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Study of the quenching mechanism of bovine serum albumin with presence of chloramphenicol and enrofloxacin

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ABSTRACT

Chloramphenicol (CHL) and Enrofloxacin (EFLX) can both quench the fluorescence of bovine serum albumin (BSA) in the aqueous solution of pH=7.40. This quenching effect becomes more significant when CHL and EFLX coexist. Based on this, further studies on the interactions between CHL and EFLX using fluorescence spectrum were established. The results showed that the interaction between the drugs would increase the binding stability of the drug and protein, thus reducing the amount of drugs transported to their targets. Therefore, free drug concentration at targets would decrease, reducing the efficacy of the drugs. The results also showed that the quenching mechanism of BSA by the drugs is a static procedure. The number of binding sites is 1 in various systems. Studies utilizing synchronous spectra showed that the interaction between the drugs would affect the conformation of BSA, making protein molecules extend and decreasing their hydrophobicity.

KEYWORDS

Enrofloxacin;
Chloramphenicol;
Bovine serum albumin;
Fluorescence spectra;
Interaction.

INTRODUCTION

Enrofloxacin (EFLX) belongs to the third generation synthetics of quinolones against bacteria. It has better anti-bacterial effects for gram-negative bacillus. Furthermore, it has high degree anti-bacterial activity for gram- positive bacteria, like Staphylococcus[1]. Chloramphenicol (CHL) is a kind of broad spectrum antibiotic and can inhibit multiple kinds of coccus, bacillus and spirochete. It is an important drug used in treatment of typhoid fever, paratyphoid etc[2]. Usually, these two drugs will using coexist in the feeding of domestic animals.

In the past decades, researches on the interaction between drugs and BSA using fluorescence method have been intensively reported[3,5]. However, those researches are of single drug with BSA only. However, peoples are usually using two or more kinds of drugs in clinical practice, so the existence of the interaction between drugs have inevitable. Studies of these actions are mainly carried out through comparing the effects of administrating combined drugs and administrating certain drug alone to animals or cells of animal[6]. Researches on the interaction between drugs and BSA using fluo-

rescence method have been intensively reported. However, researches on the effect of drugs coexisting have not been reported.

This paper compares the parameters of the binding constant, binding distance, and changes in synchronous spectra between EFLX and CHL coexisting with BSA and single drug by fluorescence spectrum, to reveal the effect of drugs reduced because the interaction. In other words, the effect of drugs is reduced while combined with other drugs, the effect of the two drugs combined is less than the sum of their individual contribution (1 +1 <2)[7]. The results are identical with experiments on animal or cell. The advantage of this method is easy, fast, and cheap. The research has certain theoretical significance for rational usage of drugs, and provides a new method to study the effect of pharmacodynamic between two or multi-drugs.

EXPERIMENTAL

Apparatus

All fluorescence spectra were recorded using with a Shimadzu RF-540 spectrofluorophotometer and a Hitachi F-4500 spectrofluorophotometer; absorption was measured with an UV-VIS recording spectrophotometer (UV-265 Shimadzu Japan). All pH measurements were made with a pHS-3C precision acidity meter (Leici, Shanghai).

Reagents

CHL and EFLX were obtained from Monitor of Chinese Veterinary Medicine (no less than 99.9% pure). Stock solutions (1.0mmol/L) of CHL and EFLX were prepared by dissolving the drugs in water. The stock solutions were further diluted as working solutions prior to use. BSA (10μmol/L) was purchased from Sigma Company (no less than 99% pure). A Tris-HCl buffer (50mmol/L, pH 7.40) and 100mmol/L NaCl. All other reagents were of analytical reagent grade and all aqueous solutions were prepared with newly double-distilled water. All measurements were made at T = 298 K. The fluorescence intensities were corrected for absorption of exciting light and reabsorption of the emitted light to decrease the inner filter effect using the relationship[11]:

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

Where F_{cor} and F_{obs} are the fluorescence intensities corrected and observed, respectively, and *Aex* and *Aem* are the absorption of the system at the excitation and the emission wavelength, respectively. The intensity of fluorescence used in this paper is the corrected fluorescence intensity.

