The Interaction of Cefoperazone Sodium with Bovine Transferrin and Bovine Serum Albumin

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Abstract: The interactions of cefoperazone sodium with bovine transferrin and bovine serum albumin were studied by multi-spectroscopic methods. Results showed that the intrinsic fluorescence of proteins was quenched by the cefoperazone sodium with a static quenching procedure. The thermodynamics parameters indicated that electrostatic attraction played a major role in the interactions of drug and proteins. The results of synchronous fluorescence spectra demonstrated that the microenvironments of amino acid residues of the two proteins were disturbed by cefoperazone sodium and the binding site of cefoperazone sodium to the bovine transferrin/bovine serum albumin was closer to tryptophan residues. Circular dichroism indicated that cefoperazone sodium changed the secondary structures of the two proteins. Hill's coefficient showed that there was negative cooperation in the interaction of subsequent cefoperazone sodium with bovine transferrin/bovine serum albumin. Moreover, the results showed that cefoperazone sodium bound to bovine serum albumin with higher affinity. However, cefoperazone sodium had larger influences on the microenvironment of bovine transferrin. The interaction between cefoperazone sodium and different proteins will be helpful for extracting the common features, applying the unique characteristic of drug-proteins systems.

Keywords: Bovine Transferrin, Bovine Serum Albumin, Cefoperazone Sodium, Cooperativity,
Multi-spectroscopic Methods

1. Introduction

Currently, the interaction of drug and protein has been studied widely [1, 2]. Binding of drugs to plasma proteins has vital implications for drug disposition and action. Effect of a drug depends on the availability of free drug in plasma. The free drug in plasma can bind to the receptor sites and exerting pharmacological action [3]. The free concentration and pharmacologic activity of drug in vivo is closely related to its binding capacity towards transport protein [4]. Moreover, the interaction of drugs with proteins may significantly affect their distribution, metabolism, excretion and toxicity in vivo. It forms the basis to re-design or modify the drug molecular, and is an important way to understand the biological effects of protein. Therefore, a comprehensive understanding of the binding characteristics of drugs to proteins at a molecular level is imperative and worthwhile.

Cefoperazone sodium is a semi-synthetic third generation, piperazine β-lactam antibiotics that possesses broad spectrum activity against aerobic and anaerobic gram-positive and gram-negative bacteria [5]. But there is a relatively weak effect on gram-positive bacteria. Cefoperazone sodium is a common drug to treat respiratory infections, biliary tract infection and septicemia which caused by the above bacteria, and exhibits excellent efficacy for treatment of pseudomonas aeruginosa infection [6]. The molecular weight of cefoperazone sodium is 667.66, and its structure was shown in the figure 1.

Transferrin is a single-chain glycoprotein containing around 630 amino acids which are arranged in two homologous lobes, normally designated as the N-terminal and

C-terminal lobes [7]. Each lobe comprises two domains (N_1, N_2 and C_1, C_2), connected by a short peptide spacer [8], and each lobe contains two domains comprising a series of α-helices and β-sheets [9]. TF contains two specific Fe^{3+} binding sites, and of them only 30% is iron-bound. While in other empty binding sites, TF not only bind metal ions but also bind some drug molecules [10]. Some studies had confirmed TF had a high potential for binding to therapeutic agents and acting as a carrier delivering an extensive range of drugs such as anticancer drugs into malignant and tumor cells [11, 12]. And the receptor-mediated endocytosis of transferrin and transferrin receptor is one of the most characteristic transport processes in living cells. Therefore, transferrin has been proposed as a potential drug carrier.
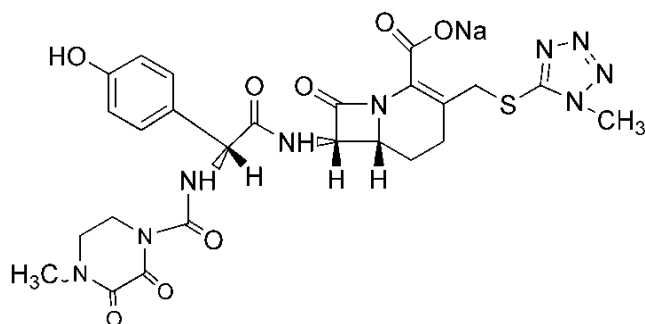

Figure 1. The molecular structure of cefoperazone sodium.

Bovine serum albumin is a major transport globular protein found in plasma. For bovine serum albumin, there is 583 amino acid residues arranged in a single polypeptide chain and 17 disulfide bridges leading to a series of nine loops [13]. Bovine serum albumin is made up of three homologous domains (I-III), each domain contains two sub-domains (A and B) [14]. Bovine serum albumin has two major drug binding sites in the sub-domains IIA (site I) and IIIA (site II) [15]. Bovine serum albumin has been studied extensively as its stability, low cost, ready availability, and outstanding ligand-binding properties. It serves as a depot and transport protein for numerous endogenous and exogenous compounds.

In this study, the reaction mechanism of cefoperazone sodium and bovine transferrin/bovine serum albumin was explored by multi-spectroscopic methods. Spectroscopic methods are common methods which are used to study the interaction of drug and protein, especially fluorescence quenching. Chemistry and fluorescence quenching is an important way to study such interaction because of its sensitivity, accuracy, and convenience of usage [16, 17]. Currently, the study of the interaction between drug and protein use one protein as a model protein mostly. However, the reports about a comparative study on the interactions of drug with two proteins are very rarely. Investigation on the interactions of drug with different proteins can study the efficacy of the drug more widely, understand the effects of drugs on the structure and function of proteins, understand the essence of the interactions between drug and proteins. And the interactions between drug and different proteins will be helpful for extracting the common features of the two systems,

applying the unique characteristic of each system [18]. Studying the interaction of bovine transferrin/bovine serum albumin and cefoperazone sodium from different perspectives is necessary to reveal more specific information about translocation and distribution of cefoperazone sodium in vivo, and it has important implication for the clinical application of cefoperazone sodium.

2. Experimental

2.1. Reagents and Apparatus

Apparatus: All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Absorption was measured with an Ultraviolet-Visible recording spectrophotometer (UV-265, Shimadzu, Japan). Circular dichroism spectra were recorded on a MOS-450/SFM300 circular dichroism spectrometer (Bio-Logic, France). All pH measurements were carried out with a pHS-3C precision acidity meter (Leici, Shanghai, China). All temperatures were controlled by a SYC-15B superheated water bath (Nanjing Sangli Electronic Equipment Factory, Sangli, Nanjing, China).

