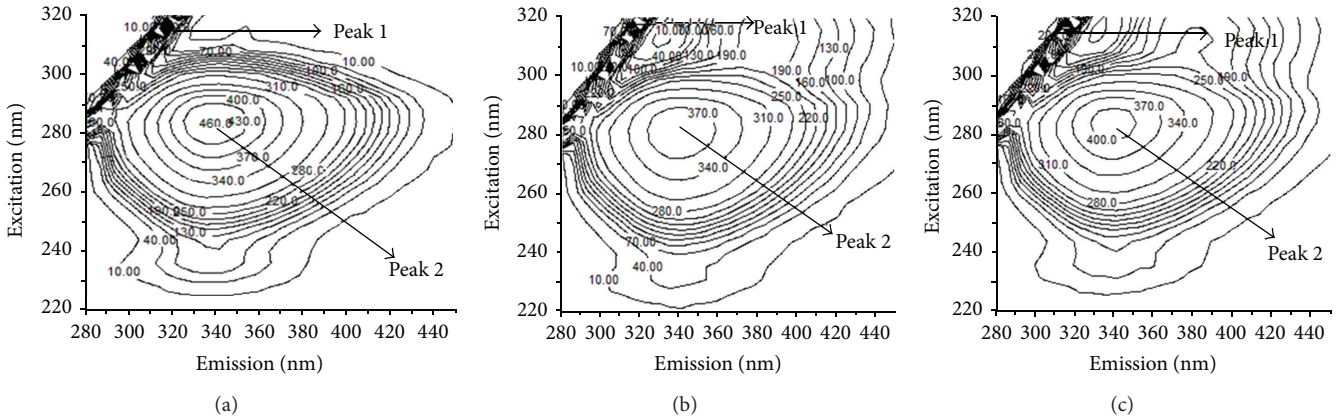


Figure 9: Contour spectra of (a) HSA, (b) HSA-warfarin, and (c) HSA-warfarin-ferulic acid. $C_{(HSA)} = C_{(warfarin)} = 4 \times 10^{-6}$ M, $C_{(ferulic\ acid)} = 6 \times 10^{-6}$ M.

($\Delta\lambda$) and density of contour have been changed after warfarin and ferulic acid were added (Table 4), which indicated that the interactions between them induced microenvironment and conformation changes in HSA. The fluorescence intensities of peak 2 decreased obviously in the presence of warfarin or ferulic acid. Redshift in the presence of warfarin or ferulic acid revealed the Trp residues were brought to a more hydrophilic environment [16].

4. Conclusions

Angelica sinensis (Oliv.) Diels is a popular traditional Chinese medicine (TCM) which is easily obtained by the public not only for medicinal use but also for a dietary supplement. Ferulic acid, [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid], is an abundant hydroxycinnamic acid and one of the most biologically active components in *A. sinensis* [26]. Warfarin, 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one, is the most widely used oral anticoagulant. This paper presents the interaction of warfarin with HSA and effect of ferulic acid on the binding by fluorescence emission and synchronous fluorescence. By decreasing binding constant and the Hill coefficient of warfarin with HSA, ferulic acid could improve the plasma concentration of free warfarin, which would increase the risk of bleeding. Under the condition of simulated human body, the free concentration of

warfarin increased at least 50%. It is very dangerous for the patient stabilized on warfarin because of its narrow therapeutic window. The studies on supermolecular interaction of warfarin with HSA and the influence of ferulic acid on the binding would contribute to the understanding of the metabolic processes of warfarin and the effect of ferulic acid. The results indicated that *A. sinensis* combined treatment with warfarin would increase the risk of bleeding. And the results provide an important theoretical support for warfarin used as oral anticoagulant.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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