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Investigation of nitroxoline-human serum albumin interactions by spectroscopic methods

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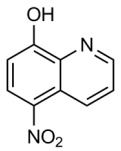
Nitroxoline is a wide spectrum antibacterial and is one of the most important urinary antiseptics. The interaction between nitroxoline and human serum albumin (HSA) has been investigated systematically by fluorescence spectroscopy, synchronous fluorescence, three-dimensional fluorescence, CD spectroscopy and UV-Vis absorption spectroscopy. The results indicated that the quenching of HSA by nitroxoline was static. The corresponding thermodynamic parameters ΔH, ΔS and ΔG calculated according to van't Hoff equation revealed that the intermolecular forces acting between nitroxoline and HSA were mainly hydrogen bonding and van der Waals forces. The conformational changes in the interaction were studied by synchronous fluorescence, CD spectroscopy and three-dimensional fluorescence spectra which showed changes in the microenvironment and conformation of HSA.

nitroxoline, human serum albumin, spectroscopic methods, interaction

1 Introduction

Nitroxoline (5-nitro-8-hydroxyquinoline, Scheme 1) has a wide spectrum of antibacterial activity and is one of the most important urinary antiseptics. It shows antibacterial activity towards both Gram-positive and Gram-negative bacteria and is also effective against certain fungi (e.g. the Candida strain) [1]. Nitroxoline is often used for the treatment of urinary tract infections (pyelonephritis, cystitis, urethritis, prostatitis etc.), the prophylaxis of infections after operations on the kidneys or urinary tract, and other illnesses involving vulnerable microorganisms. Different from other 8-hydroxyquinoline derivates, nitroxoline is rapidly absorbed by the gastrointestinal tract and is excreted unchanged by kidneys [2, 3]. Human serum albumin (HSA), as a principal extracellular protein, is a constituent of blood plasma, which plays a dominant role in the transport and disposition of a variety of endogenous and exogenous substances such as drugs. The primary pharmacokinetics function of HSA is the absorption, distribution, metabolism and excretion of drug, of which the drug distribution is primarily controlled by HSA. It was reported that most drugs diffuse through plasma and reach the target tissues by binding to HSA [4]. The investigation of drug-protein interaction is significant for through understanding the pharmacokinetic



Scheme 1 Chemical structure of nitroxoline (5-nitro-8-hydroxyquinoline).

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behavior of a drug.

Some techniques are commonly used to study the drug-protein interaction, such as fluorescence spectra [5], UV-spectrophotometry [6], circular dichroism spectra (CD) [7], Raman spectra nuclear [8], magnetic resonance (NMR) [9], electrochemistry [10], equilibrium dialysis [11] and Fourier transform infrared (FT-IR) spectra [12], etc. Among them, fluorescence is the most widely used method since it is highly sensitive, rapid, and simple; moreover, synchronous fluorescence and three-dimensional fluorescence spectra can give the information on the changes in the local environment of the fluorophore. By measuring the intrinsic fluorescence quenching of HSA, the accessibility of quenchers to albumin's fluorophores can be revealed, which can give valuable insights for albumin-drugs binding mechanisms and clues about the nature of the drug-protein interaction.

In this paper, the interaction of nitroxoline with HSA was systemically studied by fluorescence spectroscopy, synchronous fluorescence, three-dimensional fluorescence, CD spectroscopy and UV-Vis absorption spectroscopy under simulative physiological condition. Attempts were made to explore the mechanism of this interaction. This work should be able to provide some useful information on therapeutic effects of this type of compounds containing quinoline group in pharmacology and pharmacodynamics.

2 Experimental

2.1 Materials

Nitroxoline (> 99.0%) was obtained from Huanggang Huayang Pharmaceutical Co., Ltd. (China). HSA (99%) was purchased from Sigma-Aldrich Inc. (USA), and used without further purification. HSA solution was prepared on the basis of its molecular weight of 67000 and kept in a refrigerator at 4 °C and dissolved in PBS (pH 7.4) at the concentration of 1×10^{-5} mol L^{-1}. All the other starting materials used were analytical grade. Water used in all procedures was prepared using the Millipore water purification system.

