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Binding interactions of pefloxacin mesylate with bovine lactoferrin and human serum albumin*

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Abstract: The binding of pefloxacin mesylate (PFLX) to bovine lactoferrin (BLf) and human serum albumin (HSA) in dilute aqueous solution was studied using fluorescence spectra and absorbance spectra. The binding constant K and the binding sites n were obtained by fluorescence quenching method. The binding distance r and energy-transfer efficiency E between pefloxacin mesylate and bovine lactoferrin as well as human serum albumin were also obtained according to the mechanism of Förster-type dipole-dipole nonradiative energy-transfer. The effects of pefloxacin mesylate on the conformations of bovine lactoferrin and human serum albumin were also analyzed using synchronous fluorescence spectroscopy.

Key words: Pefloxacin mesylate (PFLX), Bovine lactoferrin (BLf), Human serum albumin (HSA), Fluorescence spectra, Energy-transfer efficiency

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INTRODUCTION

Pefloxacin mesylate (PFLX, Fig.1) is an antibiotic in a class of drugs called fluoroquinolones with wide spectrum of activity against various bacterial infections, such as bronchitis and urinary tract infections (Blandeau, 1999; Wang and Yuan, 1999). Due to its advantages such as good tolerance, the intaking of PFLX has no adverse effect on the pharmacokinetics features, low photo toxicity and intensive and effective antibacterial activity compared to the common quinolones, it has been widely used in clinical practice. However, few reports on the binding of PFLX to bovine lactoferrin (BLf) and human serum albumin (HSA) are available in the literature, so a detailed study on the binding reaction of PFLX will be of great interest to scientists in general and clinicians in particular.

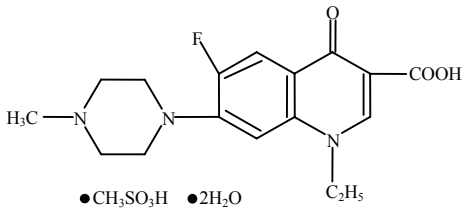

Fig.1 Chemical structure of pefloxacin mesylate

Lactoferrin is an 80 kDa iron-binding glycoprotein that is present in several biological fluids and in the secretory granules of neutrophils (Masson *et al*., 1966; 1969; Aguila and Brock, 2001) and is associated with a wide variety of biologically important processes, including host defense, regulation of cell growth, and cell differentiation (Sanchez *et al*., 1992). Lactoferrin is folded into two approximately equal lobes (N- and C-terminal), each of which is split by the iron-binding cleft into two domains (N1, N2 and C1, C2 respectively) (Baker *et al*., 1998). It is a highly basic protein, consequently interacts with many acidic molecules (Lampreave *et al*., 1990) to possibly

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modify the biological properties of lactoferrin.

Serum albumins are major soluble protein constituents of the circulatory system and have many physiological functions. The most outstanding function of albumins is that they serve as a depot protein and a transport protein for numerous endogenous and exogenous compounds. The exogenous substances bound with high affinity to protein are drugs. This interaction between protein and drug molecules results in formation of a stable protein-drug complex. Studying binding phenomena will be important for interpretation of the metabolism and transporting process, and will help to explain the relationship between structures and functions of protein.

Fluorescence spectroscopy is an appropriate method to determine the interaction between the small molecule ligand and bio-macromolecule. From measurement and analysis of the emission peak, the transfer efficiency of energy, the lifetime, and fluorescence polarization, etc., a vast amount of information will be yielded on the structural fluctuations and the microenvironment surrounding the fluorophore in the macromolecule. In this work, the binding reactions between pefloxacin mesylate and bovine lactoferrin (BLf) and human serum albumin (HSA) were investigated and the binding parameters and transfer efficiency of energy were also measured. Another main goal of this work was to check the effect of pefloxacin mesylate on BLf and HSA conformational changes. Based on the site-binding model (Klotz and Hunstone, 1971; Congdon *et al*., 1993; Baptista and Indig, 1998; Scatchard, 1949), practical fitting formulas for drugs binding to BLf and HSA were proposed. The results showed that these models can match very well with the experiments data.

EXPERIMENTAL DETAILS

Reagents and apparatus

Solutions of BLf (1.00×10^{-5} mol/L), HSA (1.00×10^{-5} mol/L) and pefloxacin mesylate (5.0×10^{-4} mol/L) and 0.05 mol/L Tris-HCl buffer pH=7.4 (0.1 mol/L NaCl used to keep the ionic strength constant) were prepared. All reagents except trimethylolmethane(tris), which was biochemical reagent were of analytical grade and double-distilled water was used throughout.

