Asian Journal of Chemistry; Vol. 27, No. 3 (2015), 818-826

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.17121

Interaction Characteristic Studies of Ciprofloxacin and/or Sulphadiazine with Bovine Serum Albumin by Spectroscopic Technique

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Received: 25 January 2014; *Accepted*: 19 June 2014; *Published online*: 19 January 2015; AJC-16678

In this paper, the interaction of ciprofloxacin (CPFX) and/or sulphadiazine (SD) with bovine serum albumin (BSA) under simulative physiological conditions was studied, by fluorescence quenching in combination with UV-visible spectroscopic method. The fluorescence quenching constants, binding distance and binding constants for BSA-CPFX and/or sulphadiazine systems were determined. The fluorescence quenching of BSA by addition of ciprofloxacin and/or sulphadiazine is due to static quenching and energy transfer. The binding constants (K_A) of BSA-SD and BSA-CPFX systems were 4.04×10^6 and 6.96×10^5 M^{-1}, respectively, showing that sulphadiazine has higher binding capability with bovine serum albumin than ciprofloxacin. In the presence of sulphadiazine or ciprofloxacin, the K_A values of BSA-CPFX or BSA-SD systems decreased to 4.63×10^3 or 6.56×10^4 M^{-1}, suggesting that free ciprofloxacin and sulphadiazine in blood increased in their co-presence. Circular dichroism spectra, synchronous fluorescence and three-dimensional fluorescence studies showed that the presence of ciprofloxacin and/or sulphadiazine could change the conformation of bovine serum albumin during the binding process. The results are of great importance in pharmacy, pharmacology and biochemistry.

Keywords: Fluorescence, Bovine serum albumin, Ciprofloxacin, Sulphadiazine, Interaction characteristics.

INTRODUCTION

Serum albumin is one of the most abundant proteins in circulatory system of a wide variety of organisms and one of the most extensively studied proteins. The albumins make a significant contribution to colloid osmotic blood pressure and aid in the transport, distribution and metabolism of many endogenous and exogenous ligands. Protein-drug binding greatly influences absorption, distribution, metabolism and excretion properties of typical drugs[1,2]. Thus, it is important and necessary to study the interaction of drug with serum albumins at molecular level. Sulphadiazine (SD) is a broad-spectrum antibiotic used for the treatment of a variety of infectious diseases. Ciprofloxacin (CPFX) is third-generation quinolone with wide antibiotic spectra, high activity, low side effect and high clinical effect and is commonly used in therapy of many bacterial infections. Its antimicrobial activity is based on the inhibition of bacterial DNA gyrase[3]. The combination of sulphadiazine with ciprofloxacin has been used in clinical practice to improve treatment purpose[4]. Protein binding has long been considered one of the most important physicochemical characteristics of drugs, playing a potential role in distribution, excretion and therapeutic effectiveness.

Several works have studied the interaction of ciprofloxacin and human serum albumin (HSA)/bovine serum albumin (BSA) using continuous ultrafiltration associated HPLC[5], capillary electrophoresis-frontal analysis[6] and affinity capillary electrophoresis[7]. Fluorescence spectroscopic method has been primarily research tools in biochemistry and biophysics due to easily achieving the determination under near physiological conditions and has been used for study on the interaction of ciprofloxacin and bovine serum albumin[8-12]. Among them, few works studied the binding site of ciprofloxacin on bovine serum albumin[8,12]. There have been few reports for studying the interaction of sulphadiazine and bovine serum albumin. The plasma protein binding of sulphadiazine was determined by ultrafiltration[13] and circular dichroism[14], but without the information for the binding site and the change of bovine serum albumin conformation. To our best of knowledge, the effect of two-coexisting sulphadiazine and ciprofloxacin on pharmacodynamics is seldom studied for drug-serum albumin systems.

In this work, bovine serum albumin was used because of its low cost and easy availability and sulphadiazine and ciprofloxacin were used as model drug. This study examined the interaction between bovine serum albumin and two-coexisting

sulphadiazine and ciprofloxacin under physiological conditions by the fluorescence quenching in combination with UV-visible spectroscopic method.

EXPERIMENTAL

Commercially available bovine serum albumin (98 %, 1×10^{-4} M) was prepared by dissolving an appropriate amount of bovine serum albumin with 0.1 M *tris*-HCl (pH 7.4) buffer solution and kept in the dark at 4 ºC. Bovine serum albumin working solutions were prepared by diluting the stock solution with water. Sodium chloride (analytical grade) solution was used to maintain the ion strength at 0.1 M. Buffer (pH 7.40) consists of *tris* (0.1 M) and HCl (0.1 M). Stock solution of ciprofloxacin and sulphadiazine (1×10^{-3} M) was prepared by dissolving the standards with water and NaOH solution, respectively. Working solutions were obtained by dilution with the buffer from the corresponding stock solution. All chemicals were of analytical reagent grade or better. Purified water was prepared by an XGJ-30 highly pure water machine (Yongcheng purification Science & Technology Co. Ltd., Beijing, China).

All fluorescence measurements were performed on an F-7000 Fluorescence spectrophotometer (Hitachi, Japan) which was equipped with a 1 cm quartz cell and thermostat bath. The spectrum data points were collected from 280 to 500 nm. The widths of the excitation and the emission slit were both set at 5 nm. Fluorescence measurements were carried out at room temperatures.

Circular dichroism (CD) spectra were obtained on a J-810 circular dichroism chiroptical spectrometer (JASCO Co. Ltd, Japan). The absorption spectra were performed on a TU-1900 double light spectrophotometer (Beijing TAYASAF Science & Technology Co., Ltd, China) using a 1 cm quartz cell in the wavelength range of 250-450 nm. All pH measurements were performed with a pHS-3C pH meter (Shanghai, China).

Determination of fluorescence intensity: Five 10 mL clean and dried test tubes were taken and 2 ml of 0.5 M NaCl, 2 mL Tris-HCl buffer (pH 7.40), 0.25 mL of 4×10^{-5} M bovine serum albumin and different volumes of ciprofloxacin or sulphadiazine standard solution of 1×10^{-3} M were added in each test tube and diluted to the mark with water. The solution of 1×10^{-6} M bovine serum albumin at pH 7.4 was marked as "control". After mixing the solutions, these were allowed to stand for 15 min for maximum binding of CPFX/SD to bovine serum albumin. The fluorescence intensity (F_0) in the absence of quencher ciprofloxacin (and/or sulphadiazine) and the fluorescence intensity (F) in the presence of quencher ciprofloxacin and/or sulphadiazine were measured at a wavelength of λ_{ex} 280 nm and λ_{em} 340 nm under temperature of 25 °C for estimating the interaction between ciprofloxacin (and/or sulphadiazine) and bovine serum albumin.

