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Study of the combination reaction between drugs and bovine serum albumin with acriflavine as a fluorescence probe

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ABSTRACT

Using Acriflavine as fluorescent probe to study the seven kinds of drugs to combination of the bovine serum albumin. These drugs are Streptomycin sulfate, Kanamycin sulfate, Gentamicin, Amikacin, Neomycin, Thiamphenicol, and Florfenicol. And after reaction of bovine serum albumin and these drugs, the fluorescence intensity of bovine serum albumin has no obvious change. Research has shown that after adding seven kinds of drugs to the bovine serum albumin and Acriflavine system, respectively, the fluorescence intensity of bovine serum albumin has recoveried. The degree of recovery are GM> TN> NM> FN> KM> SM> AM. It can be concluded that the seven kinds of drugs specifically binds to bovine serum albumin, and it determine the binding site on sub-domain IIA (site I) of the seven kinds of drugs and bovine serum albumin.

KEYWORDS

Acriflavine;
Bovine serum albumin;
Drugs;
Spectroscopy;
Bonding mechanism.

INTRODUCTION

Acriflavine (ACF) also called Acriflavinium chloride, is a kind of topical antiseptic agent derived from acridine, first synthesized in 1912 by Paul Ehrlich, a German medical researcher and was used in the early 20th century, during the First World War, as topical antibacterial and against sleeping sickness[1]. Acriflavine has proved an effective agent for gonorrhea, meningitis, intestinal infections, diphtheria, pneumonia, cholera and infected wounds; in some countries is at present formulated with urotropine as well known urinary tract antiseptic[2]. Kanamycin sulfate (KM), Streptomycin (SM), Amikacin (AM), Gentamicin (GM), Neomycin (NM) belongs to aminoglycoside antibiotics, they were the long-sought remedy for tuberculosis and other serious bacterial infections[3]. Thiamphenicol (TN), Florfenicol (FN) belongs to the chloramphenicol of broad-spectrum antibiotic. Serum albumin, the most abundant protein constituent in blood plasma, can be combined with a lot of endogenous and exogenous compounds and plays a fundamental role in the disposition and transportation of various molecules. Therefore, investigating the binding mechanism of endogenous or exogenous compounds and serum albumins has very significant implications for the life sciences, chemistry, pharmacy and clinical medicine. In recent decades investigations of the interaction between drugs and bo-

vine serum albumin (BSA) by use of the fluorescence method have been extensively reported[4-7]. The fluorescence spectrum change is obvious in the literature reported[8-11]. However, in many cases the required experimental data cannot be measured directly or calculated indirectly because of a lack of experimental results or the inconclusive nature of experimental results for the pharmaceutical molecules and the bio-macromolecules. The problem can be effectively solved by using the fluorescent probe method[12-14]. In this paper, we using ACF as a fluorescence probe to research seven drugs and BSA binding reaction and reaction mechanism under the physiological conditions. Proof of seven drugs and BSA binding in vivo, and the binding capacity were GM> TN> NM> FN> KM> SM> AM. Determine the combination regional of these seven drugs and BSA. This study provides a method for the study of reaction mechanism that some drugs(similar to TN, KM, SM, AM, GM, NM and FN) reacting with protein.

EXPERIMENTAL

Apparatus and materials

All fluorescence spectra were recorded on a Shimadzu RF-5301PC spectro-fluorophotometer. All spectrophotometric measurements were made with a Shimadzu UV-265 spectrophotometer. All pH measurements were made with a pHS-3C precision acidity meter (Leici, Shanghai). All temperatures were controlled by a CS501 super-heated water bath (Nantong Science Instrument Factory).

Bovine serum albumin was purchased from Sigma(the purity grade inferior 99%) and stock solutions (1.0×10^{-5} mol L^{-1}) were prepared by doubly distilled water. Acriflavine(2.0×10^{-3} mol L^{-1}) were prepared by doubly distilled water, respectively. All the stock solutions were further diluted as working solutions prior to use. Warfarin, ibuprofen, and digoxin were all obtained from the Chinese Institute of Drug and Biological Products and further diluted as working solution (1.0×10^{-5} mol L^{-1}). The Tris-HCl buffer (0.05 mol L^{-1}, pH=7.40) containing 0.15 mol L^{-1} NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. All other reagents were of analytical reagent grade and double-distilled water was used during the experiment. And all the stock solutions were stored at 277 K.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter using the following relationship[15]:

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

where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities respectively. A_{ex} and A_{em} are the absorbance values of aspirin at excitation and emission wavelengths, respectively. The fluorescence intensity used in this paper was corrected.