The effect of BSA with single drug (BSA-CHL or EFLX)

Successively adding 2.0 mL of 100mmol/L NaCl (to keep the pH constant and maintain the ionic strength of the solution), 1.0 mL of pH= 7.40 Tris-HCl, 1.0 mL of 10μmol/L BSA solution and 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mL of 0.2mmol/L CHL or EFLX into a 10 mL colorimetric tube. The sample was diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 10 min. The excitation and emission wavelengths for BSA were 286 nm and 340 nm, respectively, with the excitation and emission slit widths set at 10 nm. Meanwhile, make use of the fluorescence intensity of BSA without any drugs as F_0, to study the quenching effects of single drug to BSA.

The antagonistic action of CHL to EFLX

Successively adding NaCl, Tris-HCl, BSA same as above, 1.0 mL of 0.1mmol/L CHL solution and 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mL of 0.2 mmol/L EFLX into a 10 mL colorimetric tube. Meanwhile, Make use of the fluorescence intensity of BSA-CHL as F_0, to study the quenching effects of EFLX to BSA in the presence of CHL.

The antagonistic action of EFLX to CHL

Successively adding NaCl, Tris-HCl, BSA same as above, 1.0 mL of 0.1 mmol/L EFLX and 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0mL of 0.2 mmol/L CHL into a 10 mL colorimetric tube. Meanwhile, Make use of the fluorescence intensity of BSA-EFLX as F_0, to study quenching effects of CHL to BSA in the presence of EFLX.

RESULTS AND DISCUSSION

Fluorescence quenching of BSA and drugs

The fluorescence quenching of EFLX and CHL with BSA were shown in Figures 1 and 2. It is observed that

the fluorescence intensity of BSA decreases regularly with increasing concentration of EFLX or CHL, and there is a little emission wavelength shift occurring with addition of EFLX or CHL. In order to confirm the quenching mechanism, calculations are performed as follows: according to dynamic quenching, the mechanism can be described by the Stern-Volmer equation[8,9]:

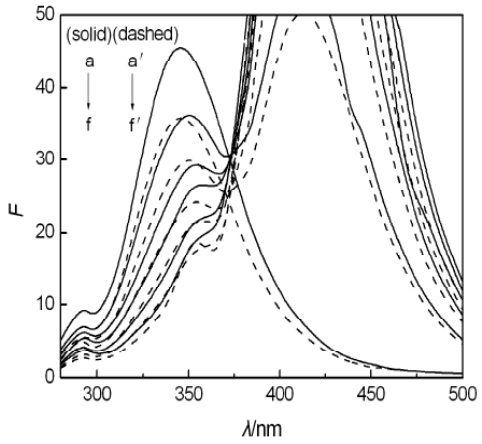
$$F_0 / F = 1 + K_q \tau_0 [C] = 1 + K_{SV} [C] \quad (2)$$

F_0 and F are the fluorescence intensities in the absence and in the presence of quencher, respectively. τ_0 is the average lifetime of the bio-molecule without quencher, which is about 10^{-8} S and K_{sv} is the dynamic quenching constant. K_q is the quenching rate constant of bio-molecule. [C] is the concentration of the quencher. The calculated results are shown in TABLE 1. For dynamic quenching, maximum scattering collision quenching constant of various quenchers with bio-molecules, K_{dif} is 2.0×10^{10} L·mol^{-1}·S^{-1}. When the fluorescence lifetime of the bio-molecule τ_0 was taken as 10^{-8} S, the quenching rate constant K_q at different system were calculated to be all at 10^{12} L·mol^{-1}·S. Obviously, the rate constant of the protein quenching by EFLX or CHL K_q is much larger than the limiting diffusion coefficient K_{dif} of the bio-molecule. This means that the quenching is not initiated by dynamic collision but static quenching by the formation of a complex compound.