Reagents: Cefoperazone sodium was of the purity grade inferior 98.5%. Bovine transferrin was purchased from Sigma-Aldrich (purity grade inferior 98.5%). Bovine serum albumin was purchased from Sigma-Aldrich (purity grade inferior 98.5%). Stock solutions of bovine transferrin (2.0 μM), bovine serum albumin (2.0 μM) and cefoperazone sodium (1.0 mM) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris-HCl buffer solution containing NaCl (0.15 M) was used to keep the pH of the solution at 7.40, and NaCl solution was used to maintain the ionic strength of the solution. All other reagents were of analytical grade, and all aqueous solutions were prepared with fresh double-distilled water and stored at 277 K.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter using the following relationship [19]:

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2} \tag{1}$$

Where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities, respectively, and A_{ex} and A_{em} are the absorbance values of cefoperazone sodium at excitation and emission wavelengths, respectively. The fluorescence intensity used in this article was corrected.

2.2. Procedures

2.2.1. Ultraviolet-Visible Absorption Measurements

1.0 mL Tris-HCl (pH=7.40), 2.0 mL bovine serum albumin or bovine transferrin solution (10.0 μM) and different concentrations of cefoperazone sodium were added into 10 mL colorimetric tube successively. The reference was different concentrations of cefoperazone sodium solution. The samples were diluted to scaled volume with double-distilled

water, mixed thoroughly by shaking, and kept static for 30 min at 298 K. The ultraviolet-visible absorption spectra of bovine serum albumin or bovine transferrin in the presence and absence of cefoperazone sodium were recorded with 1.0 cm quartz cells in the range from 190 nm to 350 nm.

2.2.2. Fluorescence Measurements

In a typical fluorescence measurement, 1.0 mL Tris-HCl (pH=7.40), 1.0 mL bovine serum albumin or bovine transferrin solution (2.0 μM) and different concentrations of cefoperazone sodium were added into 10 mL colorimetric tube successively. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 30 min at different temperatures (298, 303 and 310 K). The excitation wavelength for bovine serum albumin or bovine transferrin was 280 nm and 295 nm, respectively, with a 1.0 cm path length cell. The excitation and emission slits were set at 5 nm. The solutions were subsequently scanned on the fluorophotometer and recorded the fluorescent intensities of bovine serum albumin and bovine transferrin.

2.2.3. Synchronous Fluorescence Measurements

Solution preparation was as detailed above. The fluorescence spectra of the bovine transferrin/bovine serum albumin-cefoperazone sodium system were recorded when the wavelength interval (Δλ) value between the excitation and emission wavelengths was stabilized at 15 and 60 nm, respectively.
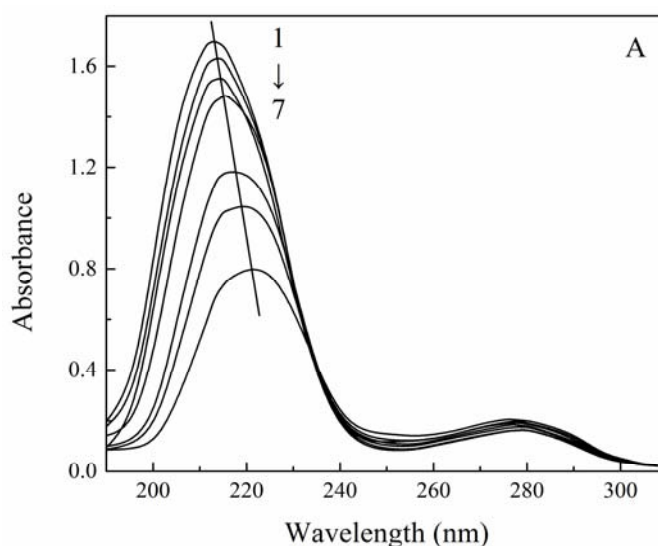
2.2.4. Circular Dichroism Measurements

1.0 mL Tris-HCl (pH=7.40), 1.0 mL bovine serum albumin or bovine transferrin solution (10.0 μM) and different concentrations of cefoperazone sodium were added into 10 mL colorimetric tube successively, which made the concentration ratio of [bovine serum albumin]/[bovine transferrin]:[cefoperazone sodium] were 1:0, 1:10, 1:20, respectively. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 30 min at 298 K. Circular dichroism measurements were carried out with a 0.1 cm path length quartz cuvette. Each spectrum was recorded at wavelengths between 200 and 300 nm and a scan speed of 1 nm sec^{-1}.

