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Study of the combination reaction between kanamycin sulfate and bovine serum albumin with Eosin Y as a fluorescence probe

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ABSTRACT

After the reaction of Bovine serum albumin and Kanamycin sulfate, the fluorescence intensity of Bovine serum albumin has no obvious change, so there is no way to directly research Kanamycin sulfate and Bovine serum albumin binding reaction with fluorescence spectrometry. This article study the combination of Kanamycin sulfate and Bovine serum albumin with Eosin Y as a fluorescence probe, using the method of fluorescence spectrum. Research has shown that after adding Kanamycin sulfate to the Bovine serum albumin and Eosin Y system, the fluorescence intensity of Bovine serum albumin has recoveried. So it can be concluded that Kanamycin sulfate specifically binds to Bovine serum albumin, and it determine the binding site on sub-domain IIA (site I) of Kanamycin sulfate and Bovine serum albumin.

KEYWORDS

Fluorescence probe;
Eosin Y;
Bovine serum albumin;
Kanamycin sulfate;
Combination reaction.

INTRODUCTION

Serum albumin is the major transport protein in the blood circulatory system; thus, it plays a major role in the transport and delivery of various endogenous and exogenous compounds, such as metabolites, drugs, and other biologically active substances[1-3]. In recent decades investigations of the interaction between drugs and bovine 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. 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, in which the mechanism of the interaction between pharmaceutical molecules and biomacromolecule is investigated by observing changes of the fluorescence spectra of bio-macromolecule or probe molecule on interaction of drug and probe molecules, thus yielding a further understanding of the functional mechanism of drug molecules in vivo.In the past few years, the competitive interaction between the pharmaceutical molecules with biologic activity and albumins which act as the models of proteins to probe molecules has been widely studied by fluorescent probe technique and other techniques[8,9]. Research on combination mechanism and model of protein and dye is conducive to study the combined action of coordination essence of biological macromolecules and small

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organic molecules. Therefore, it turns the dye as spectroscopic probes of protein molecules into a research field which attracts so much attention.

Eosin Y (EY)[10-15] is a halogenated derivative of the xanthene dye fluorescein. Its fluorescent properties are highly dependent on variations in its environment, such as solvent polarity and the presence of cationic surfactants; the environment-dependent fluorescence makes this organic dye a powerful probe of biological molecules. The applications of eosin Y include detection of submicrogram quantities of a wide range of proteins, use in dye-sensitized solar cells, photodynamic inactivation of viruses and cells and phototherapy for cancer, in which eosin Y is used as an efficient photosensitizing agent to produce singlet molecular oxygen ground state molecular oxygen. Kanamycin sulfate (Kan)[16] is an aminoglycoside antibiotic that inhibits the growth of both Gram-positive and Gram-negative bacteria. It is widely administered as a second line antibiotic in the form of injection and capsules and is also used in veterinary medicine. Because there are no obvious experimental results for the combination of BSA and Kan, so there is no way to directly research Kan and BSA binding reaction with fluorescence spectrometry. In this paper, we using EY as a fluorescence probe to research Kan and BSA binding reaction and reaction mechanism under the physiological conditions. Proof of Kan and protein binding in vivo, and through the BSA-EY system including Warfarin, Ibuprofen and Digoxin as BSA space domain of site I, II, III position of labeled drug, indicated that binding of Kan to BSA primarily occurred in sub-domain IIA (site I). With this work we provide an effective method to study the conjugation reaction between some drugs and BSA.

EXPERIMENTAL

Apparatus and reagents

All fluorescence spectra were recorded using Shimadzu RF-5301PC. Absorption was measured with an UV–vis recording spectrophotometer (UV-265 Shimadzu Japan). All pH measurements were done with a pHS-3C precision acidity meter (Leici, Shanghai). All temperatures were controlled by CS501 super heated water bath (Nantong Science Instrument Factory).