For static quenching, the relationship between the fluorescence quenching intensity and the concentration of quenchers can be described by equation 3[10,11]:

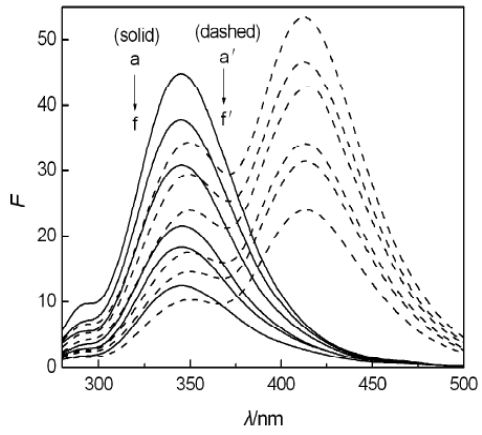
$$\lg[(F_0 - F)/F] = n \lg K_A + n \lg\{[C] - [p_t](F_0 - F)/F_0\} \quad (3)$$

Where F_0 and F are the fluorescence intensities before and after the addition of the quencher, [C] and [P_t] are the total quencher concentration and the total protein concentration, respectively. By the plot of lg (F_0 - F)/F versus lg{1/([C] - [Pt] (F_0 - F)/F_0)}, the number of binding site n and the binding constant K_A at 298 K were found. TABLE 1 gives the corresponding calculated results. TABLE 1 shows: (1) from the correlation coefficient r, it can be seen that all curves have good linearity. The number of binding sites is about 1. (2) from $K_{A\,(BSA\text{-}EFLX)}/K_{A\,(BSA\text{-}CHL)}$ =1.88, it is proved that the combining ability between EFLX and BSA are stronger than CHL and BSA. That is, the free concentration of CHL in blood is more than EFLX when the same concentrations of those drugs were injected. (3) from the ratio $K_{A(BSA\text{-}CHL\text{-}EFLX£©}/K_{A(BSA\text{-}EFLX)}$=1.21, it can be seen that the combining ability between EFLX and BSA increased due to the presence of CHL. In other words, the free drug concentration reduced and so did the effect of drugs. Similarly, from the ratio $K_{A(BSA\text{-}EFLX\text{-}CHL)}/K_{A\,(BSA\text{-}CHL)}$=1.28, it can be seen that the combining ability between CHL and BSA increased due to the



a-f: (solid line), BSA-EFLX; a′- f′: (dashed line), BSA-CHL-EFLX system. The concentration of BSA is 1.0 μmol·L^{-1}. a. Pure BSA. The concentration of EFLX: b. 10 μmol·L^{-1}, c. 20 μmol·L^{-1}, d. 30 μmol·L^{-1}, e. 40 μmol·L^{-1}, f. 50 μmol·L^{-1}. The concentrations of BSA and CHL are respectively 1.0 μmol·L^{-1} and 10 μmol·L^{-1}, a′. BSA-CHL. The concentration of EFLX: b′. 10 μmol·L^{-1}, c′. 20 μmol·L^{-1}, d′. 30 μmol·L^{-1}, e′. 40 μmol·L^{-1}, f′. 50 μmol·L^{-1}.

Figure 1 : Quenching fluorescence spectra of BSA-EFLX and BSA-CHL-EFLX system



a-f: (solid line), BSA-CHL; a′- f′: (dashed line), BSA-EFLX-CHL system. The concentration of BSA is 1.0 μmol·L^{-1}. a. Pure BSA. The concentration of CHL: b. 10 μmol·L^{-1}, c. 20 μmol·L^{-1}, d. 40 μmol·L^{-1}, e. 50 μmol·L^{-1}, f. 80 μmol·L^{-1}. The concentrations of BSA and EFLX are respectively 1.0 μmol·L^{-1} and 10 μmol·L^{-1}, a′. BSA-EFLX. The concentration of CHL: b′. 10 μmol·L^{-1}, c′. 20 μmol·L^{-1}, d′. 40 μmol·L^{-1}, e′. 50 μmol·L^{-1}, f′. 80 μmol·L^{-1}.

