Research Article

Study on the Interaction of Bovine Serum Albumin with Ceftriaxone and the Inhibition Effect of Zinc (II)

Qiaoli Yue,[1] Tongfei Shen,[1] Changna Wang,[2] Chaohui Gao,[1] and Jifeng Liu[1]

[1] *Shandong Provincial Key Laboratory of Chemical Energy Storage and Novel Cell Technology, Department of Chemistry, Liaocheng University, Liaocheng 252059, Shandong, China*
[2] *Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA*

Correspondence should be addressed to Qiaoli Yue, yueqiaoli@gmail.com

The mechanism of the interaction between bovine serum albumin (BSA) and ceftriaxone with and without zinc (II) (Zn^{2+}) was studied employing fluorescence, ultraviolet (UV) absorption, circular dichroism (CD), and synchronous fluorescence spectral methods. The intrinsic fluorescence of BSA was quenched by ceftriaxone in a static quenching mode, which was authenticated by Stern-Volmer calculations. The binding constant, the number of binding sites, and the thermodynamic parameters were obtained, which indicated a spontaneous and hydrophobic interaction between BSA and ceftriaxone regardless of Zn^{2+}. Changes in UV absorption, CD, and synchronous fluorescence spectral data are due to the microenvironment of amide moieties in BSA molecules. In the BSA-ceftriaxone-Zn^{2+} system, Zn^{2+} must first interact with ceftriaxone forming a complex, which inhibits BSA binding to ceftriaxone. The present work uses spectroscopy to elucidate the mechanism behind the interaction between BSA and ceftriaxone in the presence and absence of Zn^{2+}. The BSA and ceftriaxone complex provides a model for studying drug-protein interactions and thus may further facilitate the study of drug metabolism and transportation.

1. Introduction

The interaction between biomacromolecules, especially between plasma proteins and drugs, has been an interesting research field in life sciences, chemistry, and clinical medicine [1]. Drug-albumin complexes may be considered as models for gaining fundamental insights into drug-protein interactions.

Serum albumin is a major soluble protein constituent of the circulatory system and has many physiological functions such as acting as a plasma carrier by nonspecifically binding to several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids [2]. Albumins are characterized by a low content of tryptophan and methionine and a high content of cystine and charged amino acids [3–5]. Bovine serum albumin (BSA), an example of a mammalian albumin, has been studied extensively because of its stability, neutrality in many biochemical reactions, and low cost [6, 7]. Brown elucidated the 607 amino acid residue, primary structure of BSA in 1975, twenty one of which are tyrosine (Tyr) residues and two of which are tryptophan (Trp) residues located at positions 134 and 212, respectively [3, 6]. These two Trp residues cause BSA to have intrinsic fluorescence.

Ceftriaxone is a third-generation cephalosporin antibiotic. Cephalosporins are semisynthetic antibiotics produced by fungi *Cephalosporium* and, like penicillins, are β-lactam antibiotics, which have broad-spectrum activity against Gram-positive and Gram-negative bacteria. Ceftriaxone is often used to chelate metal ions, and we believe this improves its antibacterial activity to study the effect of metal ions on its biological activity [8, 9]. The structure of ceftriaxone contains –NH_2, –COOH, –CO, and N–C functional groups as shown in Scheme 1(a), which are all electron donors. The construction of ceftriaxone molecular model indicates that it is suitable for chelating transition metal ions such as Zn^{2+}, Cd^{2+} [10], Mn^{2+}, Fe^{3+}, Co^{2+}, Cu^{2+} [11, 12], and Pd^{2+} [13]. Ceftriaxone chelates metal ions as a bidentate monoanion

(a)

(b)
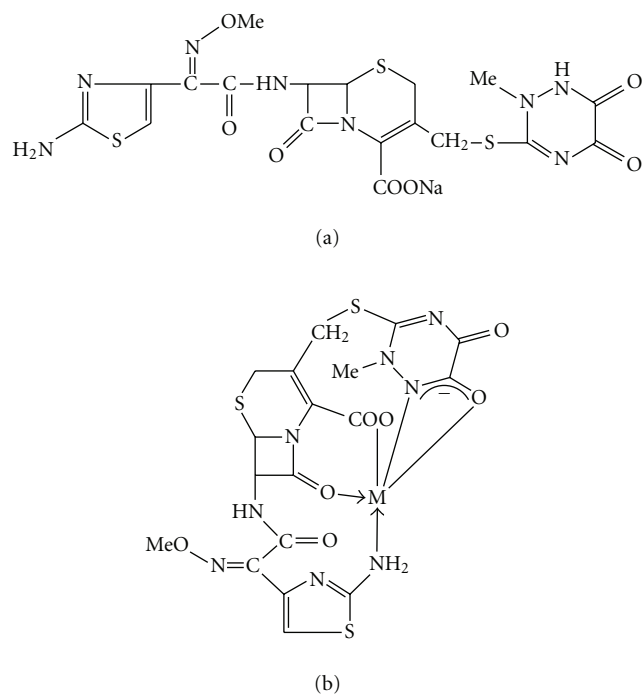
Scheme 1: Schematic drawings of the ceftriaxone (a) and ceftriaxone-M ((b), referred to in [11], Copyright 2005 Springer) molecule structure.

ligand through the β-lactam carbonyl and carboxylate group, as illustrated in Scheme 1(b). Furthermore, complexation with metal ions can make the drug more active and less toxic.