All of the fluorescence measurements were carried out on an F-4500 recording spectrofluorimeter (Hitachi, Japan) equipped with a xenon lamp source and 1.0 cm cells. A UV-260 recording spectrophotometer (Shimadzu, Japan) was used for scanning the UV spectrum. All pH measurements were made with a pHS-3C digital pH-meter (Yitong company of Jintan, Jiangsu, China).

Procedures

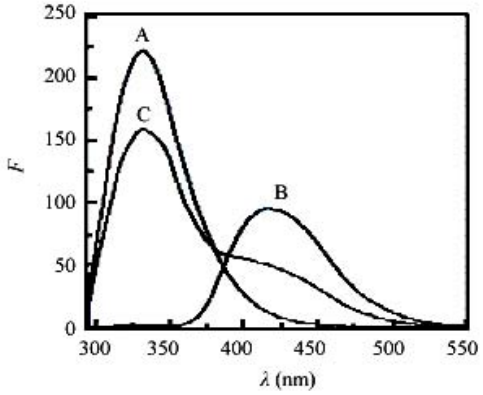
BLf and pefloxacin mesylate along with HSA and pefloxacin mesylate were dissolved in Tris-HCl buffer, the concentrations of BLf and HSA and pefloxacin mesylate were 1.00×10^{-5} mol/L, 1.00×10^{-5} mol/L and 5.0×10^{-4} mol/L, respectively. To a 1.0 cm quartz cell, BLf and HSA solutions were added to make up 2.5 ml respectively and the range of the drug solution was gradually titrateded into the cell using micro-injector. The accumulated volume was smaller than 200 μl. Under the apparatus condition of both entrance slit and exit slit width being 5 nm, and scanning speed of 240 nm/min, fluorescence quenching spectra and synchronous fluorescence spectra were obtained. Fluorescence quenching spectra were obtained at excitation and emission wavelengths of 295 nm and 300~550 nm for HSA, 290 nm and 295~550 nm for BLf respectively. For absorption spectra (UV) experiments, samples of pefloxacin mesylate were brought to 1.0 cm cuvette versus a blank of buffer. The absorbance was read and spectral scanning curves were made.

RESULTS AND DISCUSSION

Fluorescence spectra

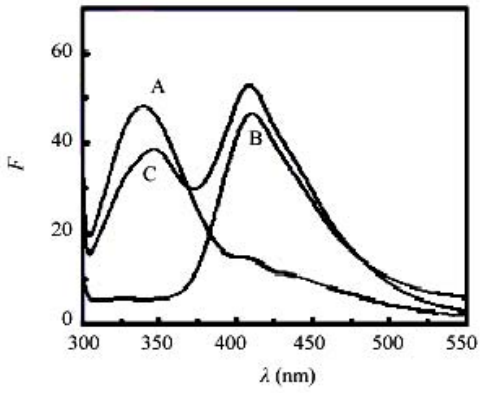
Fig.2 shows the fluorescence spectra of pefloxacin mesylate with BLf and HSA. The fluorescence emission lines of pefloxacin mesylate-BLf (1:1) mixture system and pefloxacin mesylate-HSA (1:1) mixture system can also be observed in Fig.2. It is well known that the BLf has strong fluorescence at $\lambda_{ex}/\lambda_{em}$=290/333 nm and HSA at $\lambda_{ex}/\lambda_{em}$=295/340 nm. When the solution conditions agree with our studies, Fig.2 shows the maximum strong fluorescence at 418 nm and 410 nm for pefloxacin mesylate respectively. In addition, the BLf and HSA fluorescence spectra are

somewhat quenched, indicating interaction has occurred and the energy has been transferred.



pH=7.4; λ_{ex}=290 nm; A: BLf; B: PFLX; C: BLf+PFLX (1:1)

(a)



pH=7.4; λ_{ex}=295 nm; A: HSA; B: PFLX; C: HSA+PFLX (1:1)

(b)

Fig.2 Fluorescence spectra of PFLX and BLf (a), and PFLX and HSA (b)

Binding constant and binding sites

For experiments carried out at large molar protein/dye ratios, it was assumed that only strong sites were active in binding dye. For simplicity, these strong binding sites were also assumed to be identical and to act independently. If these assumptions are valid, the site-binding model could be constructed, and the binding equation described by Scatchard is given by (Fletcher *et al*., 1970; Yang *et al*., 1997):

$$v=nK[D]/(1+K[D]). \quad (1)$$

Here v is the average number of dye molecules bound per protein molecule, n is the number of (strong) binding sites, K is the intrinsic (microscopic) binding (association) constant, and $[D]$ is the concentration of free (unbound) dye.