2.2 Fluorescence spectral measurements

All fluorescence spectra were recorded with a LS-55 spectrofluorometer (Perkin-Elmer, UK) equipped with quartz cells (1.0 cm) and a thermostat bath. To determine the fluorescence emission spectrum of the nitroxoline-HSA solution at different temperatures (298, 304 and 310 K), the width of excitation slit and emission slit was set at 15.0 and 10.0 nm, respectively. An excitation wavelength of 280 nm was used throughout to minimize the contribution of the tyrosine residues to the emission. The three-dimensional fluorescence spectra were excited at 200 nm and measured with the emission wavelength from 200 to 500 nm. The total number of scanning was 31 with an increment of 5 nm per scanning.

2.3 UV-Vis absorption spectral measurements

TU-1901 spectrophotometer (Puxi Ltd. of Beijing, China) was used to scan UV-Vis spectra. The absorption spectra were measured in the range from 600 to 250 nm at room temperature. The concentrations of all tested samples were 1×10^{-5} mol L^{-1} for HSA, nitroxoline and nitroxoline-HSA co-solution (both reactants are 1×10^{-5} mol L^{-1}).

2.4 CD measurements

CD measurements were carried out on a JASCO (J-810-150S) automatic recording spectropolarimeter (Japan), using a cylindrical cuvette with 0.1 cm of path-length. In the experiment, the concentration of HSA was kept at 2.0×10^{-6} mol L^{-1}. The spectra were recorded in the absence and presence of nitroxoline with the nitroxoline/HSA ratio 0:1, 5:1 and 10:1, respectively. The CD spectra were obtained at room temperature by employing a scan speed of 500 nm min^{-1} and a response time of 0.5 s. The CD spectra were recorded from 200 to 250 nm under constant nitrogen flush.

3 Results and discussion

3.1 Fluorescence measurements

The binding of nitroxoline to HSA was determined by fluorescence measurements. In this experiment, the HSA solution concentration was stabilized at 1×10^{-5} mol L^{-1}, and the concentrations of nitroxoline were varied from 0 to 2.0×10^{-6} mol L^{-1} with an increment of 0.20×10^{-6} mol L^{-1}. Figure 1 shows the emission spectra of HSA in the absence and presence of nitroxoline. HSA has a strong fluorescence emission peaked at 346 nm when excited with a wavelength of 280 nm. The addition of nitroxoline caused a consecutive decrease in the fluorescence intensity of HSA and the emission maximum had a slight blue-shift of 3 nm. This suggested that the microenvironment around HSA was changed after the addition of nitroxoline [13].

Fluorescence tests were performed at different temperatures to study the quenching process carefully. The two mechanisms of quenching, namely dynamic quenching and static quenching, are different in their temperature effects. Dynamic quenching depends on diffusion. The bimolecular quenching constant is expected to be higher with increasing temperature because high temperature will result in larger diffusion coefficient. In contrast, the static quenching depends on the formation of ground-state complexes, and increased temperature is likely to decrease the stability of the complex, so the value of the static quenching constant decreases with rising temperature. The possible quenching mechanism was analyzed by the Stern-Volmer equation [14]:

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

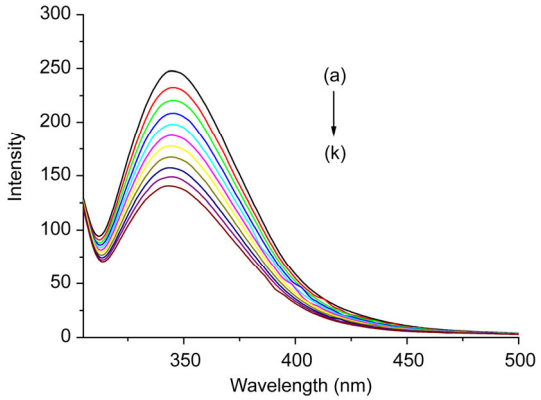

Figure 1 Emission spectra of HSA in the presence of various concentrations of nitroxoline. (a–k) c(nitroxoline) = 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 μmol L^{-1}, respectively. T = 298 K, λ_{ex} = 280 nm, c(HSA) = 1.0 × 10^{-5} mol L^{-1}.

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of quencher (nitroxoline), respectively; k_q is the bimolecular quenching rate constant; τ_0 is the life time of fluorescence in absence of quencher; K_{SV} is the Stern-Volmer quenching constant; and [Q] is the concentration of quencher. Hence the above equation could be applied to determine K_{SV} by linear regression of a plot of F_0/F against [Q]. Figure 2 displays the Stern-Volmer plots of the fluorescence quenching of HSA by nitroxoline at different temperatures. The quenching constant (Figure 2 and Table 1), K_{SV}, decreases with increasing of temperature, which indicates the interactions between nitroxoline and HSA are initiated by compound formation rather than by dynamic collision.