Procedures

Fluorescence spectra and synchronous fluorescence spectra

In the experiment we use the 1.0 mL of pH 7.40 Tris-HCl, a certain amount of 1.0×10^{-5}mol L^{-1} BSA solution(0.4 mL), and different amount of ACF (1.0×10^{-3} mol L^{-1}) was added into 10 mL colorimetric tube sequentially. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 30 minutes. The fluorescence emission spectra were measured at 293, 303, and 310 K with the width of the excitation and emission slit adjusted at 5.0 and 5.0 nm, respectively. An excitation wavelength of 280 nm was chosen and the emission wavelength was recorded from 285 to 450 nm. The synchronous fluorescence spectra were obtained by simultaneously scanning the excitation and emission monochromators. It were recorded at $\Delta\lambda$=15 nm and 60 nm in the absence and presence of various amounts of ACF over a wavelength range of 280-400 nm.

Effects of drugs on BSA-ACF system

At 293K, first we adding 0.4 mL1.0×10^{-4}mol/L ACF solution, then adding the different amount of drugs(2.0×10^{-3} mol L^{-1}), respectively. After the method according to 2.2.1 operation.

Determination of the binding sites

At 293K, adding (0.4 ml 1.0×10^{-5} mol L^{-1}) a kind of competition reagents (site marker I (WF), II (IB), or III (DG), respectively) to the mixture of BSA-ACF system. After the method according to 2.2.1 operation.

FULL PAPER

RESULT AND DISCUSSION

Fluorescence quenching spectra of BSA-ACF system

The intrinsic fluorescence of protein is a sensitive tool to study the conformation of protein when its environment or structure gets change. The quenching mechanism of fluorescence can be classified into static quenching and dynamic quenching[16]. Dynamic quenching is mainly caused by collisional encounters between the fluorophore and the quencher, static quenching is mainly resulted from the formation of stable compound between fluorophore and quencher[17]. Figure 1 shows the fluorescence emission spectra of BSA in the presence of various concentrations of ACF at 293 K. The fluorescence emission intensity of BSA decreased regularly with the gradual addition of ACF. This result indicates that ACF can interact with BSA and quench its intrinsic fluorescence, changing the microenvironment of the fluorophores.

If it is assumed that the fluorescence quenching mechanism of BSA by ACF is dynamic quenching process, fluorescence quenching can be described by Stern-Volmer equation[18].

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

where F and F_0 are the relative fluorescence intensities in the presence and absence of quencher, respectively; [Q] is the concentration of quencher, K_{sv} is the Stern-Volmer quenching constant, which measures the efficiency of quenching. k_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule in absence of quencher evaluated at about 10^{-8} s[19]. According to the Stern-Volmer plots of F_0/F versus quencher concentration at different temperatures (293, 303, and 310 K). The quenching rate constant K_q was obtained and listed in TABLE 1. It is obvious K_q decreases with rising temperatures, revealing that the quenching is initiated by static quenching process. Moreover, the values of K_q between BSA and ACF are all greater than 2×10^{10} L·mol^{-1}·s^{-1}. Therefore, ACF binding BSA was a static quenching process proved to be true[20].

For the static quenching interaction, under the assumption that there are similar and independent binding sites in the biomolecule, the binding constant and the number of binding sites can be derived from the double logarithm regression curve (Eq. (3))[21]

$$\lg[(F_0 - F)/F] = n \lg[Q] + \lg K_a \quad (3)$$

where K_a is the binding constant, n is the number of binding sites. [Q] is the total concentrations of ACF. The curve of log [(F_0-F)/F] versus log [Q] is drawn and fitted linearly, then the value of n and K_a can be obtained from the plot. And TABLE 1 gives the corresponding calculated results. The value of n almost equals to 1, indicating that there is one class of binding site for ACF to BSA molecule. In other words, ACF and BSA form a complex with molar ratio 1:1. According to the results shown in TABLE 1, the binding constants of the interaction between ACF and BSA decreases with the rising temperature, further suggested that the quenching was a static process[22].