RESULTS AND DISCUSSION

Fluorescence quenching mechanism: Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in fluorescence quenching of excited state fluorophores. These include molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. Fig. 1 shows the fluorescence spectra of bovine serum albumin in the absence and presence of ciprofloxacin and/or sulphadiazine .

The fluorescence spectra of bovine serum albumin (Fig. 1A, a) show a broad band with maximum at 340 nm; the fluorescence intensity of bovine serum albumin decreased with increasing concentration of sulphadiazine (Fig. 1A, b → f) or ciprofloxacin (Fig. 1B, b→e). The fluorescence spectra of ciprofloxacin (Fig. 1A, a') show a broad band with maximum at 410 nm, its intensity decreased with increasing concentration

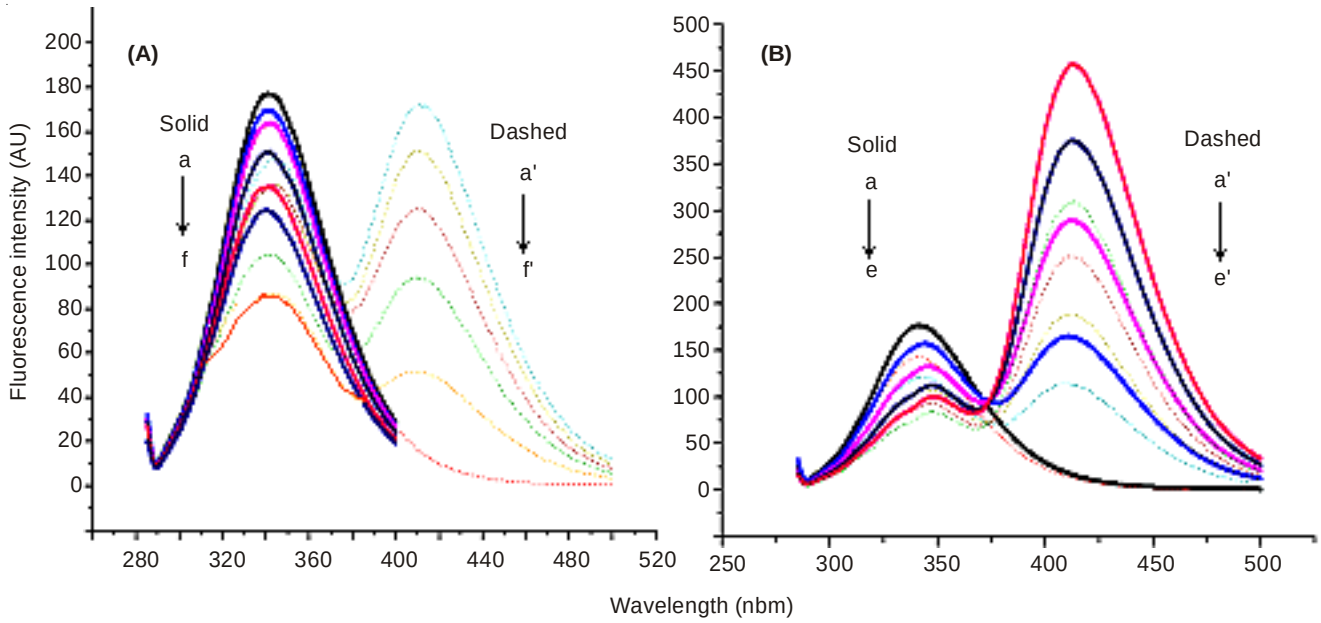

Fig. 1. Quenching fluorescence spectra of BSA–SD, BSA–CPFX–SD, BSA-CPFX, and BSA–SD–CPFX systems; (A) BSA–SD (solid line) and BSA–CPFX–SD (dashed line): bovine serum albumin (BSA), 1×10^{-6} M, ciprofloxacin (CPFX), 4×10^{-6} M; SD ($\times 10^{-6}$ M): a (a')→f (f'), 0, 1, 3, 5, 7, and 9; (B) BSA-CPFX (solid line) and BSA-SD-CPFX (dashed line): BSA, 1×10^{-6} M, SD, 5×10^{-6} M; CPFX ($\times 10^{-6}$ M): a (a')→e(e'), 0, 4.0, 8.0,12, and 16

of sulphadiazine (Fig.1A, b' → f'). Maximum fluorescence emission of bovine serum albumin underwent spectral shift from 340 to 345 nm in the presence of ciprofloxacin and no spectral shift was observed in the presence of sulphadiazine. The quenching degree of ciprofloxacin to fluorescence of bovine serum albumin was higher than sulphadiazine .

The fluorescence quenching data are analyzed by the Stern-Volmer equation[15]:

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

where F_0 and F are the fluorescence intensity in the absence and presence of quencher, respectively. K_q is the quenching rate constant, τ_0 is the fluorescence life time of biopolymer bovine serum albumin ($\tau_0 = 10^{-8}$ s)[16] and K_{SV} and [Q] are the Stern-Volmer quenching constant and concentration of quencher, respectively.

In this work, using the Stern-Volmer plots of F_0/F *vs.* concentration Q of ciprofloxacin and/or sulphadiazine was obtained. The estimated values of kinetic data along with correlation coefficient (r) are given in Table-1.

The variation of F_0/F against concentration Q fits in the equation of y = m x + c with correlation coefficient (r) greater than 0.99. Quenching rate constant (K_q) values for the tested four systems were higher than the maximum collision diffuse quenching constant of the biomolecule (2×10^{10} L mol^{-1} s^{-1}). It is indicated that the fluorescence quenching was mainly from static quenching due to complex formation[17]. No shift in emission wavelength of bovine serum albumin in the presence of sulphadiazine was observed. It is due to that the complex of bovine serum albumin and sulphadiazine has no fluorescence characteristic and the shift in emission wavelength from 340 to 345 nm for bovine serum albumin further indicates the formation of complex with fluorescence characteristic by binds of ciprofloxacin with bovine serum albumin sites.