Common metal ions, especially transition metal ions, participate in many biochemical processes. Therefore, it is necessary to investigate protein-drug interactions in the presence of metal ions owing to the inevitable effects of metal ions in a ternary protein-drug-metal ion system [14]. Due to the intrinsic fluorescence of BSA from Trp-134 and Trp-212, the interaction between BSA and ligands such as drugs is frequently investigated by fluorescence methods [7, 15–18]. The effect of metal ions such as Cu^{2+} [14], Co^{2+} [19], and Fe^{3+} [20] in the interaction of serum albumin and drugs has been studied. The goal of this work is to reveal the interaction mechanism of BSA and ceftriaxone and the effect of Zn^{2+} on the present system by spectroscopic methods. This study may facilitate the study of metabolism and transportation of antibiotics as well as the relationship between protein and antibiotics in the presence of metal ions.

2. Materials and Methods

2.1. Instruments. All fluorescence measurements were carried out on an LS-55 fluorophotometer (Perkin Elmer, USA) equipped with a computer for data collection and a 1.0 cm quartz cell, with the slit's width of Ex/Em 5.0/5.0 nm and the PMT voltage at 700 V. Circular dichroism (CD) spectra were obtained on a J-810 circular dichroism chiroptical spectrometer (JASCO Co., Ltd, Japan). The UV spectra were obtained from a Lambda-40 spectrophotometer (Perkin

Elmer, USA) coupled with a 1.0 cm quartz cell. All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device Works, China) with a combined glass-calomel electrode. The temperature was controlled by a water bath, and temperatures were kept in a certain range ($T \pm 0.1°C$) throughout the experiment.

2.2. Materials. In this work, all reagents used were of analytical-reagent grade unless specified. Doubly distilled deionized water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). BSA (Sigma) was purchased from a local market without further purification to prepare a stock solution ($50 \mu mol L^{-1}$), which was kept in a brown flask at $4°C$. A $1.0 mmol L^{-1}$ stock solution of ceftriaxone was prepared in a calibrated flask. The phosphate buffered saline (PBS) was prepared using Na_2HPO_4 and NaH_2PO_4 for controlling pH of the system at 7.4. Other reagents were purchased from a local market.

2.3. Procedures. A 3 mL solution, containing appropriate concentration of BSA, was titrated by successive additions of a 1.0 mM ceftriaxone solution. Titrations were done manually by using micropipettors.

In a typical fluorescence measurement, the fluorescence emission spectra were recorded in the presence and absence of Zn^{2+} ions ranging from 300 to 450 nm upon the excitation wavelength at 295 nm using the excitation and emission slit widths both of 5 nm. The experiments for discussing binding mechanism were conducted at three temperatures, 298, 308, and 318 K, as maintained by water bath.

Synchronous fluorescence spectra of BSA titrated with various concentrations of ceftriaxone were recorded from 250 to 320 nm ($\Delta\lambda = 60$ nm) and from 260 to 320 nm ($\Delta\lambda = 15$ nm), at which the spectrum only showed the spectroscopic behavior of Trp and tyrosine (Tyr) residues of BSA, respectively. The excitation and emission slit widths were set at 5/5 nm and 10/10 nm for $\Delta\lambda = 60$ nm and $\Delta\lambda = 15$ nm, respectively.

The UV-vis absorbance spectra of BSA-ceftriaxone system were recorded at 293 K. CD spectra were recorded from 200 to 280 nm at 0.2 nm with a scan of 50 points at 310 K in a thermostated cell holder, with three scans averaged for each CD spectrum. The results were expressed as ellipticity (mdeg), which was obtained in mdeg directly from the instrument. The molar ratio of BSA, ceftriaxone, and Zn^{2+} was varied as 1:0:0, 1:1:0, 1:0:1, and 1:1:1.

Binding studies of BSA and ceftriaxone in the presence or absence of Zn^{2+} were performed at two modes. First, the interaction between BSA and ceftriaxone without Zn^{2+} was studied. BSA concentration was fixed at 0.5 μM, and a series of ceftriaxone standard solutions was added. Second, the interaction of BSA-Zn^{2+} in the presence of various concentrations of ceftriaxone was investigated. The concentration for BSA and Zn^{2+} was kept at 0.5 μM and 1.0 μM, respectively, whereas ceftriaxone was then gradually added to the BSA-Zn^{2+} mixture. Fluorescence spectra of BSA were recorded from 300 to 500 nm upon excitation at 295 nm for both of the two modes.