Synchronous fluorescence spectra

The synchronous fluorescence spectra can provide information on the molecular microenvironment, particularly in the vicinity of the fluorophore functional groups[23]. When Δλ is 15 nm, synchronous fluorescence detects characteristics of tyrosine (Tyr) residues, but when Δλ is 60 nm, characteristic information from tryptophan (Trp) residues is highlighted[24].

The synchronous fluorescence spectra of BSA-ACF systems shown in Figure 2. As seen in Figure 2, the λ_{max} had red shifted when Δλ =15nm and Δλ=60nm(the λ_{max} has an obvious red shift). This indicated that the interaction of BSA with ACF changed

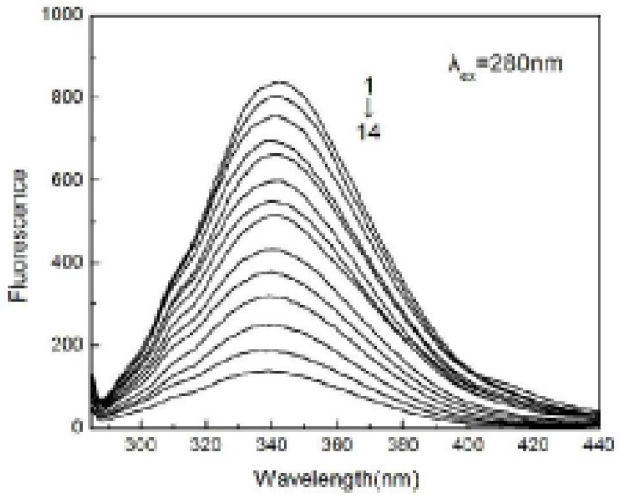

Figure 1 : Fluorescence emission spectra of BSA-ACF(T=293K); 1~14 C_{BSA}(4×10^{-7}mol L^{-1})+C_{ACF} (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0)$\times10^{-5}$ mol L^{-1}

TABLE 1 : Quenching reactive parameter of BSA and ACF at different temperatures

T/(K)	K_q/(L mol^{-1}s^{-1})	r_1	SD_1	K_a/(L mol^{-1})	r_2	SD_2	n
293	6.37×10^{12}	0.9984	0.021	5.18×10^{4}	0.9951	0.051	0.93
303	5.80×10^{12}	0.9961	0.012	4.95×10^{4}	0.9978	0.032	1.09
308	4.38×10^{12}	0.9988	0.007	4.30×10^{4}	0.9936	0.043	0.92

r_1r_2 are the linear relative coefficient of $F_0/F\sim[Q]$,$lg(F_0-F)/F\sim lg[Q]$, respectively. SD_1, SD_2 are the standard deviation of $F_0/F\sim[Q]$, $lg(F_0-F)/F\sim lg[Q]$, respectively.