3. Results and Discussion

3.1. Ultraviolet-Visible Absorption spectra Studies

In order to validate the quenching mechanism of fluorescence of bovine transferrin/bovine serum albumin-cefoperazone sodium system, the absorption spectra of bovine transferrin/bovine serum albumin in the presence and absence of cefoperazone sodium were recorded and presented in figure 2. Static quenching, a compound is formed between ground state of fluorescence substance and quencher, therefore the absorption spectra of fluorescence substance would be influenced [20]. In contrast, the dynamic quenching only affects the excited state of the fluorophore and thus no changes in the absorption spectra are expected [21]. As can be seen from figure 2, bovine transferrin had two absorption peaks at 213 nm and 280 nm, bovine serum albumin had two absorption peaks at 210 nm and 280 nm. The intensities of the absorption peak of bovine transferrin (213 nm) and bovine serum albumin (210 nm) obviously decreased with the addition of cefoperazone sodium, and the peaks both shifted toward longer wavelength. After analyzing the spectra plot, it could be demonstrated that the absorption spectrum of bovine transferrin/bovine serum albumin (curve 1) is different from the spectra of bovine transferrin/bovine serum albumin-cefoperazone sodium system (curves 2-7). Meanwhile, the difference cannot be treated as within experimental error. So, the static quenching mechanism for bovine transferrin/bovine serum albumin-cefoperazone sodium system can be confirmed.
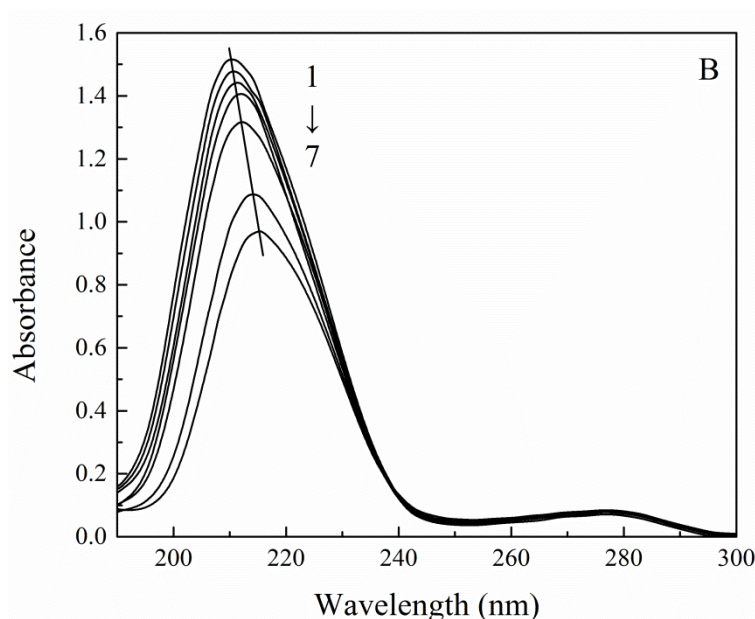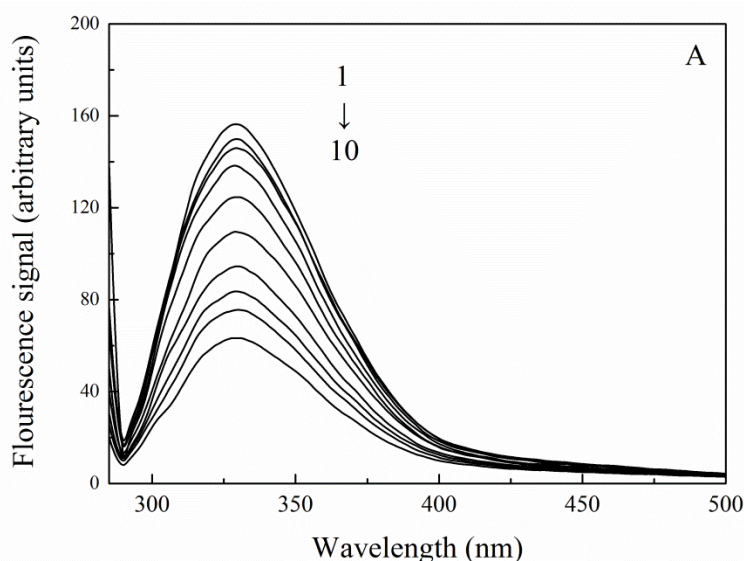
Figure 2. *Absorption spectra of bovine transferrin-cefoperazone sodium (A) and bovine serum albumin-cefoperazone sodium (B) systems (T=298 K) $C_{bovine\ serum\ albumin}=C_{bovine\ transferrin}=2.0\ \mu M$, $C_{cefoperazone\ sodium}=(0, 4.0, 6.0, 8.0, 20.0, 40.0, 50.0)\ \mu M$.*

3.2. Analysis of Fluorescence Quenching Spectroscopy

Fluorescence spectroscopy, which is a sensitive technique and easy to use, is suitable for assessing interactions between micro-molecules and macro-molecules [22]. Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample such as excited state reactions, energy transfers, ground-state complexes formation and collisional process [23]. It is well known that there are two quenching mechanisms involved in quenching process, which are usually classified as dynamic quenching and static quenching. They can be distinguished by their different dependence on temperature [24]. Dynamic quenching is expected to increase the quenching constants with a gradually increasing temperature, because higher temperatures results in larger diffusion coefficients. On the other hand, an increase in temperature is likely resulting in a smaller static quenching constant due to the dissociation of weakly bound complexes [25]. In this study, fluorescence quenching experiments were performed according to the experimental step of "Fluorescence measurements". The fluorescence spectra of bovine transferrin/bovine serum albumin-cefoperazone sodium system were shown in figure 3. From figure 3 it can be seen that, when the excitation wavelength (λ_{ex}) was 280 nm, bovine transferrin had a strong fluorescence emission peak at 330 nm, and bovine serum albumin had a strong fluorescence emission peak at 340 nm. Fluorescence was not observed in the cefoperazone sodium or buffer solution in the experimental conditions. The fluorescence intensity of bovine transferrin/bovine serum albumin decreased regularly with the addition of cefoperazone sodium, which indicated that the cefoperazone sodium bound to bovine transferrin/bovine serum albumin.
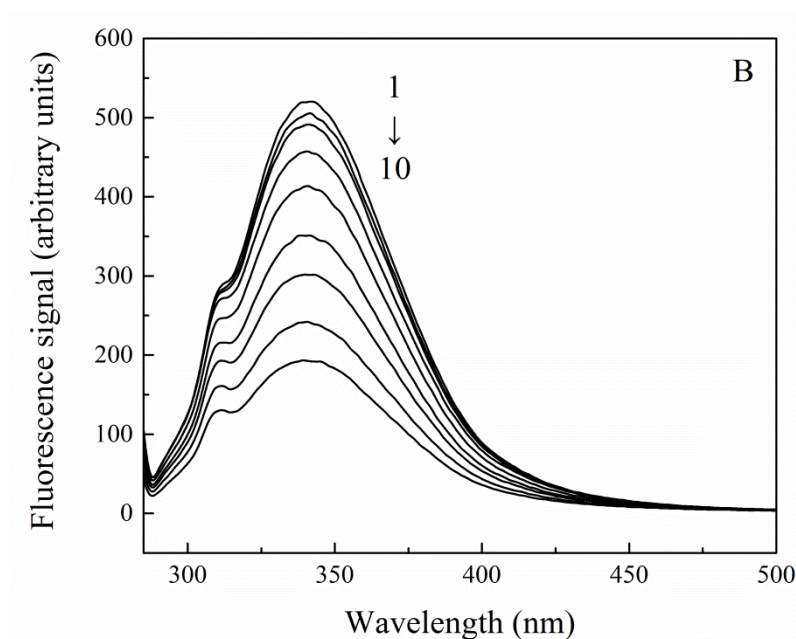
Figure 3. *Fluorescence spectrums of bovine transferrin-cefoperazone sodium (A) and bovine serum albumin-cefoperazone sodium (B) systems (T=298 K, λ_{ex}=280 nm) $C_{bovine\ transferrin}$=0.2 μM, $C_{cefoperazone\ sodium}$=(0, 0.8, 6.0, 10.0, 20.0, 40.0, 50.0, 70.0, 80.0, 100.0) μM.*