3. Results and Discussion

3.1. Fluorescence Quenching Study of BSA by Ceftriaxone in the Presence or Absence of Zn^{2+}. In order to investigate the effect of ceftriaxone for BSA and BSA-Zn^{2+}, the fluorescence spectra were drawn in PBS (pH 7.4). In the liquid fluorescence, the inner filter effect (IFE) could not be ignored. Due to the increasing concentrations of a fluorescent substrate and quencher, the increasing absorbance of excitation and/or emission radiation introduces IFE that decreases the fluorescence intensity and results in a nonlinear relationship between the observed fluorescence intensity and the concentration of the fluorophore. The fluorescence intensity was corrected by multiplying appropriate correction factors according to the experiment results reported byGu and Kenny [21], as follows:

$$F_{ideal}(\lambda_{ex}, \lambda_{em}) = F_{obs}(\lambda_{ex}, \lambda_{em}) CF_p(\lambda_{ex}) CF_s(\lambda_{em})$$
$$\approx F_{obs}(\lambda_{ex}, \lambda_{em}) 10^{(a_{em}+a_{ex})/2}, \tag{1}$$

where $F_{ideal}(\lambda_{ex}, \lambda_{em})$ is fluorescence intensity observed at emission wavelength λ_{em} when excited at excitation wavelength λ_{ex}, and CF_p is the correction factor for the primary IFE (pIFE), which depends on the total absorbance of the sample at λ_{ex}, where CF_s is the correction factor for secondary IFE (sIFE), which depends on the total absorbance of the sample at λ_{em}. a_{ex} and a_{em} are the values of absorbance of the solution per cm at λ_{ex} and λ_{em}, respectively. F_{obs} is the observed fluorescence.

Both Trp and Tyr amino acid residues give fluorescence emissions when excited at 280 nm, while Trp alone has a fluorescence emission at an excitation wavelength of 295 nm [14]. We obtained the fluorescence spectra for the system of BSA with ceftriaxone in the presence or absence of Zn^{2+} by fixing an excitation wavelength of 295 nm. In addition, the fluorescence intensity in the whole procedure was corrected by exploiting (1), though the effect of the IFE was not obvious. BSA exhibits a strong fluorescence emission with band peak at 348 nm. The fluorescence intensity of BSA decreased gradually with the addition of ceftriaxone regardless of Zn^{2+} as shown in Figure 1.

3.2. Quenching Mechanism. Fluorescence quenching can be processed via different mechanisms, usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their response to temperature [22]. Increased temperatures will quicken diffusion, produce large amount of collisional quenching, dissociate weakly bound complexes, and diminish static quenching. Based on this, the Stern-Volmer plots at different temperatures were drawn. As shown in Figure 2, the curves were linear. The quenching type may be static or dynamic, since the characteristic Stern-Volmer plot of combined quenching (both static and dynamic) has an upward curvature. Furthermore, the slope of the Stern-Volmer plots increased with the temperature. In this case, the quenching mode of ceftriaxone on the fluorescence of BSA was due to static quenching.
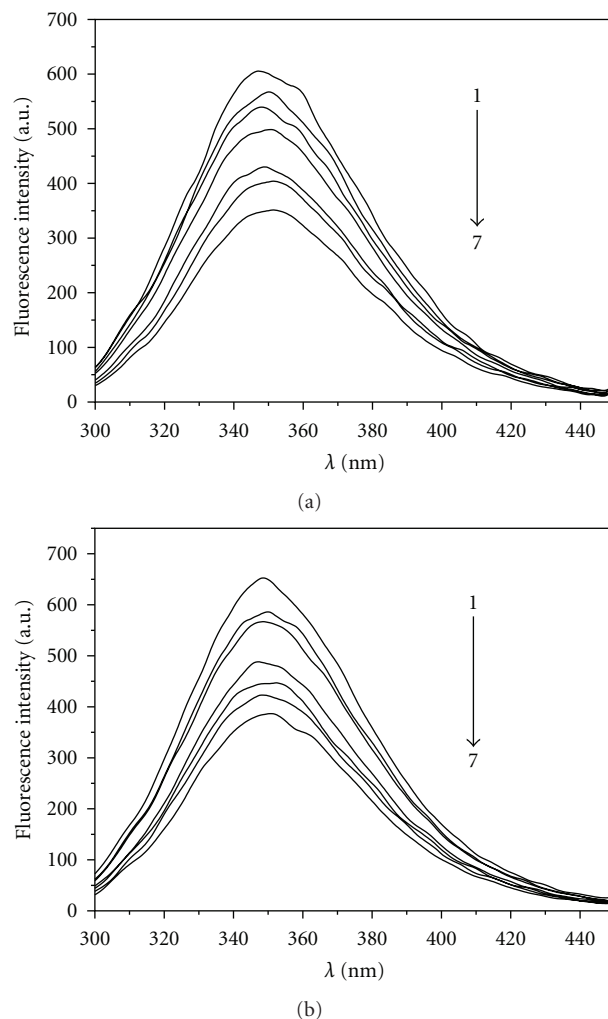
(a)

(b)

FIGURE 1: Fluorescence spectra of BSA in the absence (a) and presence (b) of Zn^{2+} with different ceftriaxone concentrations: 0, 1, 2, 4, 6, 8, and 10 μmol l^{-1} from (1) to (7), respectively. [BSA] = [Zn^{2+}] = 0.5 μmol l^{-1}.