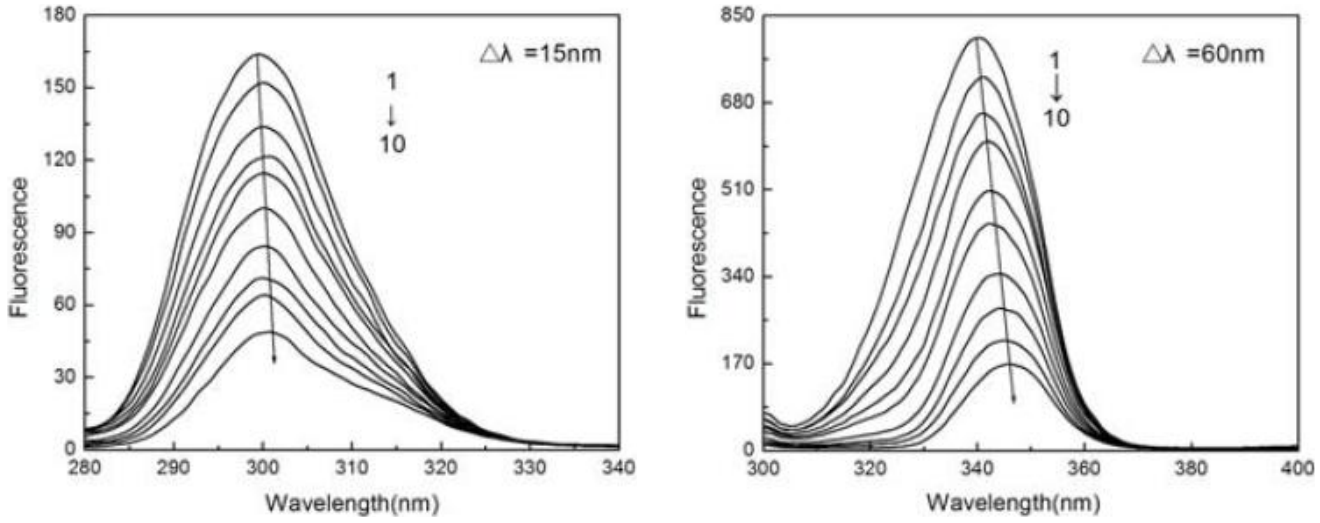

Figure 2 : Synchronous fluorescence spectra of BSA-ACF system (T=293K); 1~10 C_{BSA} (4.0×10^{-7}mol L^{-1})+C_{ACF} (0, 0.3, 0.6, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0)$\times10^{-5}$mol L^{-1}

the microenvironment of Tyr residues and Trp residues[25]. High concentrations of dyes make protein molecules extend, reducing the energy transfer between the amino acid residues, and reducing their fluorescence intensity.

Type of interaction force of BSA-ACF systems

Basically, four main types of interactions, hydrogen bonds, electrostatic forces, van der Waals forces, and hydrophobic forces play critical roles in the interactions between small molecules and macromolecules[26]. In order to characterize the force between ACF and BSA, thermodynamic parameters on the temperatures were analyzed. The thermodynamic parameters, free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) are important for confirming the binding mode. The thermodynamic parameters can be calculated using Eqs. (5) and (6)[27,28].

$$\mathbf{R \ln K = \Delta S - \Delta H / T} \quad (5)$$

$$\mathbf{\Delta G = \Delta H - T\Delta S} \quad (6)$$

In the present case, K is analogous to the effective quenching constants K_a for the quencher-acceptor system at the corresponding temperature and R is gas constant. If it is assumed that the enthalpy change (ΔH) nearly had no change within the investigated temperature, there should be a good linear relationship between ln K and 1/T. The result of ΔH, ΔS and ΔG were -17.40 KJ mol^{-1}, 31.00 KJ mol^{-1}, -26.79 KJ mol^{-1} (T=293 K), respectively. This showed that the electrostatic attraction play major roles in the binding process between ACF and BSA. The negative value of ΔG indicated a spontaneous reaction occurred between ACF and BSA.

Effect of drugs on the fluorescence emission spectra of the BSA-ACF system

The emission spectra of BSA-ACF in the absence and presence of FN are shown in Figure 3 (KM, SM, AM, GM, NM and TN are similar to FN). As shown in Figure 3, when more and more drugs added to the system, the fluorescence intensity at 340nm increased gradually, which indicative of a competitive binding reaction between drugs, ACF and BSA, and made the fluorescence intensity of BSA recovered. Comparing

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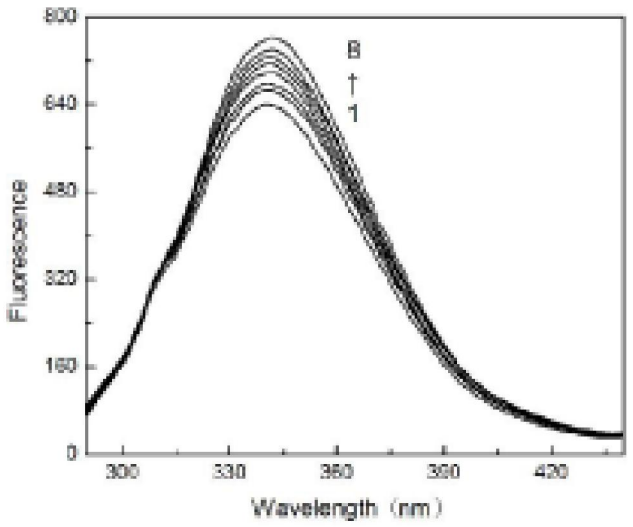

Figure 3 : Fluorescence emission spectra of BSA-ACF-FN(T=293K); 1~8 C_{BSA} (4.0×10^{-7}mol L^{-1})+C_{ACF} (4.0×10^{-6}mol L^{-1})+C_{FN} (0, 0.4, 0.8, 1.2, 1.6, 2.0, 4.0, 6.0)$\times10^{-4}$ mol L^{-1}

the recovery of BSA fluorescence intensity of different drugs at the same concentration: GM> TN> NM> FN> KM> SM> AM, it indicates that the capability of competitive binding reaction between drugs and ACF-BSA system: GM> TN> NM> FN> KM> SM> AM.