Binding constant and binding site number: The binding of ciprofloxacin with bovine serum albumin to form complex in the ground state is further understood on the basis of available binding site number and binding constant of the complex formation process. For static quenching, the following equation was used to calculate the binding constant and binding sites[18,19]:

$$\log [(F_0\text{-}F)/F] = \log K_A + n \log [Q] \quad (2)$$

where K_A and n are the binding constant and binding site number, respectively. The plots of log $[(F_0\text{-}F)/F]$ *vs.* log [Q] presented in Fig. 2 are linear. Binding constant (K_A) and the binding site number (n) could be calculated from the intercept and slope, as shown in Table-2, along with K_A values in the literatures.

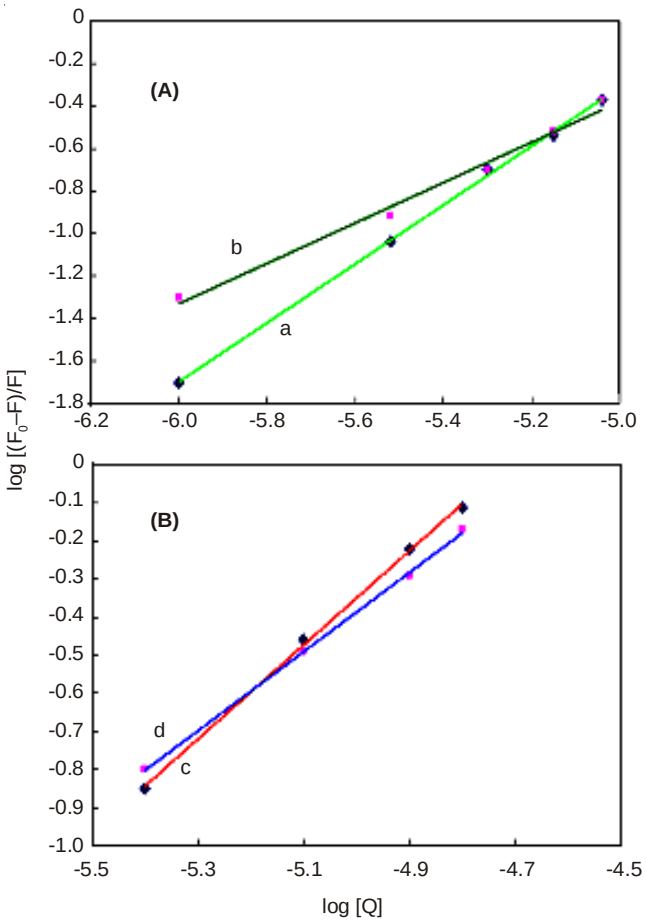

Fig. 2. Plot of log[$(F_0\text{-}F)/F$] *vs.* log[Q] at 25 ºC; (a) BSA-SD; (b) BSA-CPFX-SD; (c) BSA-CPFX; (d) BSA-SD-CPFX

It is shown that the correlation coefficient of the regression equations for the curves of log[$(F_0\text{-}F)/F$] *vs.* log[Q] is approximately equal to 1. The relative literatures reported different K_A values (10^2-10^5 M^{-1}) for BSA-CPFX because of using different methods. There was stronger combination action between CPFX/SD and bovine serum albumin. The K_A ratio of BSA-

TABLE-1
QUENCHING REACTIVE PARAMETER OF BSA–SD AND/OR CIPROFLOXACIN SYSTEMS AT 25 ºC

System	Regression equation	r	K_q (L mol^{-1} s^{-1})
BSA-SD	$F_0/F = 0.9732 + 4.76 \times 10^4$ Q	0.9907	4.76×10^{12}
BSA-CPFX	$F_0/F = 0.9720 + 5.00 \times 10^4$ Q	0.9962	5.00×10^{12}
BSA-CPFX–SD	$F_0/F = 1.003 + 4.18 \times 10^4$ Q	0.9953	4.18×10^{12}
BSA-SD-CPFX	$F_0/F = 0.9920 + 4.28 \times 10^4$ Q	0.9995	4.28×10^{12}

TABLE-2
EXPERIMENTAL VALUES OF THE BINDING CONSTANT K_A AND THE NUMBER OF BINDING SITE n OF THE INVESTIGATED COMPOUNDS AND BOVINE SERUM ALBUMIN AT 25 ºC

System	Regression equation	r	n	K_A (M^{-1})
BSA-CPFX	Log $[(F_0\text{-}F)/F] = 5.842 + 1.24$ Log [Q]	0.9995	1.24	6.96×10^5
BSA-SD	Log $[(F_0\text{-}F)/F] = 6.607 + 1.38$ Log [Q]	0.9994	1.38	4.04×10^6
BSA-CPFX-SD	Log $[(F_0\text{-}F)/F]3.665 + 0.82$ Log [Q]	0.9923	0.82	4.63×10^3
BSA-SD-CPFX	Log $[(F_0\text{-}F)/F]4.817 + 1.04$ Log [Q]	0.9997	1.04	6.56×10^4

SD to BSA-CPFX equals to 5.8, showing higher binding of sulphadiazine than ciprofloxacin to bovine serum albumin. It is shown that concentration of free ciprofloxacin in blood is higher than sulphadiazine. The K_A ratio of BSA-SD-CPFX system to BSA-CPFX system equals to 0.09 and the K_A ratio of BSA-CPFX-SD to BSA-SD equals to 0.001, suggesting that free ciprofloxacin and sulphadiazine in blood in their co-presence increased. It was proved that the interaction between the drugs reduces the binding stability of the drug and protein. At the same time, increase of the free drug concentration will increase the effect of the drugs.

Energy transfer from bovine serum albumin to DES: Fluorescence resonance energy transfer is an important technique for investigating a variety of biological phenomena including energy transfer processes[20]. Here the donor and acceptor are bovine serum albumin and SD/CPFX, respectively. It was observed that there is spectral overlap between fluorescence emission of bovine serum albumin and absorption spectra of ciprofloxacin and sulphadiazine in the wavelength range of 260-500 nm, as shown in Fig. 3. The fluorescence emission (330 nm) of BSA-SD solution (d) at an excitation wavelength of 280 nm is from bovine serum albumin (a) only since sulphadiazine is a non-fluorescence drug molecule. However, at this wavelength sulphadiazine (c) has weak absorption. The fluorescence emission (337 nm) of BSA-CPFX solution (b) at an excitation wavelength of 280 nm is from the complex of bovine serum albumin and ciprofloxacin, which is a fluorescence molecule. However, at this wavelength ciprofloxacin (e) has stronger absorption. It suggested the possibility of fluorescence resonance energy transfer from bovine serum albumin to CPFX/SD molecules in solution.