The Stern-Volmer equation was utilized to further elaborate the fluorescence quenching mechanism. The relationship between quenching efficiency (F_0/F) and the concentration of quencher ($[Q]$) for the complete static or dynamic quenching mode should satisfy the following equation [23]:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + k_q\tau_0[Q], \tag{2}$$

where F_0 and F are the fluorescence intensity of fluorophore in the absence and in the presence of quencher, respectively, $[Q]$ is the concentration of quencher (ceftriaxone), K_{sv} is the Stern-Volmer constant corresponding to the slope of the plot for F_0/F versus $[Q]$ in l mol^{-1}, k_q is the quenching rate constant for biomolecular quenching in l mol^{-1}s^{-1}, and τ_0 is the average fluorescence lifetime of fluorophore without quencher evaluated at about 5 ns [24–26]. The results listed in Table 1 show that the Stern-Volmer quenching constant K_{sv} is inversely correlated with temperature, and the value of k_q is larger than the limiting diffusion constant of the
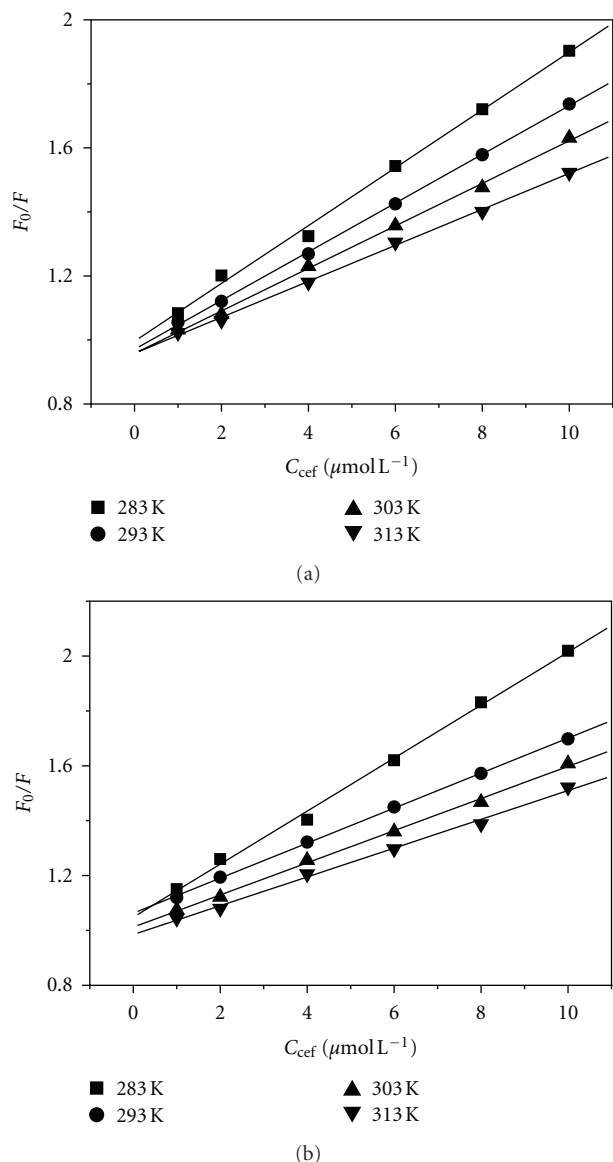
(a)

(b)

FIGURE 2: Stern-Volmer plots for the system of BSA without (a) and with (b) Zn^{2+} in the presence of ceftriaxone (with the concentrations from 0 to $10\,\mu mol\,l^{-1}$), at different temperatures. $[BSA] = [Zn^{2+}] = 0.5\,\mu mol\,l^{-1}$.

biomolecule $(2.0 \times 10^{10}\,l\,mol^{-1}s^{-1})$ [27]. This suggests that the fluorescence quenching was caused by a specific interaction between BSA and ceftriaxone regardless of Zn^{2+}. This evidence indicated that a static quenching was dominant in the system. From the results, it is also given that K_{sv} in the presence of Zn^{2+} was smaller than that in the absence of Zn^{2+}, indicating that quenching efficiency of ceftriaxone decreased by introducing Zn^{2+}. To confirm the static quenching mode, the lifetime of BSA and BSA-ceftriaxone was investigated with the results shown in Figure 3. It can be observed that the decay time of BSA fluorescence emission in the presence and absence of ceftriaxone is almost the same with the average lifetime at 5.1 ± 0.2 ns and 5.0 ± 0.1 ns, respectively. According to the literature, such slight differences indicated that the
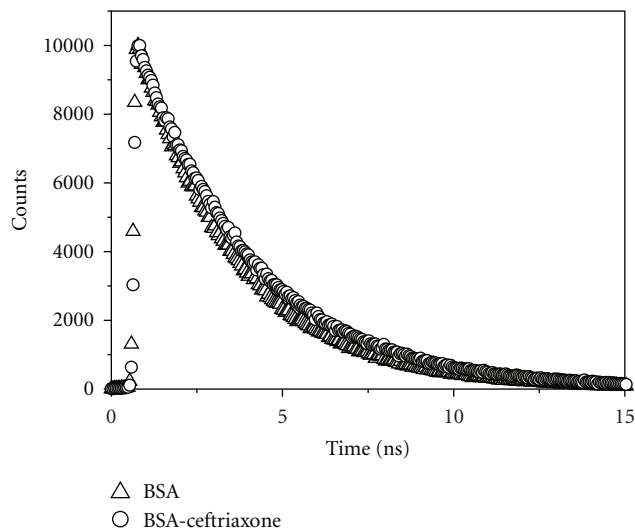

FIGURE 3: Fluorescence decay profiles of BSA in the presence and absence of ceftriaxone in 0.1 M PBS of pH 7.4. BSA $0.5\,\mu mol\,l^{-1}$, and ceftriaxone $2\,\mu mol\,l^{-1}$.

fluorescence quenching of BSA caused by ceftriaxone was attributed to the static quenching [22]. Thus, it is proposed that the complex formation takes a major role rather than dynamic collision for the interaction between BSA and ceftriaxone.