UV-Vis absorption spectra experiment is performed to confirm this static quenching process. UV-Vis absorption measurement is a very simple method to know the complex information and usually applied to explore the structural changes. Dynamic quenching only affects the excited states of the fluorophores, and thus no changes in the absorption spectra are expected. On the contrary, a HSA-drug complex forms in the static quenching, which changes the UV spectrum of HSA. The UV-Vis absorption spectra of HSA, nitroxoline and nitroxoline-HSA system are shown in Figure 3. As can be seen from the figure, the UV absorption spectrum of HSA (Figure 3(a)) and the absorption spectrum (Figure 3(d)) obtained by subtracting the absorption spectrum of nitroxoline from that of nitroxoline-HSA at the same concentration are different. This result reconfirms that the fluorescence quenching of HSA is primarily caused by complex formation between nitroxoline and HSA [15].

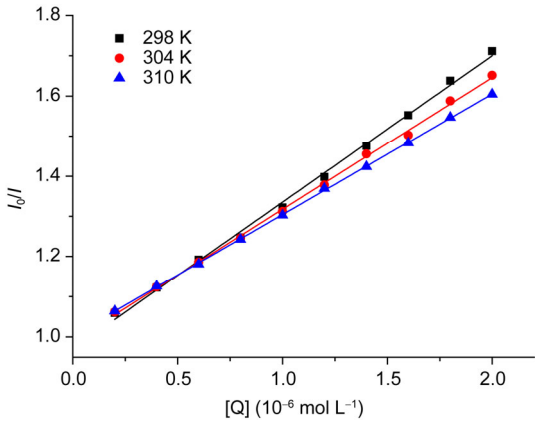

Figure 2 Stern-Volmer plots for the quenching of HSA by nitroxoline at different temperatures. pH 7.4.

Table 1 Stern-Volmer quenching constants for the interaction of nitroxoline with HSA at three different temperatures

pH	T (K)	K_{SV} (× 10^5 L mol^{-1})	k_q (× 10^{13} L mol^{-1} s^{-1})	R [a]	S.D. [b]
7.4	298	3.649	3.649	0.997	0.0064
	304	3.283	3.283	0.998	0.0040
	310	3.007	3.007	0.999	0.0013

a) Linear correlated coefficient; b) standard deviation for the K_{SV} values.

For a complex formation process, a modified Stern-Volmer equation (Eq. (2)) is used to calculate the affinity constant K_b for the binding between nitroxoline and HSA:

$$\frac{F_0}{\Delta F} = \frac{1}{fK_b}\frac{1}{[Q]} + \frac{1}{f} \quad (2)$$

where f represents the fraction of accessible fluorescence and K_b is the effective quenching constant. $F_0/\Delta F$ is linear with the reciprocal value of the quencher concentration [Q], and the slope equals to the value of $(f\,K_b)^{-1}$. Figure 4 shows the linear plots at varying temperatures based on the modified Stern-Volmer and the corresponding values of K_b are listed in Table 2.

3.2 Type of interaction force between nitroxoline and HSA

Considering that, the force between small organic molecule

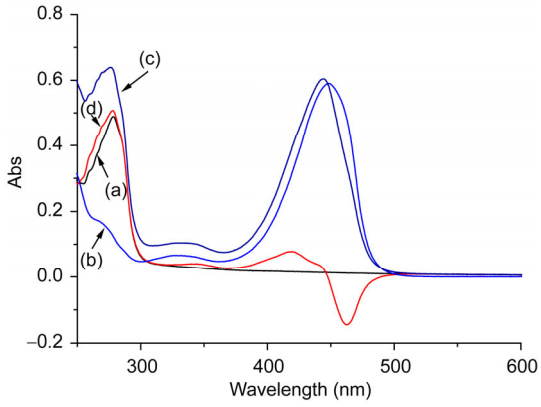

Figure 3 UV-Vis absorption spectra of HSA, nitroxoline and nitroxoline-HSA solutions. c(HSA) = c(nitroxoline) = 1.0 × 10^{-5} mol L^{-1}. (a) The absorption spectrum of HSA only; (b) the absorption spectrum of nitroxoline only; (c) the absorption spectrum of compound nitroxoline-HSA when the mole ratio is 1:1; (d) the difference absorption spectrum between nitroxoline-HSA and nitroxoline at the same concentration.

