

Synchronous Fluorescence Characteristics on the Interaction of Cefotaxime Sodium and Transferrin

Cui M, Liu B*, Li T, and Duan S

College of Chemistry & Environmental Science, Hebei University, Baoding, China

*Corresponding author: Liu B, Key Laboratory of Analytical Science and Technology of Hebei Province, College of Chemistry & Environmental Science, Hebei University, Baoding, China 071002, Tel: 0312-5079650; E-mail: lbs@hbu.edu.cn

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Abstract

At different temperatures, the spectral characteristics about the interaction between cefotaxime sodium and transferrin were analyzed by synchronous spectroscopy. Meanwhile, UV-absorption spectroscopy was used to supplement and verify the conclusion obtained from synchronous spectroscopy. Results showed that cefotaxime sodium quenched fluorescence from transferrin by the way of static quenching. New compound was generated by electrostatic force. The new compound did not stimulate or inhibit the interaction between subsequent ligand and transferrin. Tryptophan and tyrosine in transferrin both participated in the interaction. Circular dichroism spectroscopy displayed that Peptide chains of transferrin became loose and α -helical secondary structure of protein was changed.

Keywords: Synchronous fluorescence spectra; Quenching reaction; Synergy; Electrostatic force

Introduction

Transferrin consists of homologous lobes and each of them is divided into two domains, responsible for transport of metal ions and minerals [1]. The half-life of transferrin is short and the reserve of it is small. Its quantity will quickly decline upon the deficient intake of energy and protein and will quickly return to normal upon the sufficient intake of energy and protein. So, transferrin could be regarded as a good indicator assessing the nutritional condition about neonates. Transferrin plays an important role not only in hematopoiesis process but also in anti-infection by competing with cells for Fe (iron). Transferrin could be used to diagnose jaundice induced by bacterial infection. The quantity of transferrin would become less due to the bacterial infection and serious disease.

Therefore, as a sensitive biochemistry indicator, transferrin can reflect the state of jaundice about neonates [2]. Also, the detection of transferrin can offer feedback on damage of kidney caused by diabetes and can provide reference and basis for clinical treatment and early prevention. Meanwhile, deterioration of such illness can be avoided and clinical mortality can be reduced [3]. Liver damage and reserve function of liver cells declining would influence the synthesis and secretion of transferrin. The reduction of serum transferrin is relevant to the damage extent of liver cells [4]. Accordingly, the studies on

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transferrin are helpful to the diagnosis and treatment of diabetes, kidney disease, iron deficiency anemia and viral hepatitis. The relevant researches of serum albumin have been reported. Serum albumin also can be used to determine the effect of nutrition detection. However, the half-life of serum albumin in body is longer than that of transferrin and the sensitivity of serum albumin is poor. So, studies on transferrin show medical significance.

Cefotaxime sodium belongs to β -lactam antibiotics, and cepams are contained in its molecules. Cefotaxime sodium has anti-bacterial effect and can destroy the cell walls of bacteria. And, cefotaxime sodium can kill bacteria which are in breeding period. In addition, cefotaxime sodium can also selectively kill bacteria and has the advantage of low toxicity towards human body. Hence, as a kind of efficient, less toxic antibiotics, cefotaxime sodium has been widely applied in clinical practice [5]. Analysis methods of cefotaxime sodium are various. Because of its high accuracy, fine selectivity and sensitivity, fluorescence spectroscopy is being extensively applied in the area of pharmaceutical analysis. Currently, relevant analysis of transferrin is not too much. The binding between transferrin and cefotaxime sodium has not been reported. In this article, the interaction of cefotaxime sodium with transferrin was studied by synchronous spectroscopy, UV-absorption spectroscopy and circular dichroism spectroscopy. The analysis on cefotaxime sodium can offer guidance for clinical medication and pharmacokinetics analysis of drugs in the body. The molecular structure of cefotaxime sodium is shown in FIG. 1.