BSA was purchased from Sigma Company (no less than 99% pure). The eosin Y was high purity grade and imported to load separately, which was produced by Amresco Company. Stock solutions of BSA (1.0×10^{-5} mol L^{-1}) and EY (2.0×10^{-4} mol L^{-1}) were prepared. The stock solutions were further diluted as working solutions prior to use. Kanamycin sulfate (Kan), which was obtained from Beijing Xinjingke Biotechnology companies, was prepared as working solution (4.86×10^{-3} mol L^{-1}). 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}). Tris–HCl buffer (0.05 mol L^{-1}, pH=7.40), which contains 0.2 mol L^{-1} NaCl solution, was prepared. All other reagents were of analytical reagent grade and all aqueous solutions were prepared with newly double-distilled water and stored at 277 K.

Under some conditions the fluoresced light is absorbed by quenching groups on neighboring substrates or cleaved product molecules so that only a fraction of the fluoresced light impinges upon the detector system of the fluorometer. This phenomenon is known as the inner filter effect. The fluorescence intensities were corrected to avoid the inner filter effect using the relationship (Eq. (1)):[17]

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

Where F_{cor} and F_{obs} are the corrected fluorescence intensity and the observed fluorescence intensity, respectively, and A_{ex} and A_{em} are the absorption of the system at the excitation wavelength (λ_{ex}) and the emission wavelength (λ_{em}), respectively. The intensity of fluorescence used in this paper was the corrected fluorescence intensity.

Procedures

At 293, 303, and 310 K, 1.0 mL pH =7.40 Tris–HCl buffer solution was placed in a 10.0 ml colorimetry tube, followed by a known amount of 1.0×10^{-5} mol L^{-1} BSA aqueous solution 0.4ml and 2.0×10^{-4} mol L^{-1} EY aqueous solution. The mixture was diluted to 10.0 ml with distilled water and then shaken until homogenous. After a 20-min wait at the temperature of the experiment, fluorescence measurements were carried out at 340 nm keeping the excitation wavelength at 280 nm. Excitation slit and emission slit were set as 5 nm.

1.0 ml Tris–HCl buffer solution was placed in a 10.0 ml colorimetry tube, followed by a known amount of 1.0×10^{-5} mol L^{-1} EY aqueous solution 1.0 ml and 1.0×10^{-6} mol L^{-1} BSA aqueous solution. The mixture was diluted to 10.0 ml with distilled water and then shaken until homogenous. After a 20-min wait at 303 K, the absorption spectrum of the solution was recorded.

At 293, 303, and 310 K, 1.0 ml Tris–HCl buffer solution was placed in a 10.0 ml colorimetry tube, followed by 0.4 ml 1.0×10^{-5} mol L^{-1} BSA aqueous solution and 0.4 ml 1.0×10^{-5} mol L^{-1} EY aqueous solution. After a 20-min wait at the temperature of 303 K, a known amount of 4.68×10^{-3} mol L^{-1} Kan solution was added. The mixture was diluted to 10.0 ml with distilled water and then shaken until homogenous. After a 20-min wait at the temperature of 303 K, fluorescence measurements were carried out at 340 nm keeping the excitation wavelength at 280 nm. Both excitation and emission slits were at 5 nm. In addition, the absorption spectrum of the solution was recorded.

1.0 ml Tris–HCl buffer solution was placed in a 10.0 ml colorimetry tube, followed by BSA aqueous solution (0.4 ml 1.0×10^{-5} mol L^{-1}), 0.4 ml 1.0×10^{-5} mol L^{-1} site marker I (WF), II (IB), or III (DG) for different series, a known amount of 1.0×10^{-5} mol L^{-1} EY aqueous solution. The mixture was diluted to 10.0 ml with distilled water and then shaken until homogenous. After a 20-min wait at the temperature of the experiment (303 K), fluorescence measurements were carried out.