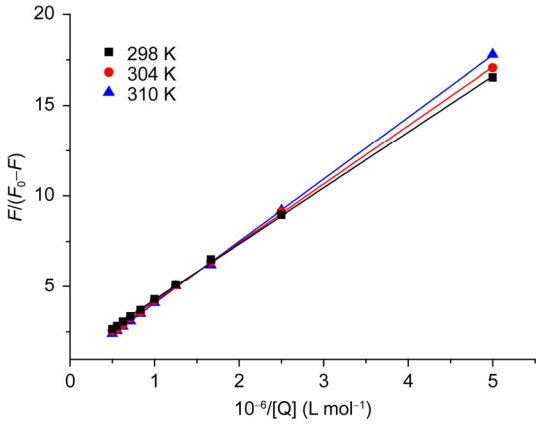

Figure 4 Modified Stern-Volmer plots of nitroxoline and HSA at different temperatures.

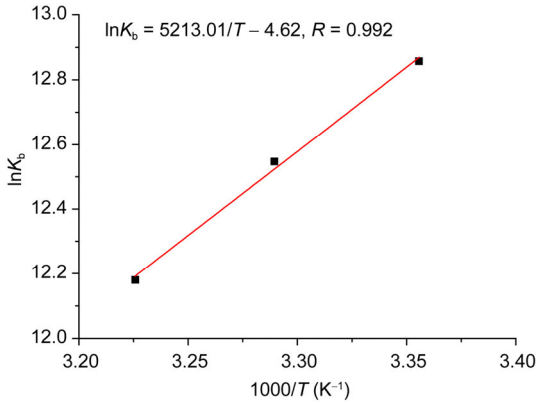

Figure 5 Arrhenius plot for interaction between HSA and nitroxoline.

and biological macromolecule includes hydrophobic force, hydrogen bond, van der Waals force and electrostatic interactions [16], etc., the thermodynamic parameters, enthalpy change (ΔH) and entropy change (ΔS) of the reaction are important for the study of the interaction force. The values of ΔH and entropy change ΔS can be estimated from the following van't Hoff equation:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

And the free energy changes (ΔG) at different temperatures can be calculated from the following relationship:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K \quad (4)$$

In Eq. (3), K is analogous to the effective quenching constants K_b at the corresponding temperature. The enthalpy change (ΔH) can be regarded as a constant since the temperature only changes a little (12 K) and it can be calculated from the slope of the van't Hoff relationship.

The thermodynamic parameters were calculated from Eqs. (3) and (4) (Figure 5) and summarized in Table 2. We can see that the binding reaction of nitroxoline to HSA is exothermic ($\Delta H < 0$). This suggests that higher temperatures will weaken the binding, which can also be as indicated by the decreasing values of K_b (Table 2). The negative ΔH and ΔS values indicated that both van der Waals forces and hydrogen bonding play vital role in the binding of nitroxoline to HSA [17]. The negative ΔG at all temperatures studied reveals that the interaction process between nitroxoline and HSA can occur spontaneously.

3.3 Conformation investigations

To further investigate whether the conformation and microenvironment of HSA is affected by the binding of nitroxoline to HSA, the methods of synchronous fluorescence spectroscopy, CD, and three-dimensional fluorescence spectroscopy were utilized.

Synchronous fluorescence spectroscopy studies

As introduced by Llody [18], the synchronous fluorescence spectroscopy is a very useful method to study the microenvironment of amino acid residues by measurement of the emission wavelength shift [19]. When the *D*-value ($\Delta\lambda$) between excitation and emission wavelength are stabilized at 15 or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine or tryptophan residues, respectively [20]. As shown in Figure 6, when $\Delta\lambda$ was set to 15 nm (Figure 6(A)), the fluorescence intensity decreased steadily with the addition of nitroxoline while no significant blue shift or red shift of the maximum emission wavelength is observed. This revealed that there was no change in the microenvironment of the tyrosine residue. When $\Delta\lambda$ = 60 nm (Figure 6(B)), the fluorescence intensity decreased more and there is a slight blue shift of the maximum emission wavelength, which indicated that the microenvironment around the tryptophan residues was disturbed and the hydrophobicity of the tryptophan residues increases in the presence of nitroxoline. The fluorescence intensity decreased steadily with the addition of

Table 2 Apparent binding constants K_b at different temperatures and relative thermodynamic parameters of the nitroxoline-HSA system

pH	T (K)	K_b (× 10^5 L mol^{-1})	R [a)]	S.D. [b)]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
7.4	298	3.836	0.999	0.0016	–43.32	–31.87	–38.41
	304	2.950	0.999	0.0016		–31.64	
	310	1.948	0.999	0.0021		–31.41	

a) Linear correlated coefficient for the K_b values; b) standard deviation for the K_b values.