The linear form equation is as follows:

$$v/[D]=-Kv+Kn. \quad (2)$$

Eq.(2) is the usual form of Scatchard equation (Fletcher *et al*., 1970; Yang *et al*., 1997).

In the case of fluorescence only caused by a protein at the selected wavelength, the relationship between the concentration of protein and the fluorescence intensity can be described by

$$F_0/F=[P_t]/[P]. \quad (3)$$

According to the definition of n, another equation is also known,

$$v=([D_t]-[D])/[P_t]=n([P_t]-[P])/[P_t]=n(F_0-F)/F_0, \quad (4)$$

where, $[P_t]$ is the total protein concentration, $[D_t]$ is the final dye concentration, F_0 and F are, respectively, the fluorescence intensity in the absence of a quencher and in its presence at $[D]$ concentration.

The following equation would be obtained by combining Eq.(1) with Eq.(4):

$$F_0/F=K[D_t]F_0/(F_0-F)-nK[P_t]. \quad (5)$$

It indicates that the binding constant K and binding sites n can be obtained at the same time using least-squares algorithm for data-fitting according to Eq.(5).

The fluorescence quenching spectra of BLf and HSA in Tris-HCl buffer with increasing pefloxacin mesylate concentration and fixed BLf and HSA concentrations (both are 1.00×10^{-5} mol/L) are shown in Fig.3. It is obvious that the concentration of pefloxacin mesylate gradually increases with the titration, the fluorescence intensities of BLf and HSA decrease regularly, at the same time, the fluorescence emission peak of pefloxacin mesylate is gradually enhanced. The well-defined isobestic points are observed at 382 nm for BLf and 368 nm for HSA, which are the direct evidences for drug-protein complex formation.

After the fluorescence quenching on BLf at 333 nm and HSA at 340 nm were measured, the linear fit of fluorescence intensity changes of BLf-PFLX system and HSA-PFLX system were assessed by Eq.(5). Fig.4 shows the fitting curves and Table 1 shows the fitting results.

It is indicated that there are strong binding forces between pefloxacin mesylate and BLf as well as pefloxacin mesylate and HSA, and that about two binding sites would be formed for BLf which was the same number as that for iron, but it is uncertain that the binding sites for lactoferrin are equal to those for iron and the two binding sites should have the same binding constant according to the Scatchard equation, and that about one binding site for HSA. Scatchard plot obtained in the present work were both one straight line, which can validate the rationality for the binding model.

Table 1 The binding parameters for the systems of pefloxacin mesylate-BLf and pefloxacin mesylate-HSA

Compound	Binding constant, K (L/mol)	Binding site, n	Correlation coefficient, γ
BLf	1.06×10^5	1.85	0.9977
HSA	7.04×10^4	1.13	0.9972

Binding distance between the drug and the amino acid residues of BLf and HSA

According to Förster non-radioactive energy transfer theory (Yang and Gao, 2002; Horrocks and Collier 1981), the energy transfer effect is related not only to the distance between the acceptor and donor