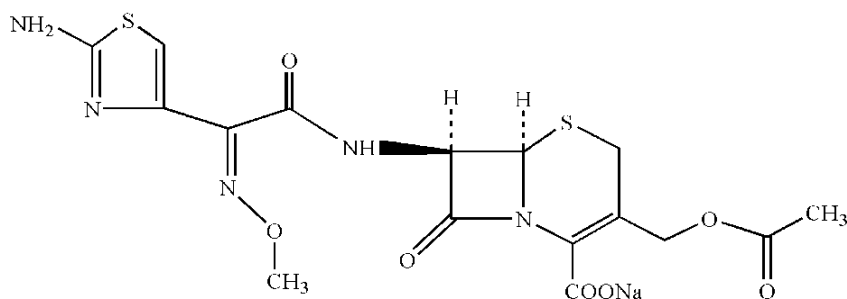


FIG. 1. Chemical structure of cefotaxime sodium.

Experimental

Apparatus

Synchronous fluorescence spectra were recorded by with a Shimadzu RF-5301PC spectrofluorophotometer. Absorption was measured by an UV-vis recording spectrophotometer (UV-265, Shimadzu, Japan). Temperature was controlled by a CS501 superheated water bath (Nantong Science Instrument Factory). The pH measurements were carried out with a pH-3C precision acidity meter (Leici, Shanghai, China). Circular dichroism spectra were recorded on a MOS-450/SFM300 circular dichroism spectrometer (Bio-Logic, France).

Materials

Transferrin was purchased from Sigma-Aldrich (purity grade inferior 98%, Shanghai, China). Cefotaxime sodium was of the purity grade inferior 98.5%. Stock solutions of transferrin (1.0×10^{-5} M) and cefotaxime sodium (1.0×10^{-3} M) were prepared. 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 double-distilled water and stored at 277 K.

Moreover, when the interaction between drugs and protein is investigated by fluorescence spectroscopy, some drugs absorb light at the excitation and emission wavelengths of protein that influence the determination of fluorescence intensity. It is known as the inner-filter effect. In order to remove the inner filter effects of protein and ligand, absorbance measurements were performed at excitation and emission wavelengths of the fluorescence measurements. Fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emission light to decrease the inner filter using the following relationship [6]:

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

Where F_{cor} and F_{obs} is respectively the corrected and measured fluorescence intensities, and A_{ex} and A_{em} is respectively the absorbance values of cefotaxime sodium at excitation and emission wavelengths. The fluorescence intensity used in this article has been corrected.

Procedures

Tris-HCl 0.5 mL (pH=7.40), 0.5 mL transferrin solution (2.0×10^{-6} M) and different concentrations of cefotaxime sodium were added into 5 mL colorimetric tubes successively. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking and kept static for 40 min at 298 K. The excitation and emission slits were set at 5 nm. We respectively scanned the fluorescence spectra of the transferrin-cefotaxime sodium system with a 10 mm path length cell when the $\Delta\lambda$ value between the emission and excitation wavelengths was fixed at 15 nm and 60 nm. The above experiment was separately repeated at 310 K and 318 K.

Tris-HCl 0.5 mL (pH=7.40), 1 mL transferrin solution (2.0×10^{-5} M) and cefotaxime sodium of different concentrations were added into a series of 5 mL colorimetric tubes. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking and kept static for 40 min. Cefotaxime sodium solution of different concentrations was as the corresponding reference. The UV-vis absorption spectra of each transferrin-cefotaxime sodium system were scanned from 190 nm to 350 nm with 10 mm quartz cells by UV-vis recording spectrophotometer.

The alterations in the secondary structure of the protein in the presence of cefotaxime were recorded on a MOS-450/SFM300 circular dichroism spectrometer with 1 cm quartz cell at room temperature. For circular dichroism experiments, transferrin concentration was 1 μ M. The spectropolarimeter was sufficiently purged with 99.9% dry nitrogen before measurement. Each spectrum was baseline corrected, and the final plot was taken as an average of three accumulated plots in the range of 200 nm to 250 nm. The circular dichroism spectra were collected with an interval of 1 nm and with a scan speed of 100 nm/min.

Results and Discussion

Synchronous fluorescence spectra studies of the transferrin-cefotaxime sodium system