Fluorescence recovery mechanism

After adding drugs in BSA-ACF system, the fluorescence of the system recoveried (as seen in Figure 3). It showed that, these was a competition binding reaction between the drugs and ACF for BSA. Eqs. (6) and (7) show the competitive reaction (take FN for example):

$$\text{BSA-ACF} + \text{FN} \rightarrow \text{BSA-FN} + \text{ACF} \quad (6)$$

$$\text{Or BSA-ACF} + \text{FN} \rightarrow \text{ACF-FN} + \text{BSA-FN} \quad (7)$$

Assume that the reaction carried out in accordance with the Eq. (6), as a result of FN could not quench the fluorescence of BSA, so with the concentration of the generated BSA-FN increased gradually, fluorescence intensity of the system wound restore gradually. This was consistent with the results of Figure 3. In the meantime, the concentration of the dissociative ACF increased gradually, the absorbance value also increased gradually and the position of the peak remained unchanged at 436 nm (as shown in Figure 4). Figure 4 showed that drugs, BSA had no absorption in the range of 350-500 nm wavelength, and the absorbance of the system along with the increasing of drugs' concentrationat 436 nm increased and had no peak shift phenomenon. It proved that the reaction was conducted according to the Eq. (6). If the reaction carried out in accordance with the Eq. (7), because of FN not quench thefluorescence of BSA, therefore, with the concentration of BSA-FN increased gradually,the fluorescence intensity of the system wound restore gradually. It inosculated with the results of Figure 3. A new compound ACF-FN wound generate at the same time, the formation of new compound is often accompanied by the displacement of the absorption peak. In Figure 4, beacuse of no peak displacement, it also proved that the reaction was conducted according to the Eq. (6). Namely in the presence of ACF, these is a competition binding reaction between the drugs and BSA.

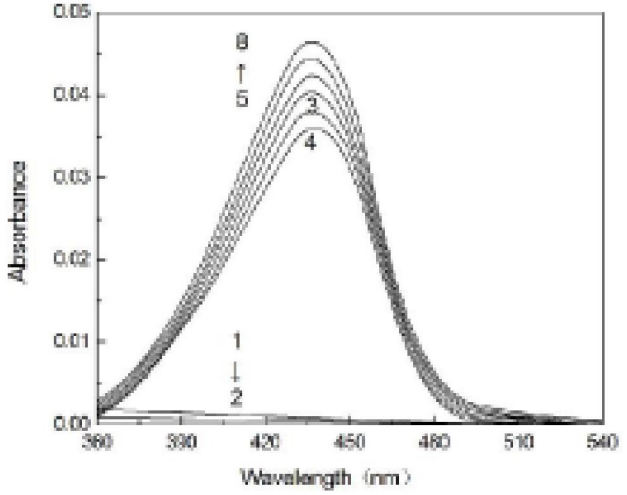

Figure 4 : Absorption spectra of BSA-ACF-FN(T=293K); 1, C_{BSA} (4.0×10^{-7}mol L^{-1}); 2, C_{FN} (4.0×10^{-3}mol L^{-1}); 3, C_{ACF} (1.0×10^{-6}mol L^{-1}); 4~8, C_{ACF} (1.0×10^{-6}mol L^{-1})+C_{BSA} (4.0×10^{-7}mol L^{-1})+C_{FN} (0,1.0, 2.0, 3.0, 4.0)$\times10^{-3}$mol L^{-1}

Identification of the binding site

At 280 nm wavelength the Trp and Tyr residues in BSA are excited, whereas the 295 nm wavelength excites only Trp residues. In BSA sub-hydrophobic domain, IIA (containing both Trp 212 and Tyr 263) and IIIA (containing only Tyr: Tyr 401, Tyr411, Tyr 497) is the major binding site of small molecule ligands[29]. Based on the Stern-Volmer equation, comparing the fluorescence quenching of BSA excited at 280 nm and 295 nm allows to estimate the participation of Trp and Tyr groups in the system[30]. As seen in Figure 3, in the presence of ACF, the quenching curves of BSA excited at 280 nm and 295 nm didn't overlap and the

quenching curves of BSA at 280 nm was much greater than 295 nm. This phenomenon showed that Trp and Tyr residues played an important role in the interaction between ACF and BSA. Therefore, it implied that the primary binding site for ACF was sub-hydrophobic domain IIA[31].