3.3. Influence of Zn^{2+} on the Binding Constant. Several equations can be used for the calculation of binding constant of the interaction between a protein and drug. One of the most frequently used equations is the Scatchard procedure equation since it can provide the actual stoichiometry for the binding sites of protein and ligands [28]

$$\frac{r}{D_f} = nK - rK, \tag{3}$$

where r is the moles of ceftriaxone bound per mole of BSA, D_f is the molar concentration of free ceftriaxone, n is the binding stoichiometry per class of binding sites, and K is the equilibrium binding constant.

The linearity of Scatchard plots for the ceftriaxone-BSA system was obtained as shown in Figure 4. Table 2 summarizes the binding constant, K, values and the number of binding sites per BSA at four temperatures acquired in the absence and presence of Zn^{2+}. The values of n suggest that ceftriaxone binds to a single class of binding sites on BSA. From the values of K, it was illustrated that there was a moderate binding force between ceftriaxone and BSA. Serum albumin has a limited number of binding sites for endogenous and exogenous ligands. These ligands are typically reversibly bound and have binding constants ranging from 10^4 to 10^8 [29, 30]. A K value larger than 10^4 indicates a significant interaction between ligand and protein. In addition, one category of binding sites of BSA for ceftriaxone in the absence and presence of Zn^{2+} was obtained.

TABLE 1: Dynamic quenching constant between ceftriaxone and BSA without and with Zn^{2+}.

System	Temperature K	Stern-Volmer equation	K_q L mol^{-1} s^{-1}	r
Without Zn^{2+}	283	$y = 0.0903[Q] + 0.9969$	1.81×10^{13}	0.9982
	293	$y = 0.0671[Q] + 1.0039$	1.52×10^{13}	0.9966
	303	$y = 0.0592[Q] + 1.0074$	1.33×10^{13}	0.9991
	313	$y = 0.0517[Q] + 0.9965$	1.12×10^{13}	0.9981
With Zn^{2+}	283	$y = 0.0966[Q] + 1.0483$	1.93×10^{13}	0.9987
	293	$y = 0.0638[Q] + 1.0631$	1.28×10^{13}	0.9998
	303	$y = 0.0586[Q] + 1.0120$	1.17×10^{13}	0.9989
	313	$y = 0.0525[Q] + 0.9985$	1.05×10^{13}	0.9979

TABLE 2: Binding constant between ceftriaxone and BSA.

| Temperature K	$K \times 10^5$ mol L^{-1}	n	ΔG kJ mol^{-1}	ΔH kJ mol^{-1}	ΔS J mol^{-1} K^{-1}
283	1.56	0.91	-13.28		
293	1.99	0.92	-13.84	2.49	55.72
303	2.37	0.92	-14.39		
313	2.93	0.92	-14.95		

In addition, it also can be observed from Table 2 that the presence of Zn^{2+} decreased the binding constants and binding sites of ceftriaxone-BSA complex, indicating that Zn^{2+} would affect the binding capacity of ceftriaxone binding to BSA. The smaller binding constant could result from two aspects: (1) competitive binding to BSA between Zn^{2+} and ceftriaxone and (2) Zn^{2+} binding to ceftriaxone inducing the conformational changes of ceftriaxone.

3.4. Thermodynamic Analysis.

In general, intermolecular interacting forces between a small molecule and a biomacromolecule include hydrogen bonding, van der Waals force, and electrostatic and hydrophobic interactions. The thermodynamic parameters enthalpy change (ΔH) and entropy change (ΔS) of the reaction are important for confirming the binding mode. The temperature-dependent thermodynamic parameters for the ceftriaxone-BSA system in the absence and presence of Zn^{2+} are used to characterize the intermolecular forces between ceftriaxone and BSA. $\Delta H°$ and $\Delta S°$ for a binding reaction can be derived from the following van't Hoff equation:

$$\ln k = \frac{-\Delta H°}{RT} + \frac{\Delta S°}{R}, \tag{4}$$

$$\Delta G° = \Delta H° - T\Delta S°, \tag{5}$$

where K is the binding constant at the corresponding temperature, and R is the gas constant. The results of K, $\Delta H°$, $\Delta S°$, and the corresponding values of Gibbs free energy ($\Delta G°$) for the system of BSA-ceftriaxone and BSA-ceftriaxone-Zn^{2+} were presented in Table 2. The sign and magnitude of the thermodynamic parameters were associated with various interactions which might take place in the protein association processes. As shown in Table 2, the negative values of ΔG along with the positive values of ΔH and ΔS are obtained for the ceftriaxone-BSA interaction. $\Delta G < 0$ reveals that the binding process is spontaneous.
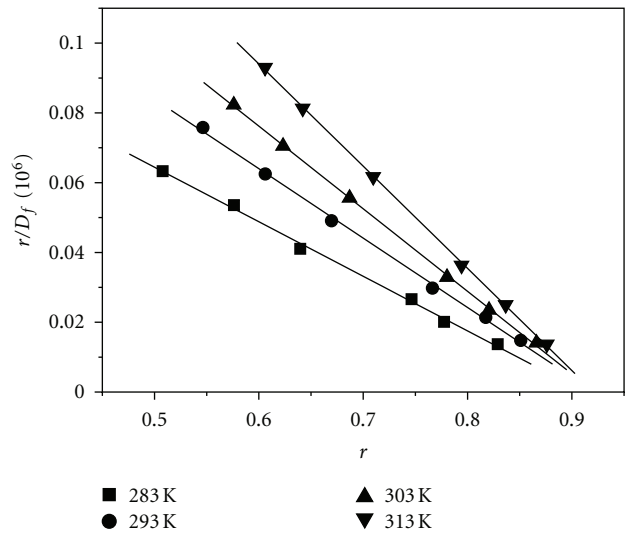
FIGURE 4: Scatchard plots for the BSA-ceftriaxone system in PBS (pH 7.4). BSA concentration: 0.5 μmol l^{-1}, λ_{ex} = 295 nm.

$\Delta S > 0$ is evidence of hydrophobic interactions [31]. Moreover, $\Delta H \approx 0$ and $\Delta S > 0$ are characteristics of electrostatic interactions in aqueous solution. Therefore, ceftriaxone bound to BSA displayed mainly hydrophobic and electrostatic interactions.

3.5. UV-Vis Absorption Studies.

UV-Vis absorption measurements are used to study structural changes and complex formation [20]. UV absorption spectra of ceftriaxone-BSA with or without Zn^{2+} systems were recorded and shown in Figure 5. The UV absorption spectrum of BSA shows a strong band with a maximum at 208 nm and a weak band with a maximum at 279 nm. The absorbance (279 nm) intensity of ceftriaxone-BSA system in the presence and absence of Zn^{2+}
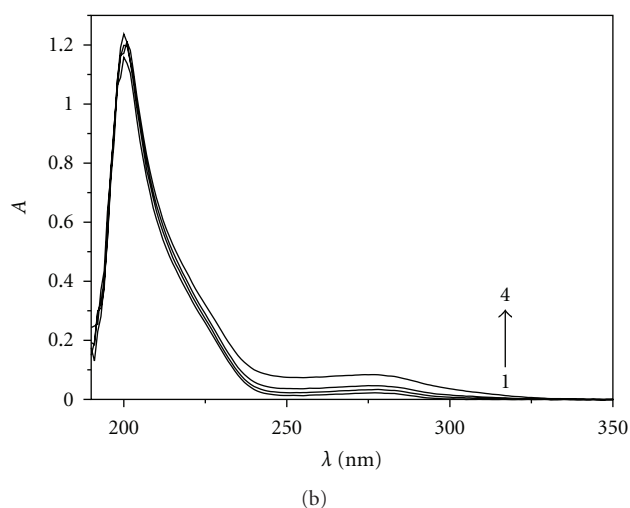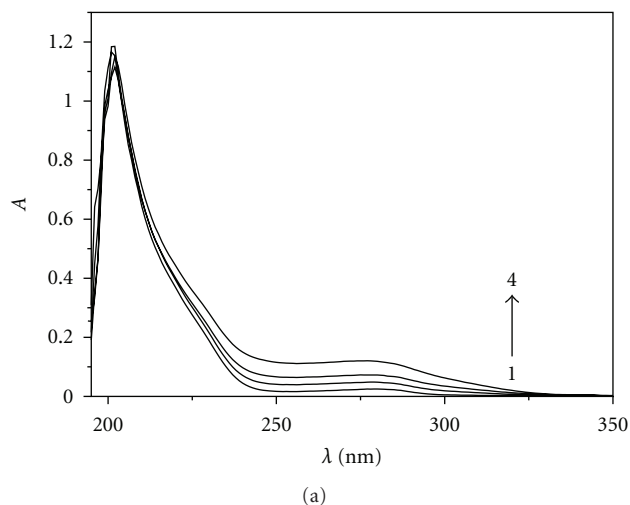
(a)

(b)

FIGURE 5: UV absorption spectra of BSA (A) and BSA-Zn^{2+} (B) in the presence of ceftriaxone. [BSA] = [Zn^{2+}] = 0.5 μmol l^{-1}, the concentration of ceftriaxone, 1–4, 0, 1, 2, and 5 μmol l^{-1}.

(a)

(b)

FIGURE 6: CD spectra of BSA (a) and BSA-Zn^{2+} (b) in the presence of ceftriaxone. 1 ceftriaxone, 2 BSA, 3 BSA-ceftriaxone, 4 BSA-Zn^{2+}-ceftriaxone, and 5 BSA-Zn^{2+}.

increased with increasing concentration of ceftriaxone, and the peak showed a slight blue shift (from 279 to 275 nm). However, the absorption at 208 nm did not significantly change. It was reported that the difference in the spectral peak of 208 nm is due to changes in the conformation of the peptide backbone associated with helix-coil transformation [32, 33]. In addition, the peak at the 279 nm region is related to the polarity of the microenvironment around Trp and Tyr residues of BSA. The interaction between ceftriaxone and BSA with or without Zn^{2+} may change the polarity of microenvironment around Trp and Tyr residues of BSA but does not cause a conformational change of BSA.