Synchronous fluorescence spectroscopy, a kind of simple and effective method, can be used to measure fluorescence quenching and the potential change of maximum emission wavelength. Also, it can reflect the change of microenvironment around chromophore [7]. So, synchronous fluorescence spectroscopy usually plays an important role in studies on interaction between proteins and small molecules. The difference value ($\Delta\lambda$) between the emission and excitation wavelengths was fixed at 15 nm and 60 nm. When $\Delta\lambda=15$ nm, the corresponding spectra reflect the spectral characteristics of tyrosine residues. When $\Delta\lambda=60$ nm, the corresponding spectra reflect the spectral characteristics of tryptophan residues [8]. FIG. 2 has shown the synchronous fluorescence spectra of $\Delta\lambda=15$ nm and $\Delta\lambda=60$ nm. As is shown in FIG. 2, when $\Delta\lambda=15$ nm, $\Delta\lambda=60$ nm, with

the increasing of cefotaxime sodium concentration, the two corresponding fluorescence intensities both decrease and the positions of two spectral peak both have red shift. This phenomenon indicated that cefotaxime sodium quenched the fluorescence of transferrin [9], with tyrosine and tryptophan both taking part in the reaction, the conformation of transferrin being changed, the polarity of microenvironment around tyrosine and tryptophan increasing and the corresponding hydrophobic decreasing.

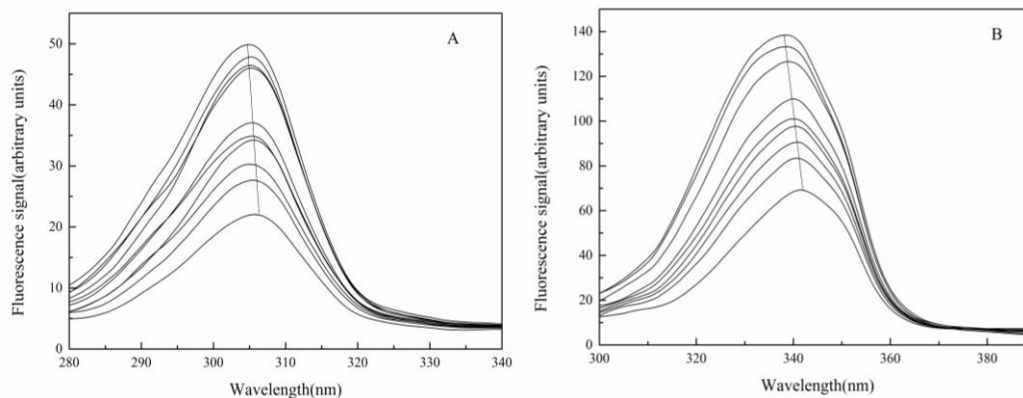


FIG. 2. Fluorescence spectrum of transferrin-cefotaxime sodium system (T=298 K) (A) $\Delta\lambda=15$ nm; (B) $\Delta\lambda=60$ nm.

$$C_{\text{transferrin}}=2.0 \times 10^{-7} \text{ M}; 1 \sim 10 C_{\text{cefotaxime sodium}}=(0, 0.4, 2.0, 4.0, 5.0, 6.0, 8.0, 9.0, 10, 14) \times 10^{-5} \text{ M}$$

Fluorescence quenching mechanism contains static quenching, dynamic quenching etc. During static quenching process, a new compound without fluorescence or a weak fluorescent compound was formed from the reaction between fluorescent molecules and quencher. However, dynamic quenching is induced by collision between fluorescent molecules and quencher. To further study the mechanism of the transferrin-cefotaxime sodium system, the data obtained from experiment would be processed by Stern-Volmer equation [10]:

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

Where, F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively. τ_0 is the average lifetime of fluorescence without quencher and is 10^{-8} s. K_{sv} is the Stern-Volmer quenching constant. K_q is the quenching rate constant, and $[L]$ is the concentration of the quencher. Based on the linear fit plot of F_0/F versus $[L]$, the K_q , K_{sv} values and Stern-Volmer curves of the transferrin-cefotaxime sodium system can be obtained. The calculated results are shown in TABLE 1. Stern-Volmer curves of the transferrin-cefotaxime sodium system are shown in FIG. 3. As is shown in TABLE 1, quenching constants K_{sv} decrease upon the rising of the temperature and curve slope becomes smaller in FIG. 3. Therefore, static quenching may be the main quenching pattern [11]. Meanwhile, all the K_q are greater than the maximum diffusion collision rate constant ($2 \times 10^{10} \text{ M}^{-1}\cdot\text{S}$). So, the process is static quenching process caused by the generation of new compound from the transferrin-cefotaxime sodium system [12].