The crystal structure of BSA is a heart-shaped helical monomer composed of three homologous domains named I, II, and III, with each domain including two sub-domains called A and B to form a cylinder[32]. The principal ligand-binding regions of albumin are hydrophobic cavities in sub-domains IIA and IIIA, which have similar chemical properties. These two binding cavities are also referred to as sites I, II, and III (site I in sub-domain IIA, sites II and III in sub-domain IIIA). To identify the binding site on BSA, site marker competitive experiments were carried out, using the drug which specially binds to a known site or region on BSA. X-ray crystallography studies have shown that warfarin (WF) binds to sub-domain IIA whereas ibuprofen (IB) and digoxin (DG) are believed to bind to IIIA binder sites II and III, respectively[33]. Information about BSA-ACF binding site can therefore be obtained by monitoring changes in the fluorescence of ACF-bound BSA caused by binding by site I (WF), site II (IB), and site III (DG) markers. Binding constants determined on the basis of Eq. (3) show the effect of WF, IB, and DG on BSA-ACF at 293 K. It is observed that binding constants for the ternary system ($K_{BSA\text{-}WF\text{-}ACF}=6.54\times10^3$ $L\cdot mol^{-1}$; $K_{BSA\text{-}IB\text{-}ACF}=1.19\times10^4$ $L\cdot mol^{-1}$; $K_{BSA\text{-}DG\text{-}ACF}=1.40\times10^4 L\cdot mol^{-1}$) are lower than that for the binary system BSA-ACF ($K_{BSA\text{-}ACF}=5.18\times10^4 L\cdot mol^{-1}$). It can be seen that the binding constant for the ternary system BSA-WF-ACF) was the most different, indicating that WF hinders the formation of BSA-ACF and can compete for the same binding site in sub-domain IIA (site I).

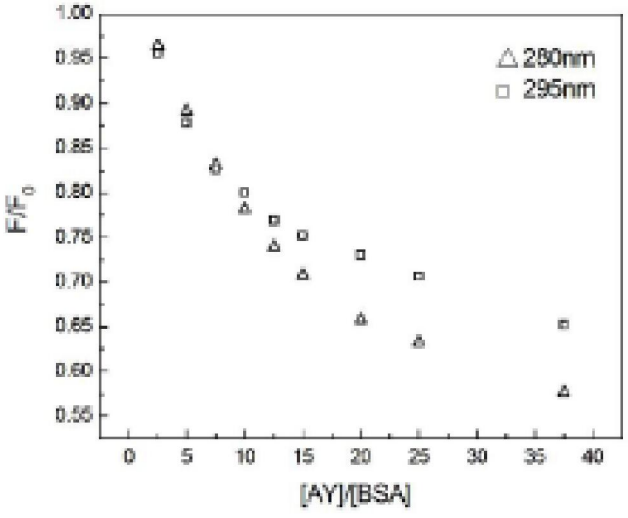

Figure 5 : Fluorescence emission spectra of BSA-ACF (T =293 K); C_{BSA} = 4.0×10^{-7} mol L^{-1}, $C_{ACF}=1.0\times10^{-6}\sim 1.5\times10^{-5}$ mol L^{-1}

CONCLUSIONS

The binding sites(sub-domain IIA site I) of ACF on BSA is determined by competitive reagent, proves the specific binding between ACF and BSA, and explains that ACF can study the combination of drugs and proteins as a probe instead of competition reagent. Due to After reaction of BSA and KM, SM, AM, GM, NM, TN, FN, respectively, the fluorescence intensity of BSA has no obvious change, so there is no way to directly research drugs and BSA binding reaction with fluorescence spectrometry. In this paper using ACF as fluorescent probe to study the drugs to respond to the combination of the BSA, it proves that there is a reaction between drugs and BSA, and it determines their binding site on sub-domain IIA (site I). The study of using fluorescent probes for the interaction between no or weak fluorescence intensity change of drug molecular and BSA opens new avenues of research, broadens the scope of drugs research.

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FULL PAPER

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