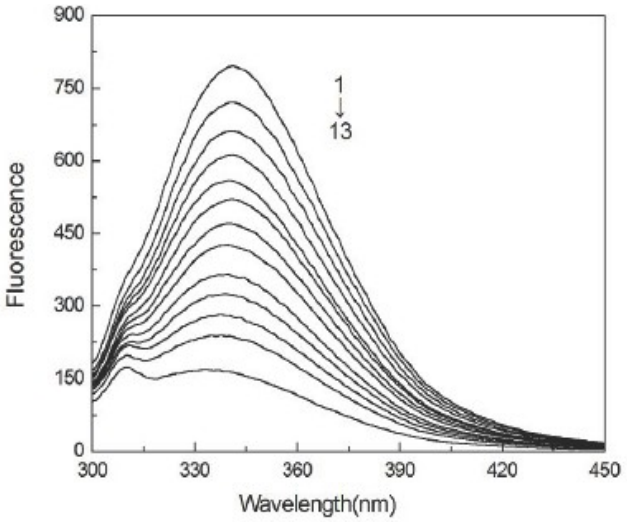
RESULTS AND DISCUSSION

Fluorescence spectroscopic studies of Eosin Y and bovine serum albumin

The fluorescence emission spectra of BSA in the absence and presence of EY are shown in Figure 1. It is observed that the fluorescence intensity of BSA decreases regularly upon the increasing concentration of EY, whereas BSA fluorescence emission band is about 340 nm with no obvious shift. This indicates that EY could bind to BSA without altering local dielectric environments[18,19]. No emission was given from EY under the same conditions.

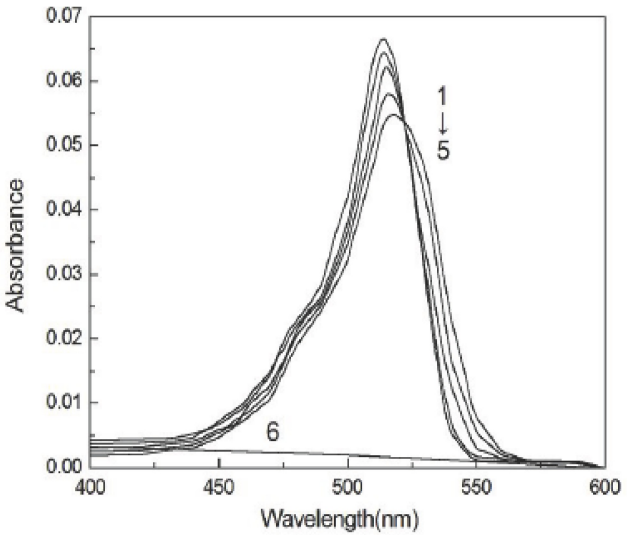
The absorption spectra of the bovine serum albumin and Eosin Y system

The absorption spectra of EY in the absence and presence of BSA are shown in Figure 2. As it can be seen from Figure 2, BSA had no absorption in the range 450–600 nm and λ_{max} of EY was at 514 nm. The absorbance of EY at 514 nm decreased with increasing concentration of BSA and there was a red shift of the absorption wavelength on addition of BSA, indicating that EY reacted with BSA forming a compound. At the same time, it proved that the fluorescence quenching was static quenching[20].



1-13 C_{BSA} (4×10^{-7} mol L^{-1})+C_{EY} (0, 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5, 0.6, 1.0)$\times10^{-6}$ mol L^{-1}

Figure 1 : Fluorescence emission spectra of BSA-EY (T=303K)



1-5 C_{EY} (1.0×10^{-6} mol L^{-1})+ C_{BSA} (0, 0.5, 1.0, 2.0, 3.0)$\times10^{-7}$ mol L^{-1}; 6, C_{BSA}=3.0×10^{-7} mol L^{-1}

Figure 2 : Absorption spectra of EY-BSA (T=303K)

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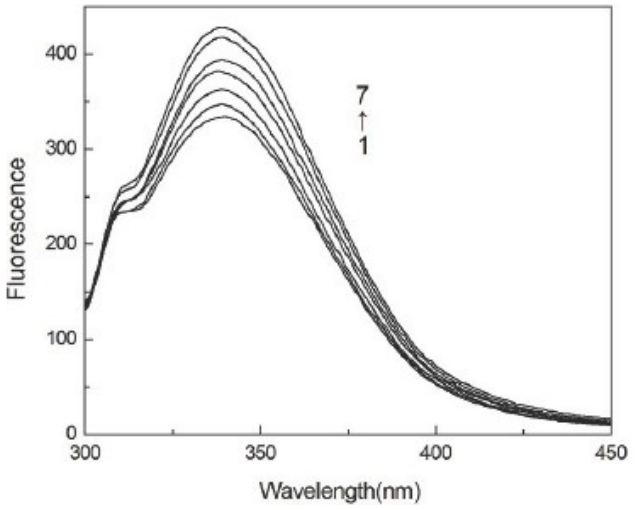
Effect of kanamycin sulfate on the fluorescence emission spectra of the bovine serum albumin and Eosin Y system

The emission spectra of BSA-EY in the absence and presence of Kan are shown in Figure 3. As shown in Figure 3, with the excitation wavelength at 280 nm, the maximum emission wavelength of BSA was 340 nm. The relative fluorescence intensity of the system gradually recovered with increasing concentration of Kan. This is indicative of a competitive reaction between Kan and BSA for EY.