To confirm the quenching mechanism between cefoperazone sodium and the two proteins, the fluorescence quenching data were analyzed using the Stern-Volmer Eq. (2) [26]:

$$F_0 / F = 1 + K_q \tau_0 [L] = 1 + K_{SV}[L] \qquad (2)$$

Where, F_0 and F represent the fluorescence signals in the absence and presence of quencher, respectively. τ_0 is the average lifetime of fluorescence without quencher, which is about 10^{-8} s. K_{sv} is the Stern-Volmer quenching constant. K_q is the bimolecular quenching constant, and $[L]$ is the concentration of the quencher. According to Eq. (2), based on the linear fit plot of F_0/F versus $[L]$, the K_{sv} values can be obtained. The results were shown in Table 1. These results indicated that the probable fluorescence quenching mechanism of bovine transferrin/bovine serum albumin by cefoperazone sodium is a static quenching procedure in the experimental concentration range, because the K_{SV} decreased with the rising temperature and the values of K_q were larger than the maximum scatter collision quenching constant values of various quenchers (2.00×10^{10} M^{-1} s^{-1}).

For static quenching, the relationship between the fluorescence intensity and the concentration of quencher can usually be described using Eq. (3) [27] to obtain the binding constants (K_a) and the number of binging sites (n):

$$\log(F_0/F - 1) = n \log K_a + n \log\{[L] - n(1 - F/F_0)[B_t]\} \quad (3)$$

Where $[L]$ and $[B_t]$ are the total concentrations of cefoperazone sodium and bovine transferrin, respectively. On the assumption that n in the bracket is equal to 1, the curve of

log $(F_0/F−1)$ versus log$\{[L]−n$ $(1−F/F_0)[B_t]\}$ is drawn and linearly fitted, then the value of n can be obtained from the slope of the plot. If the value of n is not equal to 1, it is substituted into the bracket and the curve of log $(F_0/F−1)$ versus log$\{[L]−n$ $(1−F/F_0)$ $[B_t]\}$ is re-drawn. This process is iterated until a single value is obtained for n. Based on the value of n obtained, the binding constant K_a can also be determined. The results were shown in Table 2. The results showed that all the values of n were approximately equal to 1 at different temperatures, implying that there was just one binding site for cefoperazone sodium existed in bovine transferrin or bovine serum albumin. Meanwhile, the binding constants between cefoperazone sodium and bovine transferrin/bovine serum albumin decreased with increasing temperature, which indicated that high temperature reduced the binding affinity of bovine transferrin/bovine serum albumin and cefoperazone sodium, further suggesting that the quenching was a static process [28]. In addition, as shown in Table 2, the binding constants when λ_{ex}=280 nm were greater than the binding constants when λ_{ex}=295 nm at the same temperature. This indicated that tyrosine residues and tryptophan residues were both involved in the interaction of bovine transferrin/bovine serum albumin and cefoperazone sodium. Comparing the data in Table 2, we could see the binding constants of bovine serum albumin-cefoperazone sodium system were larger than the bovine transferrin-cefoperazone sodium system significantly, which indicated that the binding between bovine serum albumin and cefoperazone sodium was stronger. This may be due to the structure of bovine serum albumin is different from bovine transferrin.

Table 1. *Quenching constants of cefoperazone sodium and bovine transferrin/bovine serum albumin at different temperatures.*

	λ_{ex} (nm)	T (K)	K_q (L·mol^{-1}·s^{-1})	K_{SV} (L·mol^{-1})	R_1	SD
		298	1.42×10^{12}	1.42×10^{4}	0.9967	0.0553
	280	303	1.28×10^{12}	1.28×10^{4}	0.9934	0.0437
bovine transferrin-cefoperazone sodium		310	1.16×10^{12}	1.16×10^{4}	0.9908	0.0415
		298	3.15×10^{11}	3.15×10^{3}	0.9928	0.0141
	295	303	2.92×10^{11}	2.92×10^{3}	0.9961	0.0328
		310	2.88×10^{11}	2.88×10^{3}	0.9995	0.0023
		298	2.51×10^{12}	2.51×10^{4}	0.9987	0.0375
	280	303	2.40×10^{12}	2.40×10^{4}	0.9992	0.0273
bovine serum albumin-cefoperazone sodium		310	2.08×10^{12}	2.08×10^{4}	0.9991	0.0210
		298	9.44×10^{11}	9.44×10^{3}	0.9949	0.0034
	295	303	9.18×10^{11}	9.18×10^{3}	0.9967	0.0045
		310	8.10×10^{11}	8.10×10^{3}	0.9955	0.0161

R_1 is the linear relative coefficient of $F_0/F \sim$ [L]; SD is the standard deviation of K_{SV}.

Table 2. *Binding constants of cefoperazone sodiumand bovine transferrin/bovine serum albumin at different temperatures.*
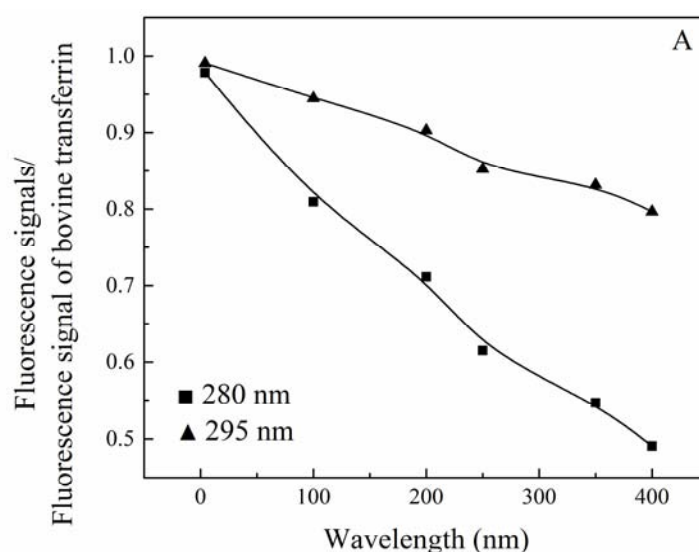
	λ_{ex} (nm)	T (K)	K_a (L·mol^{-1})	n	R_2	SD
		298	1.27×10^{4}	1.09	0.9969	0.0448
	280	303	1.16×10^{4}	1.07	0.9967	0.0395
bovine transferrin-cefoperazone sodium		310	1.06×10^{4}	0.96	0.9985	0.0383
		298	2.41×10^{3}	0.87	0.9905	0.0644
	295	303	2.34×10^{3}	0.84	0.9961	0.0137
		310	2.28×10^{3}	0.82	0.9958	0.0371
		298	2.50×10^{4}	0.97	0.9984	0.0246
	280	303	2.40×10^{4}	0.89	0.9962	0.0309
bovine serum albumin-cefoperazone sodium		310	2.33×10^{4}	0.82	0.9978	0.0212
		298	1.04×10^{4}	0.76	0.9952	0.0429
	295	303	9.58×10^{3}	0.73	0.9967	0.0450
		310	8.02×10^{3}	0.79	0.9994	0.0238

R_2 is the linear relative coefficient of log $[(F_0-F)/F] \sim$ log [L]. SD is the standard deviation of K_a.