The region of integral overlap is used to calculate the critical energy transfer distance (R_0) between bovine serum albumin (donor) and SD/CPFX (acceptor) according to Foster's non-radioactive energy transfer theory using Förster's equation[18,21]. Based on this theory, the efficiency (E) of energy transfer between donor (bovine serum albumin) and acceptor (SD/CPFX) can be calculated by eqn. 3:

$$E = R_0^6/(R_0^6 + r^6) \quad (3)$$

where, r is the binding distance between donor and acceptor and R_0 is the critical binding distance. When the efficiency (E) of energy transfer is 50 %, which can be calculated by eqn. 4:

$$R_0^6 = 8.8 \times 10^{-25} k^2 n^{-4} \Phi_D J \quad (4)$$

where, the k^2 is the spatial orientation factor of the dipole, n is the refractive index of medium, Φ_D is the quantum yield of the donor in the absence of acceptor and J is the overlap integral of the emission spectrum of the donor and the absorption spectrum of the acceptor. The J can be calculated by eqn.:

$$J = \Sigma F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \Sigma F(\lambda)\Delta\lambda \quad (5)$$

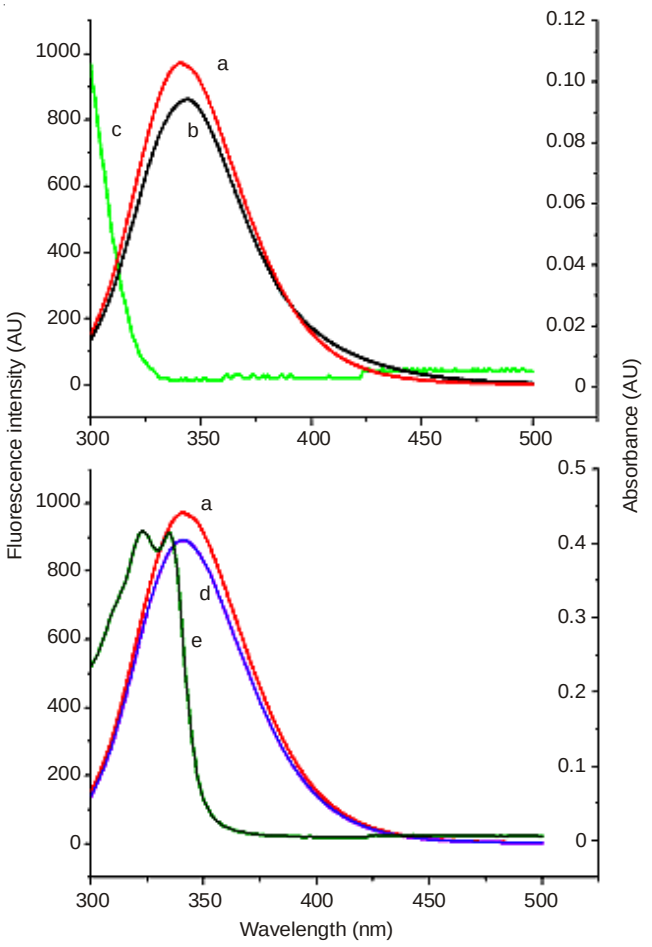

Fig. 3. Fluorescence spectra of BSA, BSA–CPFX and BSA–SD systems and UV-visible absorption spectra of SD and CPFX at 25 ºC. BSA, 3×10^{-5} M in all cases; (a) fluorescence spectrum of BSA; (b) fluorescence spectra of BSA–CPFX, CPFX, 5×10^{-6} M; (c) absorption spectrum of SD, 3×10^{-5} M; (d) fluorescence spectra of BSA–SD, SD, 4×10^{-6} M; (e) absorption spectra of CPFX, 3×10^{-5} M

where, $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength, λ, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, λ. In the present case, k^2, n and Φ_D are 2/3, 1.336 and 0.118, respectively[22].

The efficiency (E) of energy transfer can be determined by eqn. 6:

$$E = 1 - F/F_0 \quad (6)$$

where, F_0 and F are the fluorescence intensities of bovine serum albumin solutions in the absence and presence of DES, respectively.

From the overlapping R_0 was found from eqn. 4 using $K^2 = 2/3$, n = 1.336 and $\Phi_D = 0.118$ (tryptophan residue) for the aqueous solution of bovine serum albumin. J and E could be calculated from eqns. 5 and 6, respectively. At the same time, the binding distance (r) between bovine serum albumin and CPFX/SD is obtained by eqn. 3. Their values are listed in Table-3.

TABLE-3
ENERGY TRANSFER PARAMETERS FOR THE INTERACTIONS OF THE DRUGS WITH BOVINE SERUM ALBUMIN AT 25 °C

System	E (%)	J/(cm^3 mol^{-1})	R_0 (nm)	r (nm)
BSA-SD	3.90	3.20×10^{-16}	1.38	1.70
BSA-CPFX-SD	6.50	3.26×10^{-16}	1.38	2.15
BSA-CPFX	5.00	7.00×10^{-15}	2.31	3.77
BSA-SD-CPFX	4.64	2.11×10^{-14}	2.78	4.60

For BSA-SD in the presence of ciprofloxacin (BSA-CPFX-SD), the critical binding distance (R_0) did not change, but the binding distance (r) increased from 1.70 to 2.15 nm and the efficiency (E) of energy transfer increased from 3.9 to 6.5 %. For BSA-CPFX in the presence of sulphadiazine (BSA-SD-CPFX) the binding distance (r) increased from 3.77 to 4.60 nm. These data indicate that the nonradiative energy transfer from bovine serum albumin to SD/CPFX occurs, with higher possibility. It is suggested that the bindings of SD/CPFX to bovine serum albumin molecules were formed through energy transfer, which quenched the fluorescence of bovine serum albumin molecules, showing the presence of static quenching interaction between bovine serum albumin and SD/CPFX.