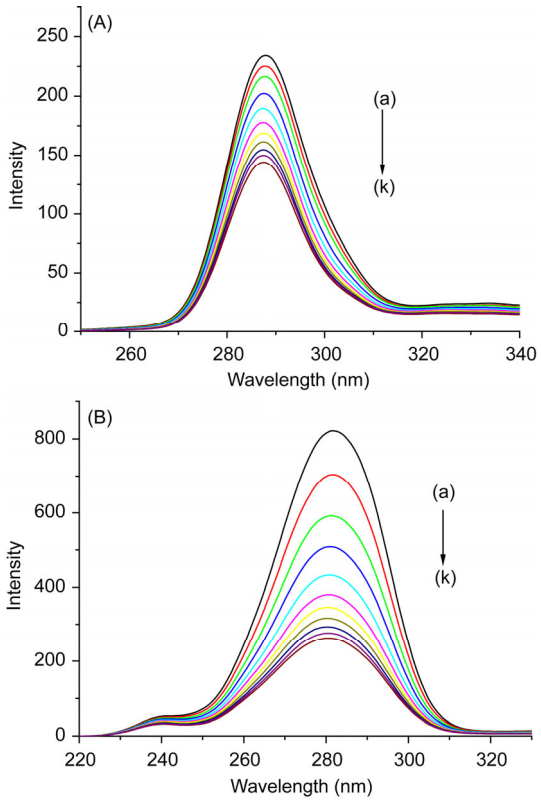

Figure 6 Synchronous fluorescence spectra of HSA. (A) $\Delta\lambda$ = 15 nm; (B) $\Delta\lambda$ = 60 nm. (a–k) c(nitroxoline) = 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 ($\times 10^{-5}$ mol L^{-1}), respectively. c(HSA) = 1.0×10^{-5} mol L^{-1}.

nitroxoline as seen in both figures, which further demonstrated the occurrence of fluorescence quenching in the nitroxoline-HSA binding process.

Circular dichroism spectra

The CD spectra of HSA with various concentration of nitroxoline in PBS (pH 7.4) are shown in Figure 7. It can be seen from the figure that HSA exhibits two negative bands at 208 and 222 nm, which is the characteristic of the α-helix structured protein. Thus, the CD spectra will change if the content of α-helix is altered. The CD results were expressed in terms of mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ according to the following equation:

$$\mathrm{MRE} = \frac{\text{observed CD (m deg)}}{C_p n l \times 10} \quad (5)$$

where C_p is the molar concentration of the protein; n is the number of amino acid residues (585 for HSA); and l is the path-length (0.1 cm). The α-helical contents of free and combined HSA were calculated from MRE values at 208 nm using the following equation:

$$\alpha\text{-helix}(\%) = \frac{-\mathrm{MRE}_{208} - 4000}{33000 - 4000} \quad (6)$$

where MRE_{208} is the observed MRE value at 208 nm; 4000 is the MRE of the β-form and random coil conformation at 208 nm; and 33000 is the MRE value of a pure α-helix at 208 nm. From the above equation, the α-helicity in the secondary structure of HSA was determined. The contents of α-helix and β-strand are calculated using algorithm SELCON3 through the CD spectroscopic data.

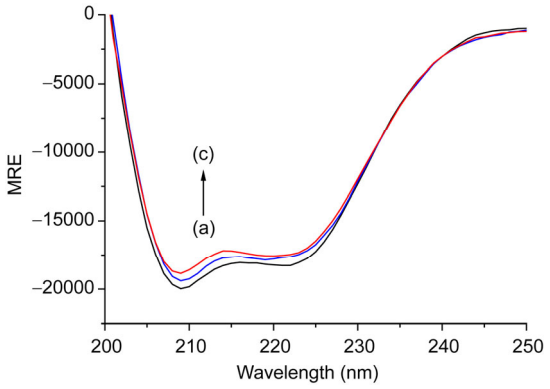

Figure 7 The far-UV CD spectra of the nitroxoline-HSA system obtained in PBS of pH 7.4 at room temperature. (a–c) c(nitroxoline) = 0, 5.0, 10.0 ($\times 10^{-6}$ mol L^{-1}), respectively. c(HSA) = 2.0×10^{-6} mol L^{-1}.