3.3. Participation of Amino Acid Residues Studies

When the excitation wavelength at 280 nm is adopted, the naturally fluoresces of protein is mainly attributed to emission of tryptophan and tyrosine residues [29]. Whereas when the excitation wavelength is fixed at 295 nm, only tryptophan residues are excited [30]. By comparing the fluorescence quenching of bovine transferrin/bovine serum albumin excited at 280 and 295 nm, the participation of tyrosine and tryptophan groups in bovine transferrin/bovine serum albumin-cefoperazone sodium system can be assessed [31].

Figure 4 is the quenching curves of bovine transferrin/bovine serum albumin-cefoperazone sodium system, from the figure 4 we can see that the quenching curves of bovine transferrin/bovine serum albumin excited at 280 and 295 nm did not overlap in the presence of cefoperazone sodium, and that the quenching curve of bovine transferrin/bovine serum albumin at 280 nm was much greater than that at 295 nm. This indicated that the tyrosine and tryptophan residues were both involved in the reaction.
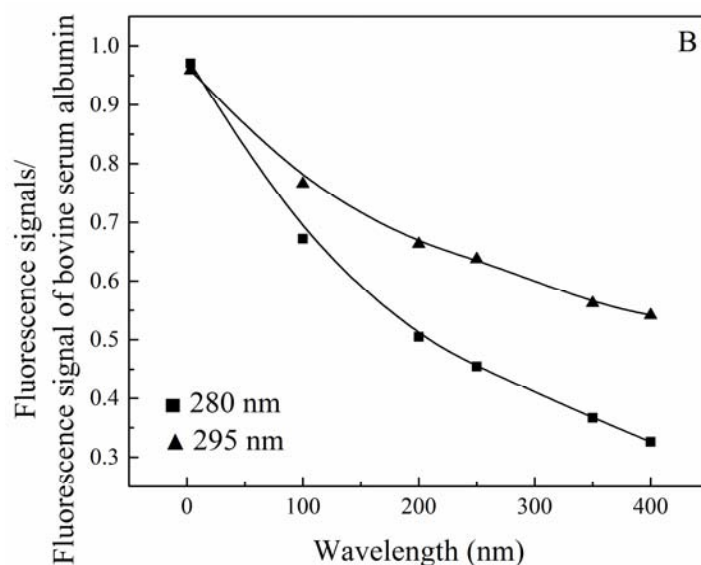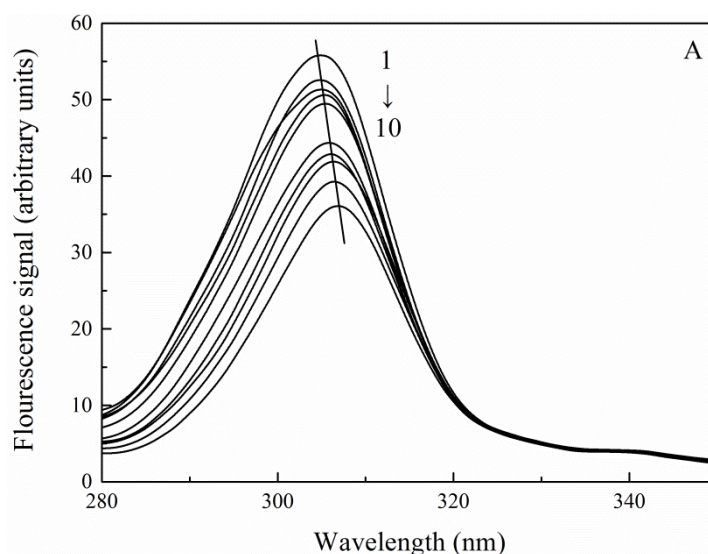
Figure 4. *Quenching curves of bovine transferrin-cefoperazone sodium (A) and bovine serum albumin-cefoperazone sodium (B) systems ($\lambda_{ex}=280/295$ nm), C bovine transferrin=C bovine serum albumin=0.2 μM, C$_{cefoperazone\ sodium}$=(0, 0. 8, 20. 0, 40.0, 50.0, 70.0) μM.*