1-7 C_{BSA} (4.0×10^{-7}mol L^{-1})+C_{EY} (4.0×10^{-7}mol L^{-1})+C_{Kan} (0, 0.097, 0.19, 0.97, 1.46, 1.94, 2.92)$\times10^{-3}$mol L^{-1}

Figure 3 : Fluorescence emission spectra of BSA-EY-Kan (T=303K)

Fluorescence quenching mechanism of bovine serum albumin by Eosin Y

Fluorescence quenching is usually classified as dynamic quenching and static quenching. Dynamic quenching, or collisional quenching, results from collision between fluorophore and quencher. Static quenching is due to the ground-state complex formation between fluorophore and dye. To determine the mechanism of binding between BSA and EY, fluorescence intensity data were analysed by the Stern–Volmer equation (Eq. (2)):[21]

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

Where F_0 and F are the steady-state fluorescence intensities of BSA at about 340 nm before and after the addition of quencher (EY); K_q is the quenching rate constant of the biomolecule; τ_0 is the average lifetime of the biomolecule without the quencher, which is approximately 10^{-8} s; K_{SV} and *[Q]* are the quenching constant and the concentration of quencher EY, respectively. In such an analysis, a plot of F_0/F versus *[Q]* will give a straight line. By Stern-Volmer equation, K_q and liner correlation coefficient r_1 at three different temperatures are obtained and summarized in TABLE 1. It is clear that K_q was decreased with the increasing temperature and it was much larger than 2.0×10^{10} L mol^{-1} s^{-1}, which implied that the quenching of BSA by EY may be a static process.

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)):[22,23]

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

where K_A is the binding constant or the apparent association constant for drug-protein interaction, *n* is the number of binding sites. By the plot of $lg[(F_0-F)/F]$ versus *lg[Q]*, the values of K_A and *n* can be obtained and were listed in TABLE 1. It was found that K_A decreased with increasing temperature, resulting in a reduction of the stability of the EY-BSA complex. The value of *n* was approximately equal to 1, indicating that there was one single binding site in BSA for EY during their interaction. So the result again confirmed that the quenching mechanism was a static quenching initiated by the formation of the ground state BSA-EY complex[24].

TABLE 1 : Quenching reactive parameter of BSA and EY 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	3.20×10^{14}	0.9917	0.068	6.18×10^{6}	0.9932	0.055	1.04
303	3.10×10^{14}	0.9911	0.049	5.26×10^{6}	0.9990	0.025	1.03
310	2.80×10^{14}	0.9972	0.022	4.13×10^{6}	0.9941	0.041	1.03

K_q is the quenching rate constant; K_A is the binding constant; *n* is the number of binding sites. r_1 is the linear relative coefficient of F_0/F~*[Q]*; r_2 is the linear relative coefficient of $lg(F_0-F)/F$~*lg[Q]*. *SD* is the standard deviation

Thermodynamic parameters and nature of binding forces

There are essentially four types of non-covalent interactions that play a key role in binding ligand to proteins. These are hydrogen bonds, Van der Waals forces, electrostatic and hydrophobic bonds interactions[25]. The thermodynamic parameters dependency to temperature must be obtained in order to elucidate the Interaction forces between EY and BSA. If the temperature does not vary significantly, the enthalpy change (ΔH) and entropy change (ΔS) can be regarded as constants. The thermodynamic parameters can be determined by the Van't Hoff equation (Eqs. (4), (5)):[26,27]

$$\mathbf{R \ln K = \Delta S - \Delta H / T} \quad \mathbf{(4)}$$

$$\mathbf{\Delta G = \Delta H - T\Delta S} \quad \mathbf{(5)}$$

Where K is the binding constant K_A and R is the gas constant. Enthalpy change (ΔH), entropy change (ΔS) and free energy change (ΔG) for the binding interaction between EY and BSA were calculated using Eqs. (4) and (5). The result of ΔH, ΔS and ΔG were -11.9 KJ mol^{-1}, 89.4 KJ mol^{-1}, -39.0 KJ mol^{-1} (T=303 K), respectively. The negative value of ΔG indicated a spontaneous reaction occurred between EY and BSA. The negative value of ΔH and positive value of ΔS showed that electrostatic attraction played an important role in the binding of EY to BSA[28].