3.6. CD Studies. Due to the influence of Zn^{2+} on the spectra of BSA-ceftriaxone, it is important to determine whether the structure of BSA changes. CD is a sensitive technique to monitor conformational changes in protein structure [16]. CD spectra of BSA, BSA-ceftriaxone, BSA-Zn^{2+}, and BSA-ceftriaxone-Zn^{2+} are shown in Figure 6. In BSA spectrum,
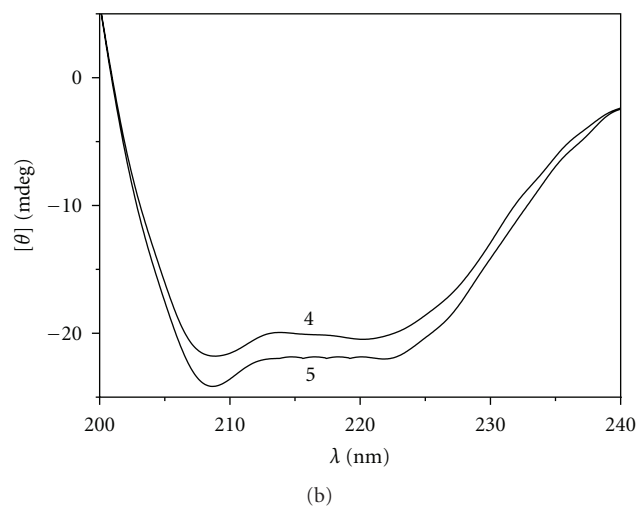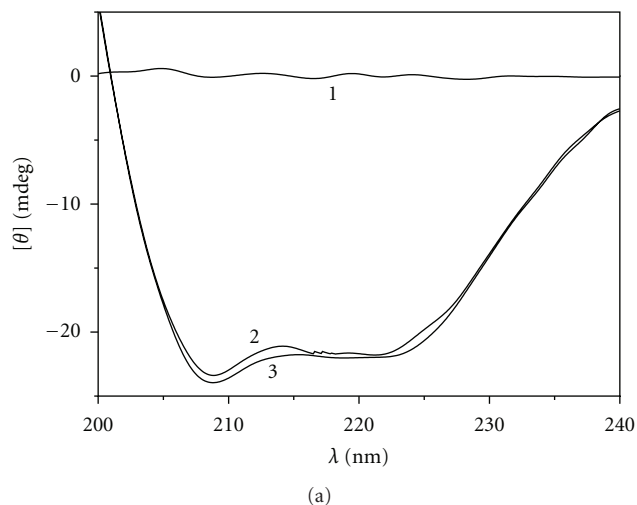
there are two minima in the ultraviolet region, one at 208 nm and the other at 222 nm, which are characteristic of the α-helical structure of a protein. Trynda-Lemiesz et al. explained that both of the negative peaks between 208-209 and 222-223 nm contribute to the transfer for the peptide bond of the α-helix [34]. Figure 6 shows that the bands of BSA are more pronounced in the presence of ceftriaxone with and without Zn^{2+}. The α-helix content in the secondary structure of BSA was determined (as listed in Table 3). From the data, it can be seen that the relative α-helix content of BSA increased in the presence of ceftriaxone, which suggested that the conformation of BSA changed by the addition of ceftriaxone. By comparison, the CD spectra for the participation of Zn^{2+} are similar to the native protein with the α-helix content of BSA decreasing slightly, which differs from previous results [14, 19]. Thus, the effect of Zn^{2+} on the BSA conformation may be ignored, and the binding of ceftriaxone is the main reason of conformational transition of BSA. In this case, Zn^{2+} may bind first to ceftriaxone forming complexes, thereby inhibiting BSA-ceftriaxone interactions. This may be the
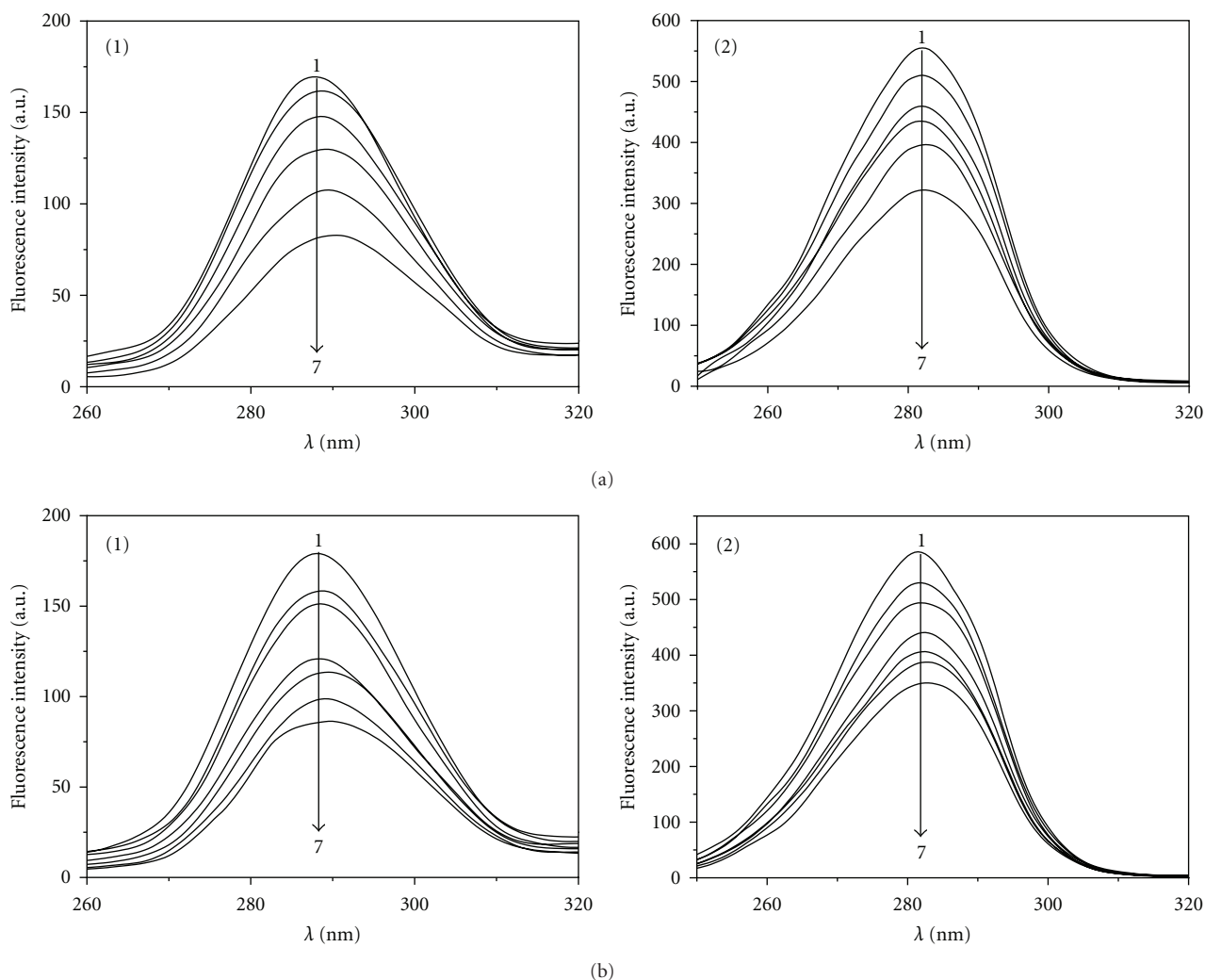
FIGURE 7: Synchronous fluorescence measurement for BSA without (a) and with (b) Zn^{2+} in the presence of ceftriaxone from (1) to (7) 0, 1, 2, 4, 6, 8, and 10 μmol l^{-1}, with $\Delta\lambda = 15$ nm (1) and $\Delta\lambda = 60$ nm (2), respectively.