3.4. Synchronous Fluorescence Spectra Studies

Synchronous spectroscopy is a powerful tool for simultaneous analysis of multiple components without sample pretreatment, it can be used for investigating the microenvironment of amino acid residues [32]. Synchronous fluorescence involves simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval (Δλ) between both. When the Δλ value between the excitation and emission wavelengths is stabilized at 15 or 60 nm, the synchronous fluorescence gives characteristic information for tyrosine residues or tryptophan residues, respectively [33]. The synchronous fluorescence spectra at these two different wavelength intervals were shown in figure 5. It was apparent from figure 5 (A) and figure 5 (B) that the emission strength of tyrosine residues and tryptophan residues of bovine transferrin decreased and the maximum emission wavelength of amino acid residues had a red shift, which indicated that the polarity around tyrosine and tryptophan residues of bovine transferrin increased [34]. Figure 5 (C) shows that the fluorescence intensity around tyrosine of bovine serum albumin decreased with increasing cefoperazone sodium content, but no obviously change of maximum emission wavelength was observed. While, it can be seen from figure 5 (D), the fluorescence intensity around tryptophan of bovine serum albumin decreased regularly and with a red shift at maximum emission wavelength. The results suggested that the polarity around the tryptophan increased but the microenvironment of tyrosine of bovine serum albumin was not been changed. The conformation of bovine transferrin and bovine serum albumin both changed with the addition of cefoperazone sodium, but the changes were different. The influence of cefoperazone sodium on bovine transferrin was larger than bovine serum albumin.
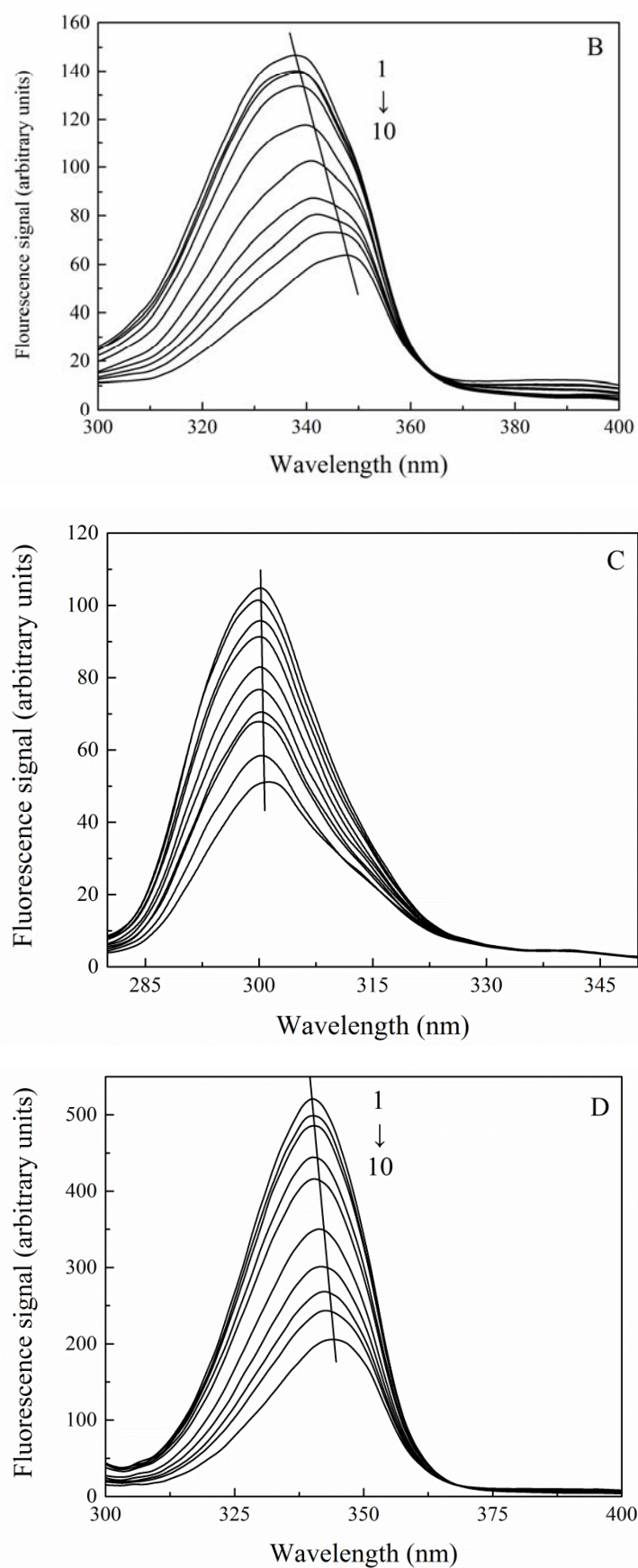
Figure 5. *Synchronous fluorescence spectrums of bovine transferrin-cefoperazone sodium (A: Δλ=15 nm, B: Δλ=60 nm) and bovine serum albumin-cefoperazone sodium (C: Δλ=15 nm, D: Δλ=60 nm) systems (T=310 K) C_bovine transferrin=C_bovine serum albumin=0.2 μM, C_cefoperazone sodium=(0, 0.8, 6.0, 10.0, 20.0, 40.0, 50.0, 70.0, 80.0, 100.0) μM.*

Since tryptophan and tyrosine residues were both involved in the reaction, the synchronous fluorescence quenching ratios at $\Delta\lambda$=15 nm and 60 nm were compared in order to further confirm the specific binding site of the cefoperazone sodium to bovine transferrin/bovine serum albumin. For comparison, the synchronous fluorescence quenching ratios (R_{SFQ}) that express the decreasing percentages of synchronous fluorescence intensity were calculated by the equation:

R_{SFQ}=1−F/F_0 [35]. The corresponding ratios of synchronous fluorescence quenching (R_{SFQ}) were illustrated in figure 6. From figure 6 it can be seen that the R_{SFQ} for $\Delta\lambda$=60 nm were bigger than corresponding ones for $\Delta\lambda$=15 nm in the two systems, which revealed that the binding site of cefoperazone sodium to the bovine transferrin/bovine serum albumin was closer to tryptophan residues.
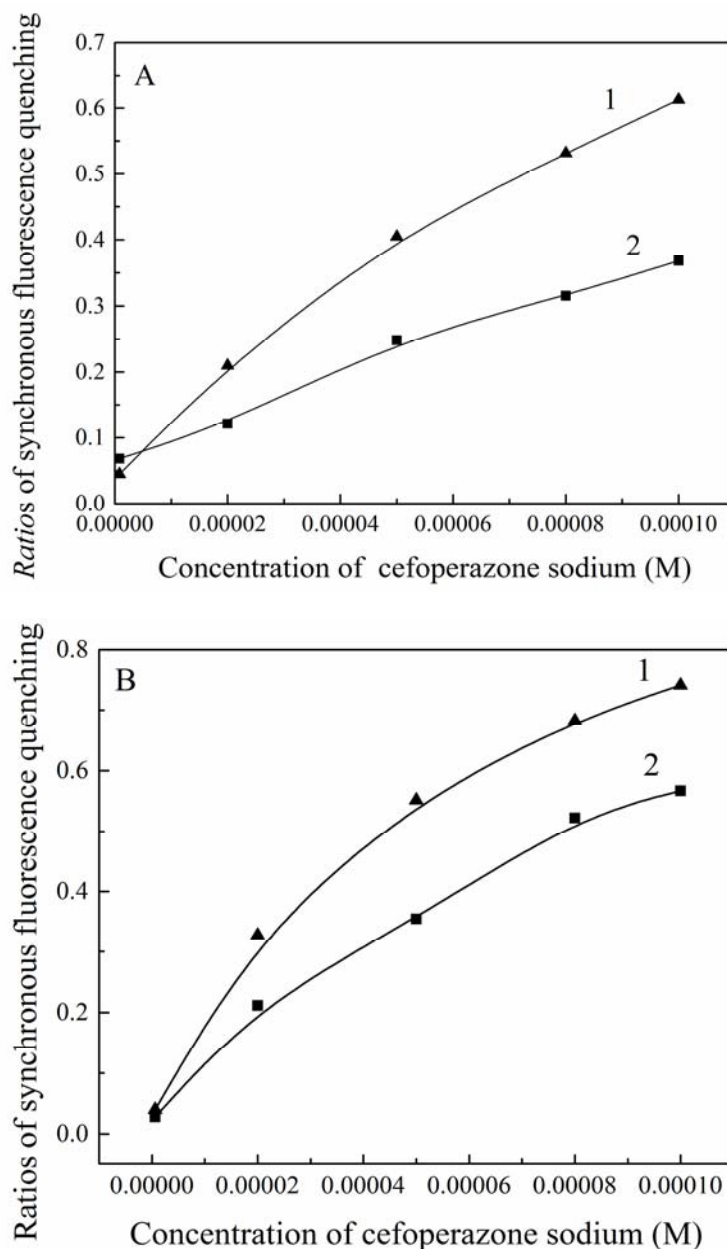