Based on static quenching theory [13], binding constant K_a and binding site number n on the transferrin-cefotaxime sodium system can be calculated by formula (3):

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

Where $[L]$ and $[B_t]$ are the total concentrations of cefotaxime sodium and transferrin, respectively. On the assumption that n in the bracket is equal to 1, the curve of $\log(F_0-F)/F$ versus $\log\{ [L] - [B_t](F_0-F)/F_0 \}$ is drawn and fitted linearly, then the value

of n can be obtained from the slope of the plot. If the n value obtained is not equal to 1, then it is substituted into the bracket and the curve of $\log(F_0-F)/F$ versus $\log\{[L]-[B_t](F_0-F)/F_0\}$ is drawn again. The above process is repeated again till n obtained is same value as the one in bracket. Based on the n obtained, the binding constant K_a can be also obtained. The calculated results were shown in TABLE 1. As is shown in TABLE 1, the values of n were approximately equal to 1 at different temperatures, which indicated that there was one class of binding sites in transferrin for cefotaxime sodium. Meanwhile, the binding constants K_a decreased with increasing temperature, further suggesting that the quenching was a static process [14].

TABLE 1. Quenching reactive parameters of transferrin and cefotaxime sodium at different temperatures ($\Delta\lambda=15, 60$ nm).

$\Delta\lambda$ /(nm)	T /(K)	K_q /(1/M·s)	K_{sv} /(1/M)	r_1	K_a /(1/M)	n	r_2	SD
15	298	9.08×10^{11}	9.08×10^3	0.9901	8.20×10^3	1.32	0.9945	0.0374
	310	8.39×10^{11}	8.39×10^3	0.9925	7.64×10^3	1.33	0.9945	0.0396
	318	7.75×10^{11}	7.75×10^3	0.9977	7.22×10^3	0.94	0.9934	0.0242
60	298	8.57×10^{11}	8.57×10^3	0.9903	7.13×10^3	1.42	0.9934	0.0267
	310	6.76×10^{11}	6.76×10^3	0.9928	6.37×10^3	1.00	0.9940	0.0394
	318	4.75×10^{11}	4.75×10^3	0.9904	6.23×10^3	1.00	0.9947	0.0294

r_1 is the linear relative coefficient of $F_0/F \sim [L]$; r_2 is the linear relative coefficient of $\log(F_0-F)/F \sim \log\{[L]-n[B_t](F_0-F)/F_0\}$; SD is the standard deviation values of binding constants.

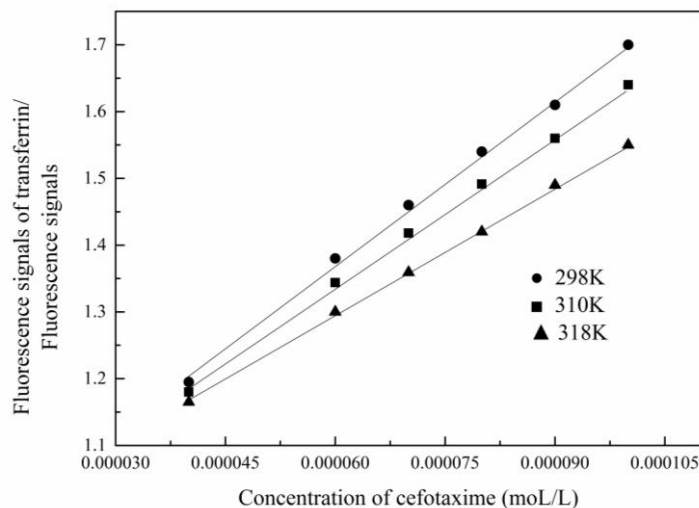


FIG. 3. Stern-volmer curves of the transferrin-cefotaxime sodium system ($\Delta\lambda=60$ nm).

$$C_{\text{transferrin}}=2.0 \times 10^{-7} \text{ M}; 1 \sim 9 C_{\text{cefotaxime sodium}}=(4.0, 6.0, 7.0, 8.0, 9.0, 10) \times 10^{-5} \text{ M}$$

Analysis of UV/vis absorption spectra

UV-visible absorption spectroscopy is simple method and applicable to know the change in hydrophobicity and to know the formation of complex between the drug and protein [15]. Dynamic quenching is mainly caused by collision and only changes the excited state of fluorescence molecules but not the absorption spectra. Because of the formation of new compound, Static quenching changes the absorption spectra of fluorescent substances [16]. Absorption spectra of the transferrin-cefotaxime

sodium system are shown in FIG. 4. As is shown in FIG. 4, with gradual addition of cefotaxime sodium to transferrin solution, the intensity of the peak at 212 nm decreases with red shift of 3 nm; the results indicate that the interaction between transferrin and cefotaxime sodium leads to the loosening and unfolding of the protein skeleton and decreases the hydrophobicity of the microenvironment of the aromatic amino acid residues [17]. Above phenomenon indicates that the change of absorption spectra of transferrin is induced by the generation of new compound and the process is static quenching.