The secondary-structure contents of protein are closely related to the biological activity. The data in Table 3 show that the content of α-helix decreases while the content of β-strand and unordered structure increases with increasing concentration of nitroxoline. This result suggests that the secondary-structure of HSA has been changed during its interaction with nitroxoline [21].

Three-dimensional fluorescence spectra

To further elucidate the conformational change of HSA induced by nitroxoline, the three-dimensional fluorescence spectra were applied to monitor the changes in the secondary structure of protein. Figure 8 shows the three-dimensional fluorescence spectra of HSA (Figure 8(a)) and nitroxoline-HSA system (Figure 8(b)), respectively. Two peak regions (Peak 1 at 280/345 nm and Peak 2 at 225/343 nm) were observed in which Peak 1 shows the spectral characteristics of tryptophan and tyrosine residues while Peak 2 relates to the fluorescence spectra of polypeptide backbone structures. As can be seen from Figure 8, both peaks in the three-dimensional fluorescence spectra of HSA are quenched (Peak 1: from 407.4 to 299.8; Peak 2: from 337.9 to 249.2). This indicates that the interaction of nitroxoline with HSA has induced some micro-environmental and conformational changes in HSA [22].

Table 3 Parameters of CD spectral study of nitroxoline-HSA system

HSA/nitroxoline	α-Helix	β-Strand	Unordered
1:0	0.596	0.062	0.235
1:5	0.583	0.070	0.245
1:10	0.556	0.072	0.248

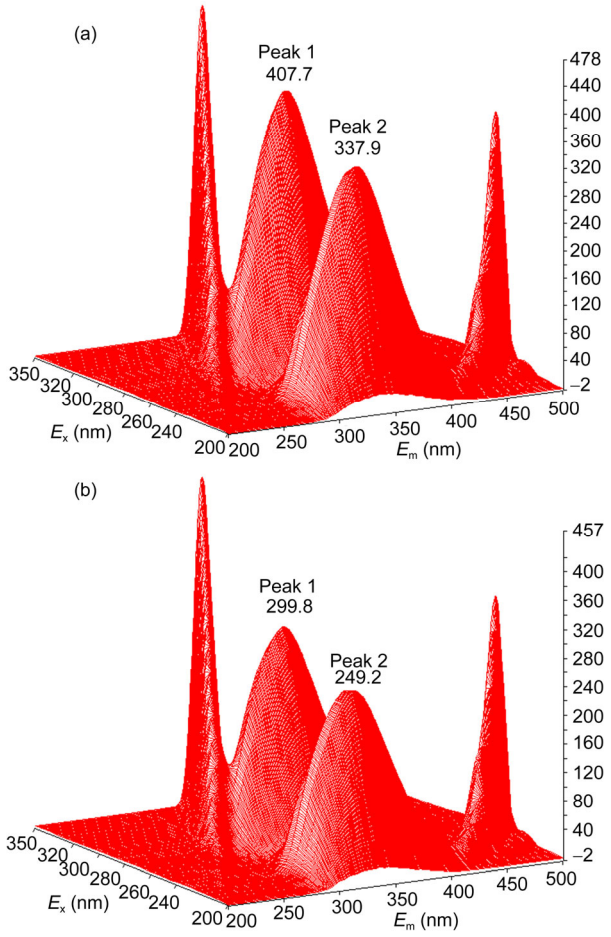

Figure 8 Three-dimensional fluorescence spectra of HSA (a) and nitroxoline-HSA (b) system. (a) c(HSA) = 2.0 × 10^{-6} mol L^{-1}, c(RNZ) = 0 mol L^{-1}; (b) c(HSA) = 2.0 × 10^{-6} mol L^{-1}, c(RNZ) = 2.0 × 10^{-6} mol L^{-1}.

On the basis of the discussion of synchronous fluorescence spectra, CD spectra and 3D fluorescence spectra, we can conclude that the binding of nitroxoline with HSA induces some minor conformational changes in the protein.

4 Conclusions

To provide some useful information on therapeutic compounds containing quinoline group, the interaction between nitroxoline and HSA was studied by fluorescence spectroscopy combined with UV-Vis and CD spectroscopy. The results demonstrated that the interaction between nitroxoline and HSA is mainly based on a complex formation process. The binding of nitroxoline with HSA was spontaneous and mainly mediated by van der Waals forces and hydrogen bonding. The experimental results also showed that this binding induces some micro-environmental and conformational changes in HSA. Thus, this paper provides a new strategy to explore the biological interaction of nitroxoline with HSA at molecular level.

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