Figure 6. Ratios of synchronous fluorescence quenching (R_{SFQ}) of bovine transferrin-cefoperazone sodium (A) and bovine serum albumin-cefoperazone sodium (B) systems (T=310 K) $C_{bovine\ transferrin}$=$C_{bovine\ serum\ albumin}$=0.2 μM, $C_{cefoperazone\ sodium}$=(0. 8, 20.0, 50.0, 80.0, 100.0) μM.

3.5. Type of Interaction Force

The acting forces between small molecules and biomolecules include hydrophobic association, vander Waals force, hydrogen bonds and electrostatic interaction [36]. The thermodynamic parameters relying on the temperatures, free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) were used to clarify the interaction of drug with protein. When the temperature varies over a small range, the enthalpies change (ΔH) can be considered to be constant. Then its value and that of entropy change (ΔS) can be

determined from the van't Hoff equations [37]:

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (4)$$

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a \qquad (5)$$

R is the gas constant (R=8.314 J·mol^{-1}·K^{-1}). T is the absolute temperature. ΔH was obtained from the slope of the linear plot (Eq. (4)) based on $R\ln K_a$ versus $1/T$. Then the values of ΔS and ΔG were calculated by Eq. (5). All the thermodynamic parameters were listed in Table 3. The negative values of ΔG indicated that the binding process is spontaneous, while the negative values of ΔH and positive values of ΔS mean that the electrostatic attraction was the main impetus in the intermolecular interaction between bovine transferrin/bovine serum albumin and cefoperazone sodium [38]. Comparing the values of ΔG in the two systems, it can be known ΔG of bovine serum albumin-cefoperazone sodium system was smaller than bovine transferrin-cefoperazone sodium system, indicating that the degree of spontaneous reaction of bovine serum albumin-cefoperazone sodium system was larger than bovine transferrin-cefoperazone sodium system. So the reaction of bovine serum albumin-cefoperazone sodium system was more likely to occur. Therefore, the binding constants of bovine serum albumin-cefoperazone sodium system were larger.

Table 3. *The thermodynamic parameters of bovine transferrin/bovine serum albumin-cefoperazone sodium system at different temperatures.*

		T/(K)	K_a (L·mol^{-1})	ΔH (KJ·mol^{-1})	ΔS (J·mol^{-1}·K^{-1})	ΔG (KJ·mol^{-1})
		298	1.27×10^4		40.04	-23.41
	λ_{ex}=280 nm	303	1.16×10^4	-11.48	39.92	-23.58
bovine transferrin-cefoperazone sodium		310	1.06×10^4		40.03	-23.89
		298	2.41×10^3		52.97	-19.30
	λ_{ex}=295 nm	303	2.34×10^3	-3.51	52.92	-19.54
		310	2.28×10^3		52.96	-19.93
		298	2.50×10^4		69.30	-25.09
	λ_{ex}=280 nm	303	2.40×10^4	-4.44	69.21	-25.41
bovine serum albumin-cefoperazone sodium		310	2.33×10^4		69.29	-25.92
		298	1.04×10^4		20.50	-22.92
	λ_{ex}=295 nm	303	9.58×10^3	-16.81	20.73	-23.09
		310	8.02×10^3		20.52	-23.17

3.6. Circular Dichroism Spectra Studies

Circular dichroism is an excellent method for determining the conformational changes of proteins. The circular dichroism spectra of bovine transferrin/bovine serum albumin in the absence (1) and in the presence of cefoperazone sodium (2 and 3) were shown in figure 7. From the figure 7 we can see that the circular dichroism spectra of bovine transferrin/bovine serum albumin exhibited two negative bands in the UV region at 208 nm and 222 nm, which were characteristic of α-helicity of protein [39]. The experiments were performed at the [bovine transferrin]/[cefoperazone sodium] molar ratios of 1:0, 1:10 and 1:20. And the molar ratios of [bovine serum albumin]/[cefoperazone sodium] were also 1:0, 1:10 and 1:20. The circular dichroism results are expressed as mean residue ellipticity (*MRE*) in deg cm^2 dmol^{-1} according to the following equation [40]:

$$MRE_{208} = \frac{Intensity\ of\ CD\ (mdeg)\ at\ 208\ nm}{10\ Cp\ nl} \qquad (6)$$

Where C_p is the molar concentration of the protein, n is the number of amino acid residues and l is the path length. The α-helical contents can be calculated using the following equation:

$$\alpha\text{-}Helix(\%) = \frac{-MRE_{208} - 4000}{33000 - 4000} \times 100 \qquad (7)$$

4000 is the *MRE* of the β-form and random coil conformation cross at 208 nm, and 33,000 is the *MRE* value of the pure α-helix at 208 nm.