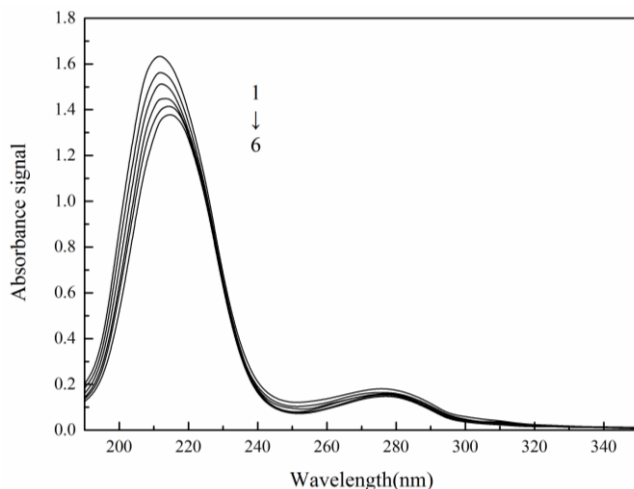


FIG. 4. UV absorption spectra of transferrin-cefotaxime sodium system (T=298K).

$$C_{\text{transferrin}}=2.0 \times 10^{-6} \text{ M}, 1\sim 6 C_{\text{cefotaxime sodium}}=(0, 0.4, 2.0, 4.0, 8.0, 10) \times 10^{-5} \text{ M}$$

Analysis of binding distance of the transferrin-cefotaxime sodium system

Förster resonance energy transfer is a mechanism describing energy transfer from one donor molecule to another acceptor molecule through non-radiative dipole-dipole coupling, which is widely used to estimate the spatial distances between the donor (protein) and the acceptor (the interacted ligand) and to investigate the structure, conformation, spatial distribution and assembly of complex proteins [9]. According to Förster's non-radiative energy transfer theory [18], efficiency of energy transfer E , critical energy-transfer distance R_0 ($E=50\%$), distance r between the energy donor and the energy acceptor, the overlap integral J between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor can be calculated by the following formulas [19,20]:

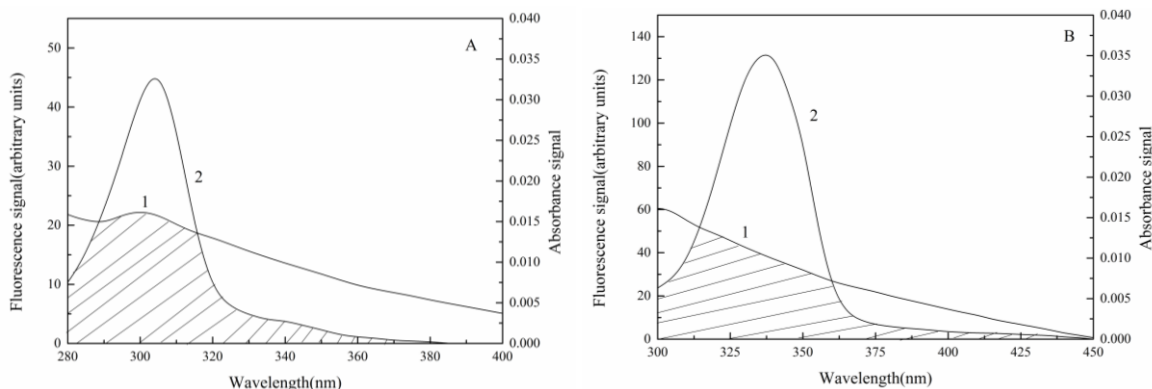
$$E=1-F/F_0=R_0^6/(R_0^6+r^6) \quad (4)$$

$$R_0^6=8.78 \times 10^{-25} K^2 \Phi N^{-4} J \quad (5)$$

$$J=\sum F(\lambda)\epsilon(\lambda)\lambda^4\Delta\lambda/\sum F(\lambda)\Delta\lambda \quad (6)$$