TABLE 3: The contents of α-helix in the BSA in the presence of ceftriaxone without or with Zn^{2+}.

System	The content of α-helix (%)
2 μM BSA	49.6
2 μM BSA-2 μM ceftriaxone	52.4
2 μM BSA-2 μM Zn^{2+}	48.0
2 μM BSA-2 μM ceftriaxone-2 μM Zn^{2+}	48.7

reason why there is a distinct decrease of K in ceftriaxone-BSA system with Zn^{2+}.

3.7. Synchronous Fluorescence of BSA with Ceftriaxone in the Absence and Presence of Zn^{2+}.
Synchronous mode fluorescence spectroscopy was applied to study the conformational changes of the protein due to this binding reaction by measuring the emission wavelength shift [1]. According to Miller [35], characteristic Trp residue information is obtained when $\Delta\lambda$ value between excitation and emission wavelength is maintained at 60 nm, while $\Delta\lambda$ value for Tyr at 15 nm. Figure 7 showed the effect of ceftriaxone on the fluorescence emission for Tyr (1) and Trp (2) in BSA structure without (A) or with Zn^{2+} (B). From Figure 7, it can be seen that the fluorescence for $\Delta\lambda = 15$ nm was much weaker than that of $\Delta\lambda = 60$ nm, and the position of the emission peak for $\Delta\lambda = 60$ nm shifted to blue side compared with that for $\Delta\lambda = 15$ nm in the presence and absence of Zn^{2+}. These observations that the fluorescence intensity of both Trp and Tyr decreases and that there is a notable red shift at maximum emission upon addition of ceftriaxone indicate that the conformation of protein has changed. It is likely due to the hydrophobic amino acid structure surrounding tryptophan and tyrosine residues in BSA, which tends to collapse slightly, so tryptophan and tyrosine residues are exposed more to the aqueous phase. According to He et al.'s explanations, the interaction of ceftriaxone with BSA affects the polarity around Trp and

Tyr residue microregions regardless of Zn^{2+} [36], which was in accordance with the result obtained from UV-Vis spectroscopy method mentioned above.

When Zn^{2+} was added to the BSA-ceftriaxone system, Zn^{2+} preferentially combined with ceftriaxone over BSA. In other words, when Zn^{2+} and ceftriaxone coexist in the blood, Zn^{2+} will react first with ceftriaxone molecules, which prevents BSA from effectively enwrapping ceftriaxone. The study of the binding phenomena is important in providing basic information on the pharmacological actions, biotransformation, and biodistribution.

Acknowledgments

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