Figure 2 : Quenching fluorescence spectra of BSA-CHL and BSA-EFLX-CHL system

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TABLE 1 : The quenching reactive parameters of BSA and drugs

System	K_q/(L·mol^{-1}·s^{-1})	R	SD	K_A/(L·mol^{-1})	R	SD	n
BSA- EFLX	3.89×10^{12}	0.9950	0.0417	3.56×10^{4}	0.9978	0.02534	1.19
BSA-CHL	2.12×10^{12}	0.9984	0.04673	1.89×10^{4}	0.9984	0.02593	1.04
BSA-CHL- EFLX	3.38×10^{12}	0.9979	0.04487	4.29×10^{4}	0.9976	0.02823	1.28
BSA-EFLX -CHL	2.74×10^{12}	0.9989	0.04041	2.41×10^{4}	0.9998	0.01581	1.23

R is the correlation coefficient for the *Kq* and K_A values. .SD is the standard deviation for the *Kq* and K_A values.

presence of EFLX. At the same time, the free drug concentration reduced and so did the effect of drugs. In other words, the effect of drugs will reduce due to the existence of interaction between CHL and EFLX.

Binding distance between the drugs and BSA

According to Fŏrster resonance energy transfer (FRET), the energy donor and the energy acceptor distance r is related with the critical energy transfer distance R_0 and energy efficiency E can be calculated by the formula[12]:

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

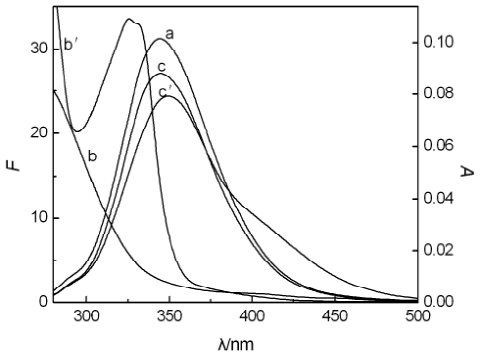
Where F and F_0 are the fluorescence intensities of BSA in the presence and absence of quencher, E is the energy transfer efficiency, r is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%. The quantity R_0^6 is calculated using the equation:

$$R_0^6 = 8.78\times10^{-25}K^2\Phi N^{-4}J \quad (5)$$

Where K^2 is the orientation factor, Φ is the fluorescence quantum yield of the donor, N is refractive index of the medium, and J is the overlap integral between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor (Figure 3), and J is given by:

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

Where F (λ) is the fluorescence intensity of the fluorescence donor at wavelength λ, and ε (λ) is the molar absorption coefficient of the acceptor at this wavelength. In the present case, K^2=2/3, N=1.336[13], Φ=0.118[14]. Thus J, E, R_0 and r were calculated and shown in TABLE 2. The donor-to-acceptor distance r‹7 nm, thus this reaction belongs to FRET[15]. In the experimental conditions, the fluorescence of BSA comes from the tryptophan residues in the 212 site[16]. This residue is situated in the hydrophobic cavity of BSA. r is the distance between binding sites and this residues. EFLX and CHL are less fat-soluble and difficult to enter the hydrophobic cavity; the binding position is relatively far from this tryptophan. So the value r is greater than the hydrophobic drug of Ligustrazine Hydrochloride (r=1.8 nm)[17]. The binding distance r of BSA-CHL-EFLX are reduced compared with BSA- EFLX, the result indicated that with presence of CHL, the binding distance between BSA and EFLX reduced while the combination ability increased. At the same time, free drug concentration reduced and so did the effect of drugs. Similarly, comparing BSA-EFLX-CHL with BSA-CHL will lead to the same conclusion.



a. fluorescence of BSA, b. absorption of CHL, b′. absorption of EFLX, the concentrations of BSA, CHL and EFLX are all 7.0 μmol·L^{-1}. c. fluorescence of BSA-CHL, the concentrations of BSA and CHL are respectively 7.0 μmol·L^{-1} and 10 μmol·L^{-1}. c′. fluorescence of BSA-EFLX, the concentrations of BSA and EFLX are respectively 7.0 μmol·L^{-1} and 10 μmol·L^{-1}.