Where, F_0 represents the fluorescence intensity of transferrin. F represents the fluorescence intensity of the whole system as the concentration of transferrin and cefotaxime sodium is the same one ($2 \times 10^{-7} \text{ M}$). K_2 is the orientation factor being equal to $2/3$. N is the refractive index of solvent, the average value (1.336) of water and the solution. Φ is the fluorescence quantum yield (0.118) of the donor in the absence of the acceptor. The overlap integral represents the area of shadow part in FIG. 5. $\epsilon(\lambda)$, $F(\lambda)$ respectively represents molar absorptivity of cefotaxime sodium and fluorescence intensity of transferrin as the wavelength is at λ . By the above formulas, E , J , R_0 , r can be available. They are presented in TABLE 2. As is shown in TABLE 2, r is smaller than 7 nm. High probability of non-radiative energy transfer between transferrin and cefotaxime

sodium can be verified [21]. Larger transferrin-cefotaxime sodium distance, r compared to that of R_0 observed in the present study also revealed the presence of static quenching mechanism between drug and protein [22,23].



(A) $\Delta\lambda=15$, (B) $\Delta\lambda=60$ nm, $T=298K$; $C_{\text{transferrin}}=C_{\text{cefotaxime sodium}}=2.0 \times 10^{-7}$ M

FIG. 5. a) UV absorbance spectra for cefotaxime sodium and b) Fluorescence spectra for only transferrin.

TABLE 2. Parameters of E , J , r , R_0 between transferrin and cefotaxime sodium at different temperatures ($\Delta\lambda=15, 60$ nm).

$\Delta\lambda/(nm)$	$T/(K)$	$E/(%)$	$J/(cm^3/M)$	$R_0/(nm)$	$r/(nm)$
15	298	4.32	4.85×10^{-14}	3.19	5.34
	310	6.38	4.91×10^{-14}	3.20	5.00
	318	5.92	2.39×10^{-14}	2.83	4.49
60	298	3.38	5.06×10^{-14}	3.21	5.62
	310	6.56	5.08×10^{-14}	3.21	5.00
	318	5.81	5.05×10^{-14}	3.21	5.11

Thermodynamic parameters and binding force

Thermodynamic parameters can be applied in judging the type of binding force. ΔG (the standard free energy change), ΔH (enthalpy change), ΔS (entropy change) can be calculated by the van't Hoff equation [24]:

$$R \ln K = \Delta S - \Delta H / T \tag{7}$$

$$\Delta G = -RT \ln K = \Delta H - T \Delta S \tag{8}$$

Where K is the binding constant and R is gas constant. When the temperature change is not very enormous, the ΔH of a system can be regarded as a constant. Data results are listed in TABLE 3. From TABLE 3, $\Delta H < 0$, $\Delta S > 0$ suggests that electrostatic force plays a major role in the interaction [25]. In addition, it can be seen that the system is a spontaneous molecular interaction in which $\Delta S > 0$ and $\Delta G < 0$.

Hill's coefficient of the transferrin-cefotaxime sodium system

The binding of a ligand molecule at one site of a macromolecule often influences the affinity for other ligand molecules at additional sites. This is known as cooperative binding. Hill's coefficient offers a way to quantify this effect and is calculated by the following formula [26]:

$$\lg \frac{Y}{1-Y} = \lg K + n_H \lg [L] \tag{9}$$

Where Y is the saturation fraction of binding; K is the binding constant and n_H is the Hill's coefficient. Hill's coefficient being greater than 1 implies positive cooperativity. Conversely, Hill's coefficient being smaller than 1 implies negative cooperativity and Hill's coefficient being equal to 1 implies a non-cooperative reaction [27].

For fluorescence measurement:

$$\frac{Y}{1-Y} = \frac{Q}{Q_m - Q} \quad (10)$$

$$Q = \frac{F_0 - F}{F_0} \quad (11)$$

Where $1/Q_m$ is the intercept of the plot $1/Q$ versus $1/[L]$. Synchronous fluorescence intensity (F) is substituted into the above equation and Hill's coefficients can be obtained via successive calculation. Hill's coefficients are listed in TABLE 4. The values of n_H are approximately equal to 1 at different temperatures, and these results indicate that there is non-cooperative reaction between transferrin and cefotaxime sodium.