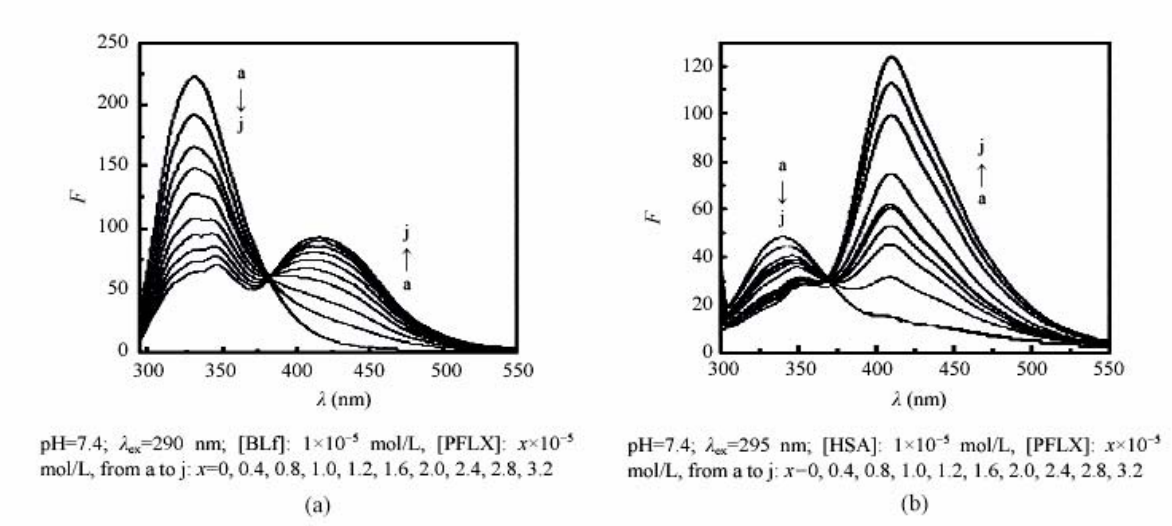


Fig.3 The effect of pefloxacin mesylate on quenching of BLf (a) and HSA (b) fluorescence

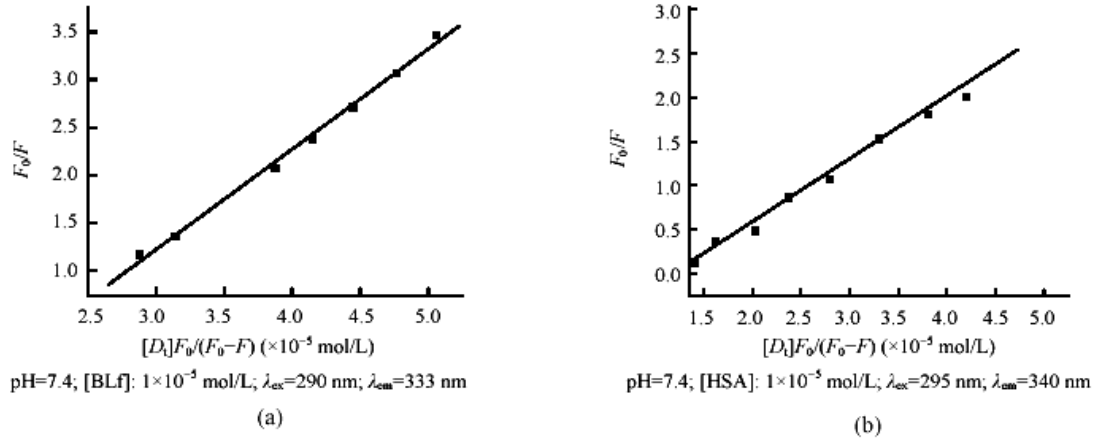


Fig.4 The fitting curves of pefloxacin mesylate-BLf (a) and pefloxacin mesylate-HSA (b) solution system

(r), but also to the critical energy transfer distance (R_0) (Horrocks and Collier, 1981; Ma *et al*., 1999),

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

Here R_0 is the critical distance when the transfer efficiency is 50%.

$$R_0^6=8.8\times10^{-25}K^2N^{-4}\Phi J, \quad (7)$$

where K^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor (Jiang *et al*., 2002; Shaklai *et al*., 1977). Therefore,

$$J=\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4\mathrm{d}\lambda\Big/\int_0^\infty F(\lambda)\mathrm{d}\lambda. \quad (8)$$

Here $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, and $\varepsilon(\lambda)$ is the molar absorptivity of the acceptor at wavelength λ, so the energy transfer efficiency is:

$$E=1-F/F_0. \quad (9)$$

The overlap of the absorption spectra of pefloxacin mesylate and the fluorescence emission spectra of BLf and HSA are shown in Fig.5. The overlap integral J can be evaluated by integrating the spectra in Fig.5. In this paper, J is given by the following Eq.(10) and was calculated to be 3.97×10^{-15} ($cm^3\cdot dm^3$)/mol for BLf and 6.15×10^{-15} ($cm^3\cdot dm^3$)/mol for HSA,

$$J=\sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda/\sum F(\lambda)\Delta\lambda. \quad (10)$$

Under these experimental conditions, the distance corresponding to 50% energy transfer from BLf and HSA to pefloxacin mesylate can be estimated to be R_0=2.11 nm and R_0=2.27 nm from Eq.(7) using K^2=2/3, N=1.36, Φ=0.13 (Horrocks and Collier, 1981). Moreover, the energy transfer effects are E=0.299 for BLf and E=0.209 for HSA from Eq.(6) and the binding distances between pefloxacin mesylate and amino acid residues in BLf and HSA are r=2.82 nm and r=2.83 nm respectively.