Figure 3 : Fluorescence spectra and absorbance spectra

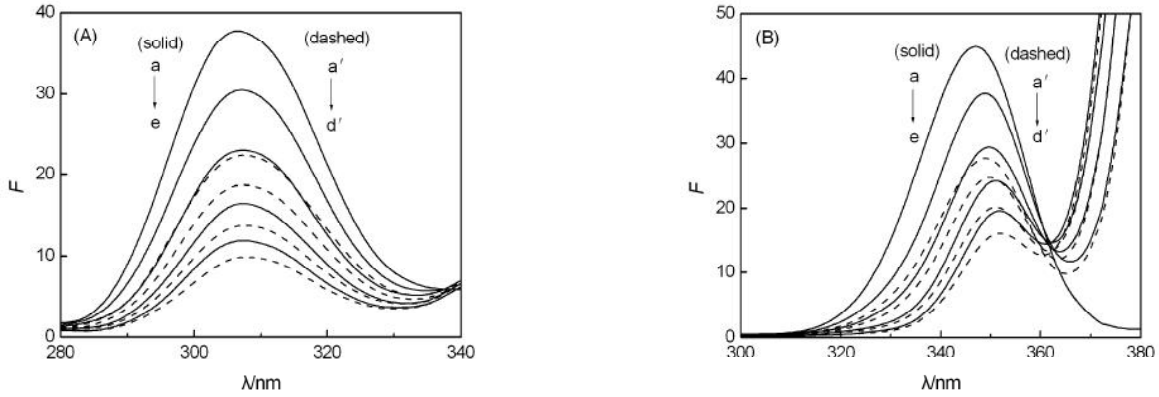
TABLE 2 : Parameters of E%, J, r, R_0 between drugs and BSA

System	E/(%)	J/(cm^3·L·mol^{-1})	R_0/(nm)	r/(nm)
BSA- EFLX	14.9	3.67×10^{-15}	2.07	2.77
BSA-CHL-EFLX	15.5	2.49×10^{-15}	1.94	2.57
BSA-CHL	7.74	1.63×10^{-15}	1.81	2.74
BSA-EFLX-CHL	7.50	1.28×10^{-15}	1.74	2.64

Conformation investigation of BSA

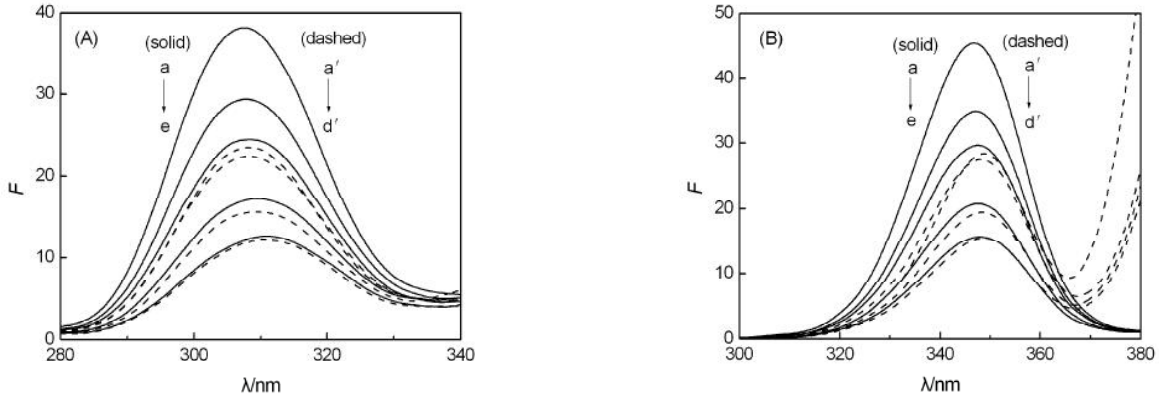
Synchronous fluorescence spectra were used to investigate the protein conformational change, as it has been