Binding site of ciprofloxacin and sulphadiazine on bovine serum albumin: The binding site of ciprofloxacin and/or sulphadiazine on bovine serum albumin was examined. The fluorescence quenching of bovine serum albumin by ciprofloxacin and/or sulphadiazine was observed under excitation at both 280 and 295 nm. The fluorescence of bovine serum albumin is mostly from tyrosine residue (Tyr) and tryptophan residue (Trp) at 280 nm excitation wavelength and the fluorescence of bovine serum albumin is only from tryptophan residue (Trp) at 295 nm excitation wavelength. The two curves were overlapped nearly, it is suggested that tryptophan residue mainly participates the binding reaction between CPFX/SD and bovine serum albumin.

On the basis of the probe-displacement method, there are at least three relatively-high specific drug-binding sites on the bovine serum albumin molecules. These sites, commonly called warfarin, ibuprofen and digoxin-binding sites, are also denoted as site I, site II and site III, respectively[23,24]. The competitive experiments suggested that the primary binding site of ciprofloxacin on bovine serum albumin was located at site I in sub-domain II_A of bovine serum albumin[8,12]. To further determine the binding site of ciprofloxacin and sulphadiazine, the competitive experiments were carried out at 25 °C using warfarin, ibuprofen and digoxin as a site I, site II- and site III-specific probe, respectively. The plots of log $[(F_0\text{-}F)/F]$ *vs.* log $[C_{DES}]$ in the absence and presence of site specific probe were prepared. The values of binding constant (K_A) and the binding site number (n) were calculated from the intercept and slope based on the eqn. 2. Binding constant and the binding site number were obtained.

Table-4 showed that the binding constants and binding site number were 4.04×10^6 M^{-1} and 1.38, respectively for SD-BSA system, while in the ibuprofen probe case, the binding constants decreased to 2.84×10^4 M^{-1}, two orders of magnitude lower than that for SD-BSA system without any specific probe and binding site number decreased from 1.38 to 0.98. It is shown the competition of ibuprofen with sulphadiazine at a same site. Otherwise, for CPFX-BSA system, in the ibuprofen probe case, the binding constant decreased from 6.96×10^5 M^{-1} (without any specific probe) to 2.43×10^4 M^{-1} and binding site number decreased from 1.24 to 0.95. It is shown the competition of ibuprofen with ciprofloxacin at a same site. The competitive experiments suggested that the primary binding site of sulphadiazine and ciprofloxacin on bovine serum albumin was located at site II in sub-domain III_A of bovine serum albumin and the second binding site was located at site I in sub-domain II_A of bovine serum albumin.

Effect of sulphadiazine and/or ciprofloxacin on the conformation of bovine serum albumin

Synchronous fluorescence studies: Synchronous fluorescence is a kind of simple and sensitive method to measure the fluorescence quenching. It can provide the information of polarity change around the chromophore micro-environment. $\Delta\lambda$, representing the difference between excitation and emission wavelengths, is an important operating parameter. When $\Delta\lambda$ is 15 nm, synchronous fluorescence is characteristic of tyrosine residue, while when $\Delta\lambda$ is 60 nm, it provided the characteristic information of tryptophan residues[25]. Because synchronous fluorescence spectroscopy is characteristics of spectrum simplification, spectrum band reduction, reducing spectrum band overlap and scattering minimal, they was used to investigating the change of conformation. In this work, the synchronous fluorescence spectra of tyrosine residue and tryptophan residues in bovine serum albumin with addition of sulphadiazine or ciprofloxacin were observed (Fig. 4). When wavelength interval is 15 nm, the spectrum characteristic of tyrosine residues in bovine serum albumin was observed. When $\Delta\lambda$ = 60 nm, the spectrum characteristic of tryptophan residues in bovine serum albumin was manifested.

It is shown in Fig. 4 (solid line) that when the drug sulphadiazine was gradually added, the main peak of tyrosine residues ($\Delta\lambda$ = 15 nm) and tryptophan residues ($\Delta\lambda$ = 60 nm) in bovine serum albumin was red shifted. It is shown in Fig. 5 (solid line) that when the drug ciprofloxacin was gradually added, the main peak of tyrosine residues ($\Delta\lambda$ = 15 nm) and tryptophan residues ($\Delta\lambda$ = 60 nm) in bovine serum albumin was red shifted. It is indicated that the presence of sulphadiazine and ciprofloxacin could change the conformation of bovine serum

TABLE-4
LINEAR EQUATION, BINDING CONSTANT K_A AND BINDING SITE NUMBER n BETWEEN SD/CPFX AND BOVINE SERUM ALBUMIN IN THE PRESENCE OF SITE-SPECIFIC PROBE

System	Linear equation	r	K_A (M^{-1})	n
SD-BSA	$Log[(F_0\text{-}F)/F] = 6.60685 + 1.38\ Log[C_{SD}]$	0.99944	4.04×10^6	1.38
SD-BSA-Warfarin	$Log[(F_0\text{-}F)/F] = 5.07652 + 1.14\ Log[C_{SD}]$	0.99335	1.19×10^5	1.14
SD-BSA-Ibuprofen	$Log[(F_0\text{-}F)/F] = 4.45301 + 0.98\ Llog[C_{SD}]$	0.99289	2.84×10^4	0.98
SD-BSA-Digoxin	$Log[(F_0\text{-}F)/F] = 6.48323 + 1.40\ Log[C_{SD}]$	0.99818	3.04×10^6	1.40
CPFX-BSA	$Log[(F_0\text{-}F)/F] = 5.84238 + 1.24\ Log[C_{CPFX}]$	0.99954	6.96×10^5	1.24
CPFX-BSA-Warfarin	$Log[(F_0\text{-}F)/F] = 5.20613 + 1.13\ Log[C_{CPFX}]$	0.99995	1.61×10^5	1.13
CPFX-BSA-Ibuprofen	$Log[(F_0\text{-}F)/F] = 4.38548 + 0.95\ Log[C_{CPFX}]$	0.99782	2.43×10^4	0.95
CPFX-BSA-Digoxin	$Log[(F_0\text{-}F)/F] = 4.78333 + 1.03\ Log[C_{CPFX}]$	0.99675	6.07×10^4	1.03