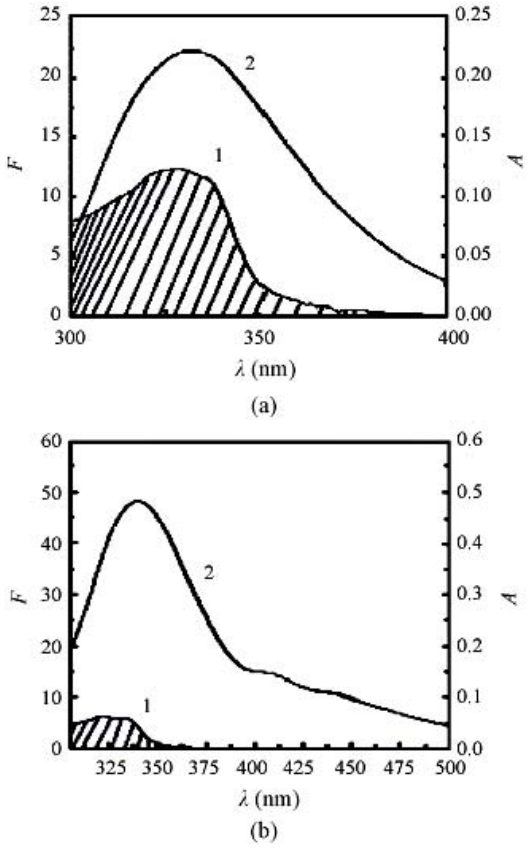


Fig.5 Overlap of (a) the absorption spectrum of pefloxacin mesylate (1) with the fluorescence emission spectrum of BLf (2), and (b) the absorption spectrum of pefloxacin mesylate (1) with the fluorescence emission spectrum of HSA (2)

Effect of the drug on the conformations of BLf and HSA

The conformational changes of BLf and HSA were evaluated by the measurement of the synchronous fluorescence intensity of protein amino acid residues before and after the addition of pefloxacin mesylate. Fluorescence measurements give information on the molecular environment in the vicinity of the fluorophore functional groups. In the synchronous spectra, the sensitivity associated with fluorescence is maintained while offering several advantages: spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. In this work, synchronous fluorescence spectroscopy was used to study the synchronous fluorescence characteristics of

BLf and HSA at different scanning interval $\Delta\lambda$ ($\Delta\lambda$=$\lambda_{emission}-\lambda_{excitation}$). When $\Delta\lambda$=15 nm, the spectrum characteristic of the protein tyrosine residues was observed, and when $\Delta\lambda$=60 nm, the spectrum characteristic of protein tryptophan residues was observed (Ma *et al*., 1999). The authors suggested a useful method to study the environment of amino acid residues is to measure the possible shift in the wavelength emission maximum λ_{max} (Yuan *et al*., 1998; Chen *et al*., 1990). The shift in the position of emission maximum corresponds to the changes in the polarity around the chromophore molecule. Thus, the conformation changes of BLf and HSA can be evaluated by the measurement of λ_{max}.

With the concentration of proteins being unchanged, and the concentration of pefloxacin mesylate increasing by titration, the synchronous spectroscopy were scanned at $\Delta\lambda$=15 nm, $\Delta\lambda$=60 nm (Figs.6 and 7).

The effect of pefloxacin mesylate on the tyrosine and tryptophan residues fluorescence intensities of BLf and HSA indicates that the main contribution to the fluorescence intensities of BLf and HSA are tryptophan residues. Figs.6 and 7 also show the effect of pefloxacin mesylate on the emission maximum with the progression of titration. A little stronger blue-shift of tryptophan fluorescence upon addition of drug was observed, and the emission maximum of tyrosine kept the position. This shift indicates that tryptophan residues were placed in a more hydrophobic environment and less exposed to the solvent. This may be due to the fact that the insertion of pefloxacin mesylate rearranged the tryptophan micro-