TABLE 3. The thermodynamic parameters of the transferrin-cefotaxime sodium system at different temperatures ($\Delta\lambda=15, 60$ nm).

$\Delta\lambda$ (nm)	T/(K)	K_a /(1/M)	ΔH /(KJ/mol)	ΔS /(J/mol·K)	ΔG /(KJ/mol)
15	298	8.20×10^3	-5.014	58.11	-22.33
	310	7.64×10^3		58.15	-23.04
	318	7.22×10^3		58.10	-23.49
60	298	7.13×10^3	-5.316	55.92	-21.98
	310	6.37×10^3		55.69	-22.58
	318	6.23×10^3		55.93	-23.10

TABLE 4. Hill's coefficient of the transferrin-cefotaxime sodium system at different temperatures ($\Delta\lambda=15, 60$ nm).

T/(K)	$\Delta\lambda=15$ nm		$\Delta\lambda=60$ nm	
	n_H	r_3	n_H	r_4
298	1.09	0.9821	0.96	0.9872
310	1.00	0.9807	1.09	0.9915
318	1.27	0.9976	1.04	0.9841

r_3, r_4 is the linear relative coefficient of $\log[Y/(1-Y)] \sim \lg[L]$.

Circular dichroism spectra analysis of the transferrin-cefotaxime sodium system

Circular dichroism can be used to display the conformation change of protein [28]. FIG. 6 is the circular dichroism about the system of transferrin-cefotaxime sodium. As known in FIG. 6, there are two negative characteristic peaks located near 211 nm and 219 nm, which are respectively due to $\pi-\pi^*$ and $n-\pi^*$ transfer for the peptide bond of the α -helix [29]. They are the characteristic peak of α -helix. As concentration ratio of transferrin and cefotaxime sodium is 1:0, 1:20 and 1:30, the intensity of negative peak becomes weak, but the shape and position of negative characteristic peak both have no change. Hence, α -

helix structure becoming loose has induced the quenching of fluorescence from transferrin, and the existence of cefotaxime sodium has changed the conformation of transferrin. However, α -helix still is the main structure form of transferrin [12].

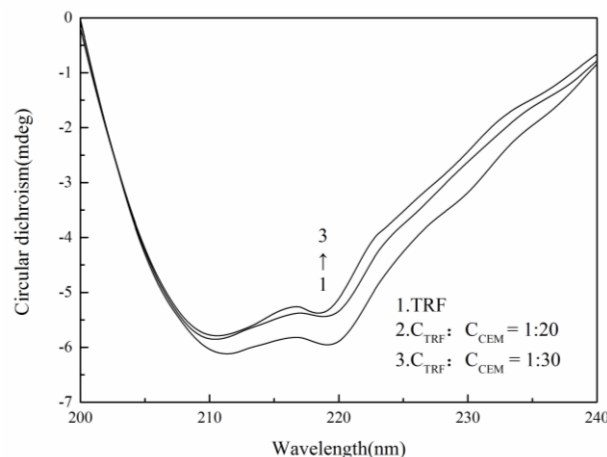


FIG. 6. The circular dichroism spectra of the transferrin-cefotaxime sodium system (T=298K).

$$C_{\text{transferrin}}=2.0 \times 10^{-6} \text{ M}; C_{\text{cefotaxime sodium}}=(4.0, 6.0) \times 10^{-5} \text{ M}$$

Conclusions

In simulated physiological conditions, the interaction mechanism of the transferrin-cefotaxime sodium system was studied. With the increasing of the temperature, binding constant (K_a) decreased and the fluorescence of the system was quenched by a way of static quenching. $\Delta S > 0$, $\Delta G < 0$, $\Delta H < 0$ indicated the system is a spontaneous molecular interaction and electrostatic force is the main binding force type. Binding distance (r) between transferrin and cefotaxime sodium is smaller than 7 nm, which indicated the existence of non-radiative energy transfer between transferrin and cefotaxime sodium. That Hill's coefficients were approximately equal to 1 implied a non-cooperative reaction. Series of data analysis can provide information for understanding drug distribution in body, transport process and interaction mechanism. Synchronous spectroscopy has sensitivity and accuracy. Synchronous spectroscopy and circular dichroism spectroscopy both displays that α -helical secondary structure of transferrin, namely conformation, was changed. So, as different study methods, they have enriched research methods of association mechanism about drugs and proteins.

Acknowledgements

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