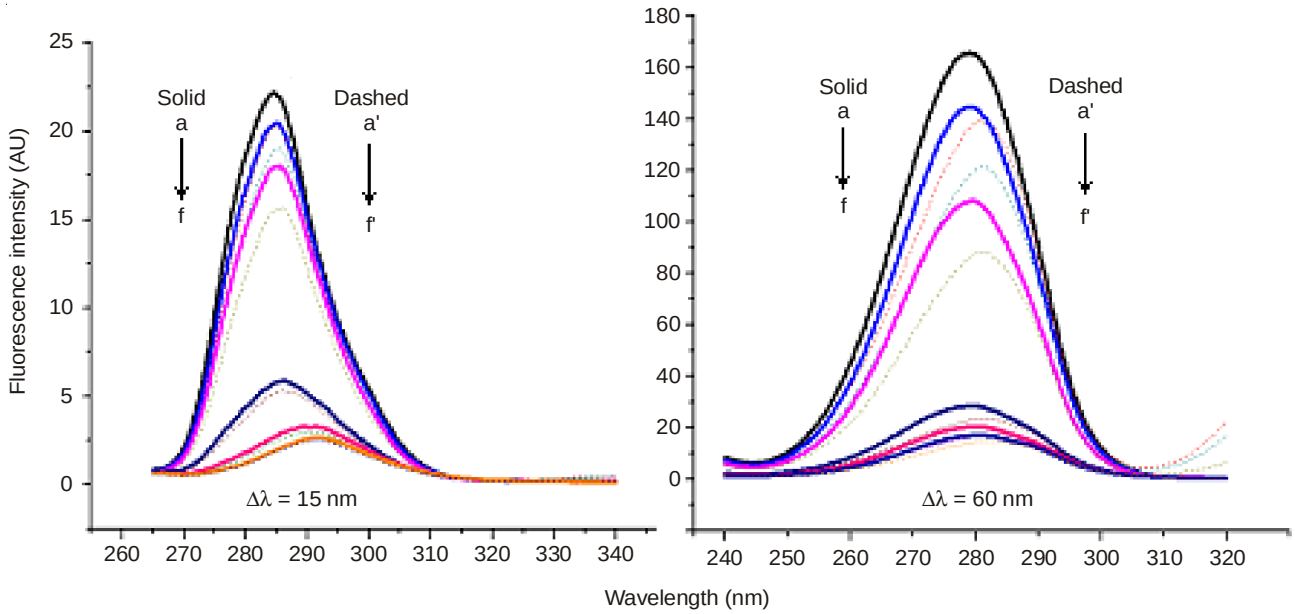

Fig. 4. Synchronous fluorescence of BSA-SD and BSA-CPFX–SD at Δλ = 15 and Δλ = 60 nm. BSA-SD (solid line): BSA, 1×10^{-6} M; SD, (10^{-5} M, a→f): 0, 0.5, 1.0, 1.5, 2.0, and 2.5; BSA-CPFX–SD (dashed line): BSA, 1×10^{-6} M; CPFX, 1×10^{-5} M, SD, (10^{-5} M, a'→f'): 0, 0.5, 1.0, 1.5, 2.0, and 2.5

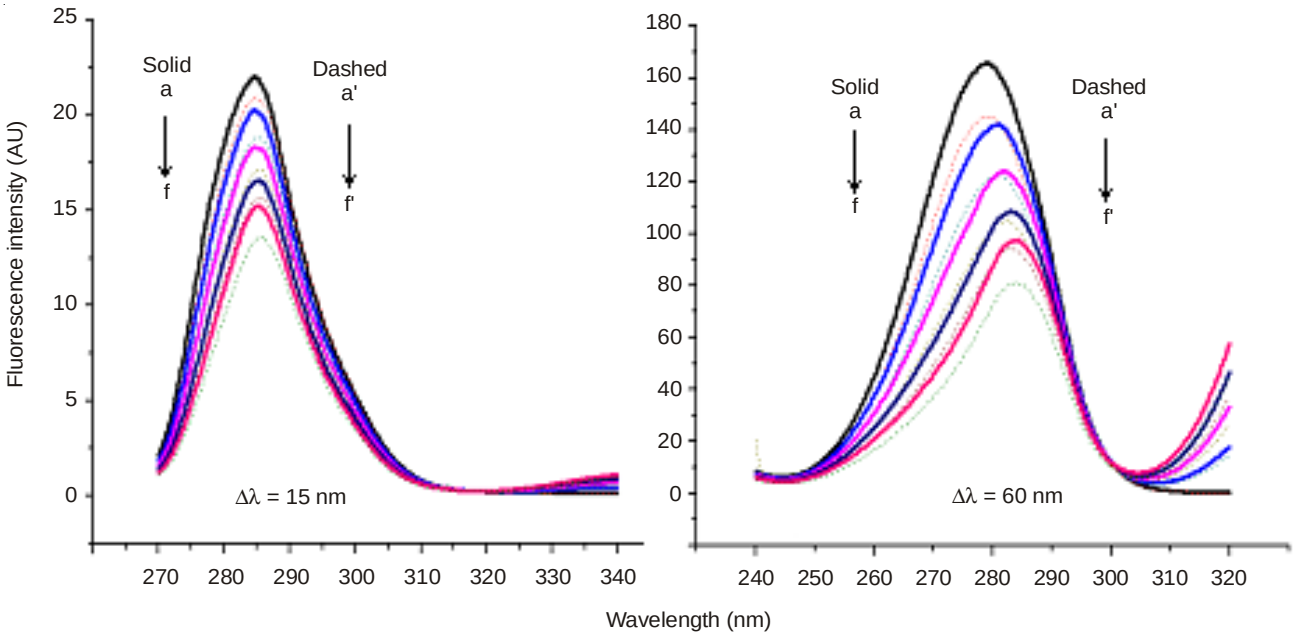

Fig. 5. Synchronous fluorescence of BSA-CPFX and BSA-SD-CPFX at Δλ = 15 and Δλ = 60 nm. BSA-CPFX (solid line): BSA, 1×10^{-6} M; CPFX, (10^{-5} M, a→e): 0.0, 0.4, 0.8, 1.2, and 1.6; BSA–SD–CPFX (dashed line): BSA, 1×10^{-6} M; SD, 5×10^{-6} M, CPFX, (10^{-5} M, a→e'): 0.0, 0.4, 0.8, 1.2, and 1.6

albumin. It is also shown that the microenvironment around the tyrosine residue and tryptophan residue changed during the binding process.