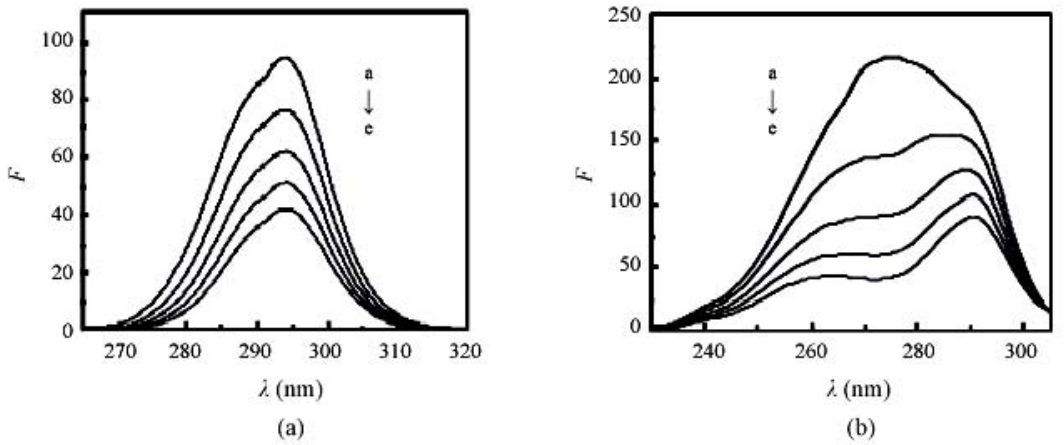


Fig.6 The effect of drug on the synchronous fluorescence spectra of BLf. (a) $\Delta\lambda$=15 nm; (b) $\Delta\lambda$=60 nm
[BLf]: 1×10^{-5} mol/L; [PFLX]: $x\times10^{-5}$ mol/L, from a to e: x=0, 0.8, 1.6, 2.4, 3.4

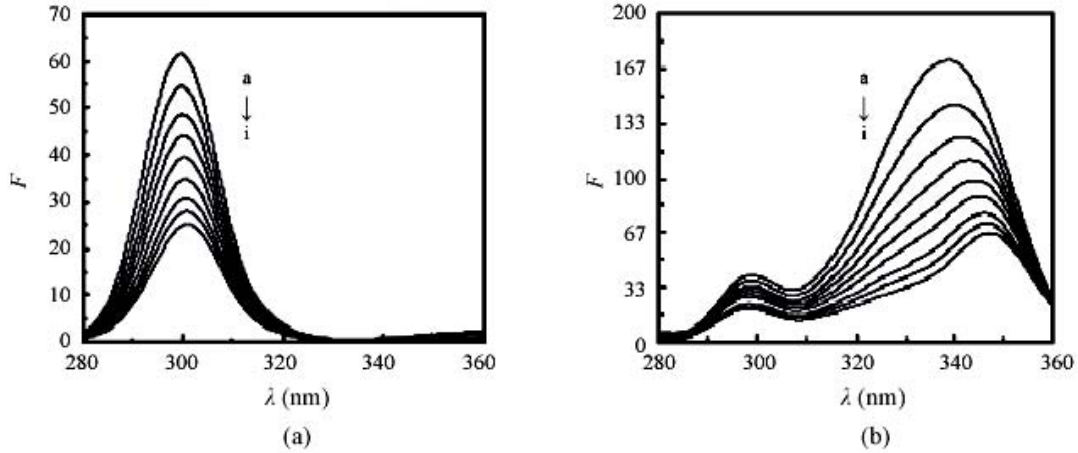


Fig.7 The effect of drug on the synchronous fluorescence spectra of HSA. (a) $\Delta\lambda$=15 nm; (b) $\Delta\lambda$=60 nm
[HSA]: 1×10^{-5} mol/L; [PFLX]: $x\times10^{-5}$ mol/L, from a to i: x=0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2

environment. It also suggested that the environment of tryptophan residues in pure protein solution is relatively polar. Binding of the pefloxacin mesylate changes the environments to apolar ones. The shift in polarity brought about conformational changes by the interaction between protein and the ligand molecule.

CONCLUSION

The binding interactions of pefloxacin mesylate with BLf and HSA in dilute aqueous solution were studied using fluorescence spectra and absorbance spectra. The results showed that the binding constant (K), binding sites (n) and the binding distances (r) are K=1.06×10^5 L/mol, n=1.85, r=2.82 nm for BLf and K=7.04×10^4 L/mol, n=1.13, r=2.83 nm for HSA, respectively. The effect of pefloxacin mesylate on the conformations of BLf and HSA was also analyzed, with the result indicating that pefloxacin mesylate can affect the conformations of BLf and HSA to some degree.

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