Binding distance between bovine serum albumin and Eosin Y

According to the theory of Föster non-radiative energy transfer, if the emitted fluorescence from donor can be absorbed by acceptor, energy may transfer from the donor to the acceptor[29,30]. Then the distance between the donor (BSA) and the acceptor (EY) can be calculated by (Eq. (6)):

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

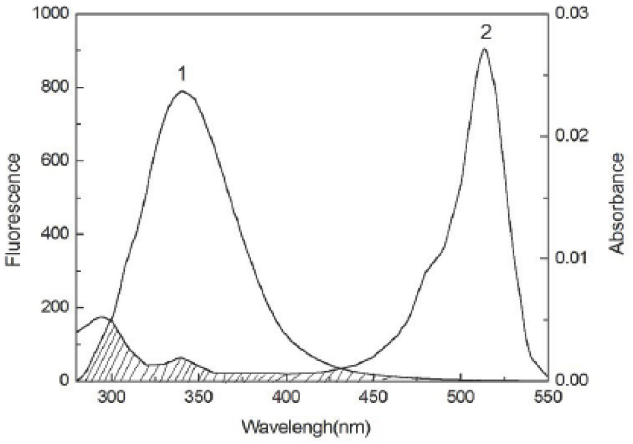
Where E is the efficiency of energy transfer, r is the distance between acceptor and donor and R_0 is the critical distance when the transfer efficiency is 50%, F_0 and F were the fluorescence intensities of BSA at 340 nm in the absence and presence of EY, respectively, R_0 can be calculated by (Eq. (7)):

$$\mathbf{R_0^6 = 8.78 \times 10^{-25} K^2 \Phi N^{-4} J} \quad \mathbf{(7)}$$

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. Herein, K^2, N and Φ are 2/3, 1.336 and 0.118 for BSA, respectively[31,32]. J is the overlap integral of the fluorescence emission spectrum of the donor (BSA) with the absorption spectrum of the acceptor (EY) (Figure 4), which is given by (Eq. (8)):

$$\mathbf{J = \Sigma F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \Sigma F(\lambda)\Delta\lambda} \quad \mathbf{(8)}$$

Where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength, λ; and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength, λ. J can be evaluated by integrating the spectra in Figure 4. The values of E, J, R_0, and r were summarized in TABLE 2. According to Föster's nonradiative energy transfer theory, the value of r is less than the academic value (7 nm), indicating that the fluorescence quenching of BSA by EY was a non-radiation transfer process and the energy transfer from BSA to EY occurred with high possibility. Therefore, it indicates that the energy transfer quenches the fluorescence of BSA when EY binds to BSA.



$C_{BSA} = C_{EY} = 4.0 \times 10^{-7}$ mol L^{-1}

Figure 4 : Overlap of the fluorescence emission spectrum of BSA (1) with the absorption spectrum of EY (2) (T=303K)

TABLE 2 : Parameters of E%, J, R_0, r between EY and BSA at different temperature

T/(K)	E/(%)	J/(cm^3·L mol^{-1})	R_0/(nm)	r/(nm)
293	57.2	5.65×10^{-15}	2.09	1.98
303	56.4	5.63×10^{-15}	2.08	1.99
310	55.4	5.62×10^{-15}	2.08	2.01

R_0 is the critical distance when E is 50%; r is the distance between acceptor and donor; J is the overlap integral between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor

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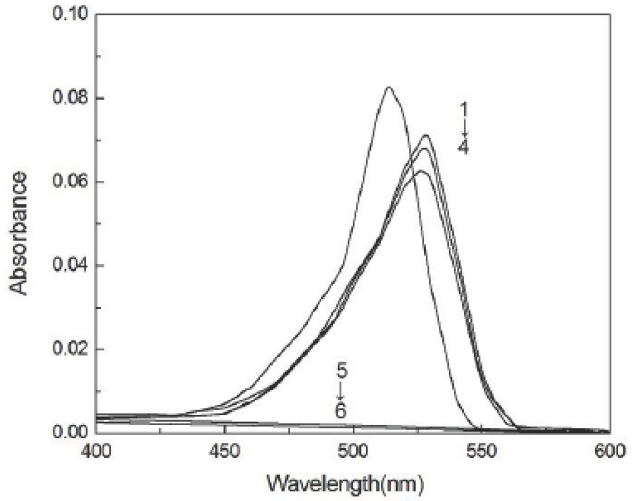
Fluorescence recovery mechanism

When Kan was added into the BSA-EY complex system the fluorescence intensity of BSA recovered (Figure 3). This phenomenon indicated there was a competitive interaction between Kan and EY for BSA. Eqs. (9) and (10) show the competitive reaction:

BSA-EY+Kan→BSA-Kan+EY (9)

Or BSA-EY+Kan→EY-Kan+BSA-Kan (10)

The absorption spectra of BSA-EY in the absence and presence of Kan were recorded to confirm the competitive reaction according to Eqs. (9) or (10) (Figure 5). As it can be seen from Figure 5, Kan and BSA had no absorption in the range 450–600 nm and λ_{max} of EY was at 514 nm. The absorption of EY decreased with increasing concentration of Kan. If the competitive reaction was according to Eq. (9), the absorption of EY should be increased with increasing concentration of Kan, which is not observed (Figure 5). If the competitive reaction was according to Eq. (10) Kan had bound EY and caused the absorption of EY to gradually decrease with increasing concentration of Kan, which is consistent with Figure 5. There was no obvious fluorescence intensity change on combining BSA and Kan, so the relative fluorescence intensity of BSA gradually recovered with increasing concentration of Kan, which is consistent with Figure 3. Therefore, the competitive reaction was according to Eq. (10).



1, C_{EY}=1.0×10^{-6}mol L^{-1}; 2-4 C_{BSA} (4.0×10^{-7}mol/L)+C_{EY} (1.0×10^{-6}mol L^{-1})+C_{Kan} (0, 0.486, 1.458)×10^{-3}mol L^{-1}; 5, C_{BSA}=4.0×10^{-7}mol L^{-1}; 6, C_{Kan}=1.458×10^{-3}mol L^{-1}

Figure 5 : Absorption spectra of BSA-EY-Kan (T=303K)

Effect of site markers on binding of eosin Y to bovine serum albumin

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[33]. 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 subdomain II, 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 subdomain IIA whereas ibuprofen (IB) and digoxin (DG) are believed to bind to IIIA binder sites II and III, respectively[34]. Information about EY–BSA binding site can therefore be obtained by monitoring changes in the fluorescence of EY-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-EY at 303 K. It is observed that binding constants for the ternary system ($K_{BSA–WF–EY}$=0.86×10^5 L mol^{-1}, $K_{BSA–IB–EY}$=1.98×10^6 L mol^{-1}, and $K_{BSA–DG–EY}$=2.11×10^6 L mol^{-1}) are lower than that for the binary system BSA-EY ($K_{BSA–EY}$=5.26×10^{6}L mol^{-1}). It can be seen that the binding constant for the ternary system BSA-WF-EY was the most different, indicating that WF hinders the formation of BSA-EY and can compete for the same binding site in sub-domain IIA (site I). The competitive interaction between Kan and EY for BSA can also be caused by location in the same binding site on sub-domain IIA(site I). Therefore, it can be concluded that Kan specifically binds to BSA.

CONCLUSIONS

The reaction of Kan and BSA can't make BSA fluorescence change, so it can not study the reaction mechanism of the two by fluorescence spectroscopy. By using EY as a fluorescent probe, and using EY and Kan competition for BSA binding reaction, it prove the existence of the binding reaction between Kan and BSA. The research on competitive drug application shows

that the binding between Kan and BSA is the binding site for the specific site I. The study of competitive reactions with a fluorescent probe broadens the scope of drug research and establishes new approaches to research on the interaction between BSA and optically inactive molecules or molecules with weak optical signal changes. This is of great significance to understanding of the absorption, distribution, and metabolism of drugs and the interaction between BSA and drugs.

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