In the presence of ciprofloxacin, the fluorescence intensity of both tyrosine residues and tryptophan residues decreased regularly with increasing in sulphadiazine content and the main peak of tyrosine residues (Δλ = 15 nm) and tryptophan residues (Δλ = 60 nm) was red shifted (Fig. 4 dashed line). In the presence of sulphadiazine, with increasing in ciprofloxacin content, the main peak of tyrosine residues (Δλ = 15 nm) was red shifted slightly and the main peak of tryptophan residues (Δλ = 60 nm) was red shifted obviously (Fig. 5 dashed line). Above result indicated that the presence of coexisting ciprofloxacin and sulphadiazine could change the conformation of bovine serum albumin.

Three-dimensional fluorescence studies: The three-dimensional fluorescence spectrum is another powerful method for studying conformation change of bovine serum albumin. In this work, the three-dimensional fluorescence spectra of bovine serum albumin, BSA-SD, BSA-CPFX and BSA-SD-CPFX systems were observed, as shown in Fig. 6 and the characteristic parameters are summarized in Table-5.

From Fig. 6, peak 1 ($\lambda_{ex}/\lambda_{em}$ = 275.0/340.0 nm) reveals the spectral characteristic of tryptophan and tyrosine residues. After the addition of ciprofloxacin or sulphadiazine in bovine serum albumin, the fluorescence intensity of bovine serum

TABLE-5
THREE-DIMENSIONAL FLUORESCENCE SPECTRA CHARACTERISTIC PARAMETERS OF THE BSA AND DIFFERENT SYSTEMS

Systems	Parameters	Peak 1	Peak 2	Peak 3	Peak 4
BSA	Peak position ($\lambda_{ex}/\lambda_{em}$, nm)	275/340	225/345	-	-
	Relative intensity	158.7	139.1	-	-
	Stokes shift $\Delta\lambda$ (nm)	65	120	-	-
BSA-SD	Peak position ($\lambda_{ex}/\lambda_{em}$, nm)	280./340	225/340	-	-
	Relative intensity	128.5	97.75	-	-
	Stokes shift $\Delta\lambda$ (nm)	60	115	60	115
BSA-CPFX	Peak position ($\lambda_{ex}/\lambda_{em}$, nm)	280/345	225/345	270/415	320/410
	Relative intensity	110.3	106.4	491.5	195.3
	Stokes shift $\Delta\lambda$ (nm)	65	120	145	90
BSA-SD-CPFX	Peak position ($\lambda_{ex}/\lambda_{em}$, nm)	280/345	225/345	270/410	320/410
	Relative intensity	89.72	78.24	151.6	68.67
	Stokes shift $\Delta\lambda$ (nm)	6	120	140	90

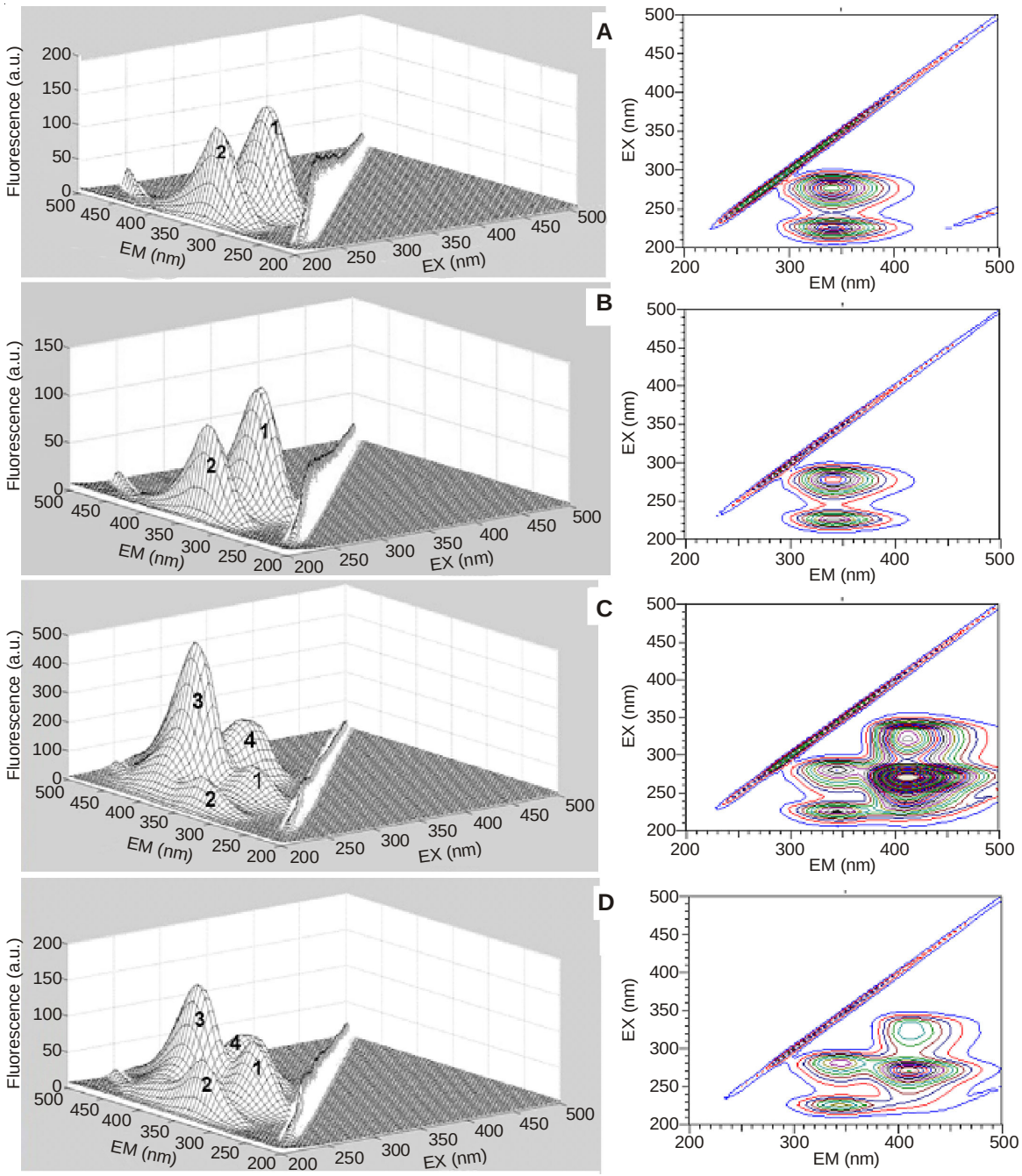

Fig. 6. Three-dimensional fluorescence spectra (left) and contour spectra (right) of (a) BSA, (b) BSA–SD, (c) BSA–CPFX, and (d) BSA–SD–CPFX systems. BSA, 1×10^{-6} M, SD, 2.5×10^{-5} M, CPFX, 2.5×10^{-5} M