shown to give narrow and simple spectra. For the synchronous fluorescence spectra of protein, when the Δλ value between the excitation and emission wavelengths is stabilized at either 15 or 60 nm, the synchronous fluorescence gives characteristic information for the tyrosine residues or tryptophan residues. Because of the red shifts of maximum emission wavelengths of both tyrosine and tryptophan with the less hydrophobic environment, these red shifts indicated that the conformation of BSA was changed[17]. The synchronous fluorescence spectra when the Δλ=15 nm and λ=60 nm were measured (Figure 4 and Figure 5). It is apparent from this figure that the red shifts occurred at maximum emission wavelength. (1) Comparing BSA-EFLX (or BSA-CHL) and BSA, the red shifts were 1 nm and 2 nm when the Δλ=15 nm, and the red shifts were 4 nm and 2 nm when the Δλ=60 nm, respectively. (2) Comparing BSA-CHL-EFLX and BSA-EFLX, the synchronous fluorescence spectra had no red shifts when the Δλ=15 nm, the red shifts were 2 nm when the Δλ=60 nm. (3) Comparing BSA-EFLX-CHL and BSA-CHL, the red shifts were 3 nm when the Δλ=15 nm, and the red shifts were 2 nm when the Δλ=60 nm. It is apparent from this data that the interaction between the drugs was existed. (4) the conformation of BSA was changed because of the interaction between EFLX and CHL. As the increase of the concentration of the drugs, the tyrosine residues and tryptophan residues microenvironment changed due to the reaction of drugs and BSA, making the hydrophobic environment of BSA more polar and less hydrophobic, and collapsing the hydrophobic structure[18]. High concentration of drugs makes protein molecules extend, thus reducing energy



a-e: (solid line), BSA-EFLX; a′- d′: (dashed line), BSA-CHL-EFLX system. The concentration of BSA is 1.0 μmol·L^{-1}. a. Pure BSA. The concentration of EFLX: b. 10 μmol·L^{-1}, c. 20 μmol·L^{-1}, d. 40 μmol·L^{-1}, e. 50 μmol·L^{-1}. The concentrations of BSA and CHL are respectively 1.0 μmol·L^{-1} and 10 μmol·L^{-1}, the concentration of EFLX: a′. 10 μmol·L^{-1}, b′. 20 μmol·L^{-1}, c′. 40 μmol·L^{-1}, d′. 50 μmol·L^{-1}. (A): Δλ=15 nm, (B): Δλ=60 nm

Figure 4 : Synchronous fluorescence spectra of BSA-EFLX and BSA-CHL-EFLX system



a-e: (solid line), BSA-CHL; a′- d′: (dashed line), BSA-EFLX-CHL system. The concentration of BSA is 1.0 μmol·L^{-1}. a. Pure BSA. The concentration of CHL: b. 10 μmol·L^{-1}, c. 20 μmol·L^{-1}, d. 40 μmol·L^{-1}, e. 60 μmol·L^{-1}. The concentrations of BSA and EFLX are respectively 1.0 μmol·L^{-1} and 10 μmol·L^{-1}, the concentration of CHL: a′. 10 μmol·L^{-1}, b′. 20 μmol·L^{-1}, c′. 40 μmol·L^{-1}, e′. 60 μmol·L^{-1}. (A): Δλ=15 nm, (B): Δλ=60 nm

Figure 5 : Synchronous fluorescence spectra of BSA-CHL and BSA- EFLX –CHL system

transfer between amino acids, reducing the fluorescence intensity[19].

CONCLUSIONS

This paper indicated that the existence of the interaction between drugs through studying the binding constant, binding distance and the change of protein conformation using fluorescence spectra. This method is simple, rapid and accurate, to provide a theoretical basis for research of drugs pharmacology, combination therapy and rational usage of drugs. The limitation of this method is that it cannot be used to study drugs that neither interacts with protein nor fluorescent.

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