The results revealed that cefoperazone sodium caused increase in α-helical content of bovine transferrin from 19.13% in free bovine transferrin to 21.94% and 26.69% in bound form. And there was an increase in the α-helical content from 44.72% in pure bovine serum albumin to 52.16% and 55.34% in the bovine serum albumin-cefoperazone sodium complexes. This indicated that the binding of cefoperazone sodium to bovine transferrin/bovine serum albumin may induce some conformational changes of proteins. It can be deduced that the α-helix structure was affected probably due to insertion of cefoperazone sodium into hydrophobic cavity of bovine transferrin/bovine serum albumin [41]. However, the similarity between the shapes of the circular dichroism spectra pertaining to bovine transferrin/bovine serum albumin in the presence and the absence of the cefoperazone sodium in all interacting systems suggested that the structure of bovine transferrin/bovine serum albumin was still predominantly α-helical [42].
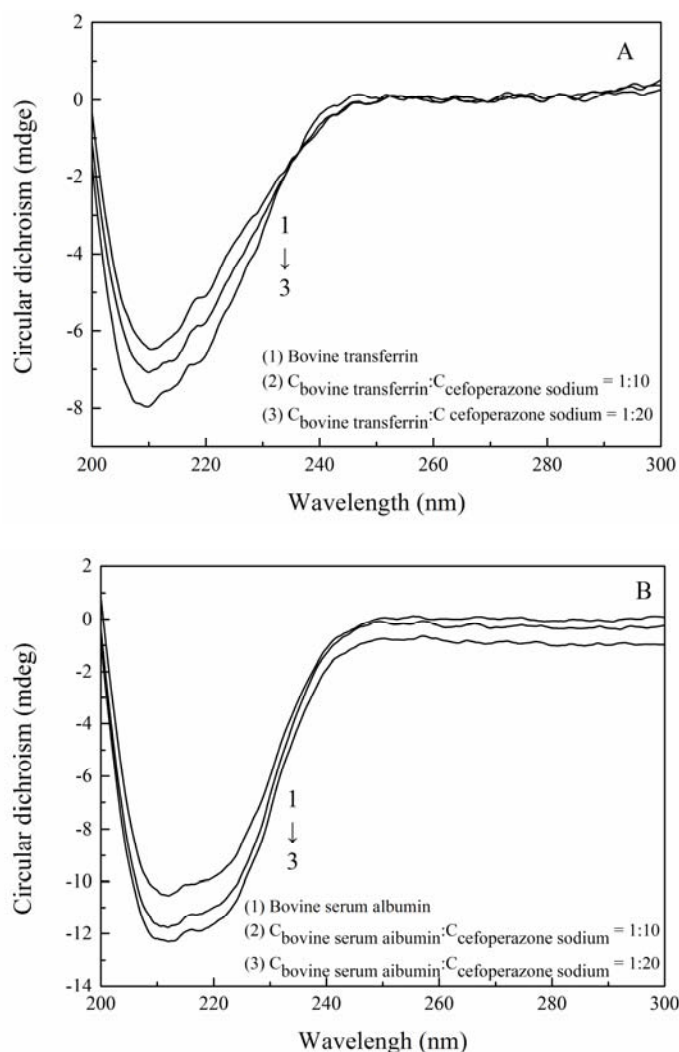
Figure 7. *The circular dichroism spectra of bovine transferrin-cefoperazone sodium (A) and bovine serum albumin-cefoperazone sodium (B) systems (T=293 K) $C_{bovine\ transferrin}=C_{bovine\ serum\ albumin}=1.0\ \mu M$, $C_{cefoperazone\ sodium}=(10.0, 20.0, 40.0)\ \mu M$.*

3.7. Hill's Coefficient Studies

In biochemistry, the binding of a ligand molecule at one site with a macromolecule often influences the affinity for other ligand molecules with this macromolecule. This is known as cooperative binding. It is classified into positive cooperativity, negative cooperativity and non-cooperativity according to the promotion or inhibition of the affinity for other ligand molecules. Hill's coefficient provides a way to quantify this effect and is calculated graphically on the basis of the following equation [43]:

$$\log \frac{Y}{1-Y} = \log K + n_H \log[L] \qquad (8)$$

Where Y is the fractional binding saturation, K is the binding constant and n_H is the Hill's coefficient. Values of Hill's coefficient > 1 indicate positive cooperativity, values < 1 indicate negative cooperativity and values=1 indicate a non-cooperative reaction.

For fluorescence measurement:

$$\frac{Y}{1-Y} = \frac{Q}{Q_m - Q} \qquad (9)$$

$$Q = \frac{F_0 - F}{F_0} \qquad (10)$$

$1/Q_m$ is the intercept of a plot of $1/Q$ vs.$1/[L]$. Hill's coefficients were presented in Table 4. As can be seen from Table 4, the values of n_H were < 1, which indicated that there was negative cooperation in the interaction of subsequent cefoperazone sodium with bovine transferrin/bovine serum albumin. This showed that the ability of subsequent ligands binding to bovine transferrin/bovine serum albumin decreased with the previous cefoperazone sodium binding to bovine transferrin/bovine serum albumin gradually. In addition, n_H were inversely correlated with increasing temperature, which is also one reason for the reduction in K_a with increasing temperature [44].

Table 4. *Hill's coefficients of bovine transferrin/bovine serum albumin-cefoperazone sodium systems at different temperatures.*

	T (K)	λ_{ex}=280 nm			λ_{ex}=295 nm		
		n_H	R_3	SD	n_H	R_3	SD
bovine transferrin-cefoperazone sodium	298	0.94	0.9985	0.0226	0.63	0.9987	0.0163
	303	0.90	0.9977	0.0272	0.59	0.9910	0.0352
	310	0.81	0.9994	0.0222	0.56	0.9966	0.0208
bovine serum albumin-cefoperazone sodium	298	0.93	0.9920	0.0526	0.98	0.9911	0.0372
	303	0.87	0.9963	0.0386	0.84	0.9940	0.0340
	310	0.85	0.9965	0.0228	0.80	0.9968	0.0240

R_3 is the linear relative coefficient of lg $[Y/(1-Y)]$~lg $[L]$.

4. Conclusions

In this experiment, the reaction mechanism of bovine transferrin/bovine serum albumin-cefoperazone sodium system was studied by fluorescence quenching method, synchronous fluorescence spectroscopy, Ultraviolet-Visible absorption spectroscopy and circular dichroism spectroscopy under imitated physiological conditions. The results showed that the quenching mechanism of bovine transferrin/bovine serum albumin-cefoperazone sodium system was static quenching. There was a closer binding between bovine serum albumin and cefoperazone sodium, thereby the binding constants of bovine serum albumin-cefoperazone sodium system were bigger. However, synchronous fluorescence spectroscopy proved that cefoperazone sodium had a greater impact on the microenvironment of bovine transferrin. These results were due to the different structures of bovine transferrin and bovine serum albumin. The obtained results suggested that both proteins could act as drug carriers, but that bovine serum albumin potentially had a higher capacity for delivering cefoperazone sodium. The valuable information obtained from these experiments will help researchers understand the pathways of drug delivery, and have practical implication for clinical application of cephalosporin drugs.

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