albumin decreased from 158.7 to 110.3 or to 128.5 and the maximum emission wavelength of bovine serum albumin was shifted. In addition, peaks 3 and 4 was observed in the presence of ciprofloxacin, which is from fluorescence spectrum of ciprofloxacin and the complex of ciprofloxacin and bovine serum albumin, respectively. After the addition of sulphadiazine and ciprofloxacin in bovine serum albumin, peak 1 decreased due to serious fluorescence quenching. The addition of them changed the polarity of this hydrophobic microenvironment and the conformation of bovine serum albumin.

In Fig. 6, peak 2 ($\lambda_{ex}/\lambda_{em}$ = 225.0/345.0 nm) reveals the fluorescence spectra behaviour of polypeptide backbone structures, which is caused by the transition of π-π* of bovine serum albumin's characteristic polypeptide backbone structure C=O[26]. After the addition of CFZ and/or LMX in bovine serum albumin, the fluorescence intensity decreased and the maximum emission wavelength of bovine serum albumin was shifted, it is due to the change of the secondary structure of bovine serum albumin.

From the contour spectra in Fig. 6, after addition of sulphadiazine alone in bovine serum albumin, fingerprint lines of contour spectra changed to be sparse and after addition of ciprofloxacin alone in bovine serum albumin fingerprint lines reduced markedly. In the presence of both ciprofloxacin and sulphadiazine the fingerprint lines of contour spectra changed to be much sparse. These revealed that the microenvironment and conformation of bovine serum albumin were changed in the binding reaction. The interaction of ciprofloxacin and/or sulphadiazine with bovine serum albumin induced the unfolding of the polypeptides chains of bovine serum albumin and conformational change of bovine serum albumin.

Circular dichroism studies: Circular dichroism (CD) is a sensitive technique to monitor conformational changes in protein structure[27]. Circular dichroism spectra of bovine serum albumin, BSA-CPFX, BSA-SD and BSA-SD-CPFX are shown in Fig. 7.

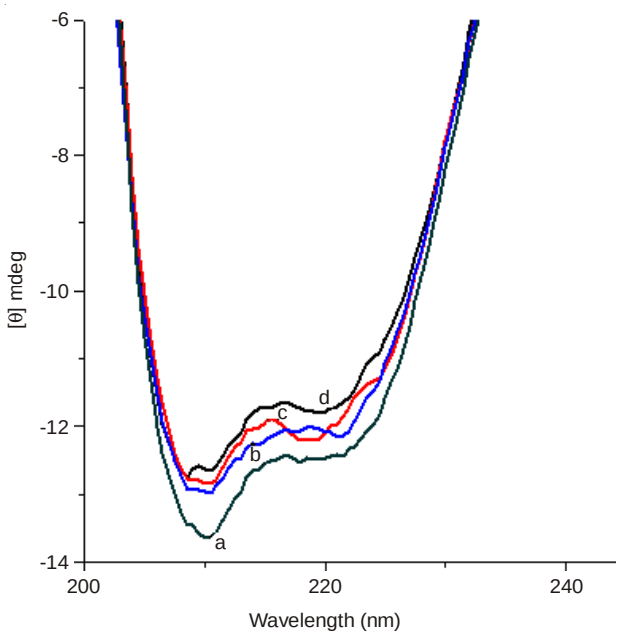

Fig. 7. CD spectra of (a) BSA, (b) BSA-SD, (c) BSA-CPFX, and (d) BSA-SD-CPFX systems. BSA, 1×10^{-6} M; SD, 5×10^{-6} M; CPFX, 5×10^{-6} M

In bovine serum albumin spectrum, there are negative peaks in the ultraviolet region, one at 209 nm and the other at 222 nm, which are characteristic of the α-helical structure of bovine serum albumin[28]. Lemiesz *et al.*[29] 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. In the presence of sulphadiazine and/or ciprofloxacin, the intensity of both the negative peaks decreased, proving the change of the α-helical structure of bovine serum albumin. However, the CD spectra of bovine serum albumin in the presence of ciprofloxacin and sulphadiazine are similar in shape, indicating that the structure of bovine serum albumin is also predominantly α-helical[30]. It is suggested that the both ciprofloxacin and sulphadiazine could alter the secondary structure of bovine serum albumin, which caused the fluorescence quenching and may affect its physiological function.

Conclusion

All the results indicated that the fluorescence quenching of bovine serum albumin by addition of ciprofloxacin and/or sulphadiazine is due to static quenching and energy transfer. The interaction of bovine serum albumin with ciprofloxacin or sulphadiazine leads to the need for more doses of the drugs to achieve therapeutic effect. The co-presence of ciprofloxacin and sulphadiazine increases the free drug concentration and therapeutic effect. The primary binding site of sulphadiazine and ciprofloxacin on bovine serum albumin was located at site II in sub-domain III_A of bovine serum albumin and the second binding site was located at site I in sub-domain II_A of bovine serum albumin. The coexisting of ciprofloxacin and sulphadiazine could change the conformation of bovine serum albumin. Circular dichroism spectra, synchronous fluorescence and three-dimensional fluorescence studies showed that the conformation of bovine serum albumin was changed dramatically with the addition of ciprofloxacin or CPFX-SD. The results are of great importance in pharmacy, pharmacology and biochemistry and are expected to provide important insight into the interactions of the physiologically important protein bovine serum albumin with drugs.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Found of Hebei Province (B2008000583) and the sustentation Plan of Science and Technology of Hebei Province (No. 10967 126D).

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