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Using fluorescent probe to study the binding reaction between Streptomycin sulfate and Bovine serum albumin

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ABSTRACT

After reaction of Bovine serum albumin and Streptomycin sulfate, the fluorescence intensity of Bovine serum albumin has no obvious change, so there is no way to directly research the binding reaction of Streptomycin sulfate and Bovine serum albumin by fluorescence spectrometry. In this paper, firstly, we study the interaction between Eosin Y and Bovine serum albumin using the method of fluorescence spectrum and ultraviolet visible absorption spectrometry. After adding the Streptomycin sulfate to the Bovine serum albumin and Eosin Y system, the fluorescence intensity of Bovine serum albumin has recovered. This shows that the specific binding reaction of Streptomycin sulfate and Bovine serum albumin has happened, and its reaction mechanism has been discussed by fluorescence probe Eosin Y.

KEYWORDS

Fluorescence probe;
UV-visible absorption spectrum;
Eosin Y;
Bovine serum albumin;
Streptomycin sulfate.

INTRODUCTION

Streptomycin sulfate (SM) is a broad-spectrum glucosamine-based antibiotic. SM is widely used to treat tuberculosis due to its high effectiveness and low cost[1-2]. Serum albumin, the most abundant protein in plasma, has strong ligand binding capacity and many physiological functions. It acts as a depot protein and a transport protein for a variety of endogenous and exogenous compounds and plays a pharmacological role in the colloid blood pressure and maintenance of blood pH[3-7]. The fluorescence spectrum change is obvious in the literature reported[8-15]. 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 (such as SM and BSA). The problem can be effectively solved by using the fluorescent probe method, in which the mechanism of the interaction between pharmaceutical molecules and bio-macromolecule 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[16,17]. In this paper, we using EY as a fluorescence probe to research SM and BSA binding reaction and reaction mechanism under the physiological conditions. Proof of SM and BSA binding in vivo, and through the BSA-EY system in-

cluding Warfarin, Ibuprofen and Digoxin as BSA space domain of site I, II, III position of labeled drug, indicated that binding of SM 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 with a Shimadzu RF-5301 spectra fluorophotometer. Absorption was measured with a UV-Vis recording spectrophotometer (UV-265 Shimadzu, Japan). 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, China).

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. Streptomycin sulfate (SM), which was obtained from Beijing Xinjingke Biotechnology companies, was prepared as working solution (2.0×10^{-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.

The fluorescence intensities were corrected for the absorption of excitation light and reabsorption of emitted light to decrease the inner filter using the following relationship: (Eq. (1)):[18-20]

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

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 fluorescence intensity used in this paper was corrected.

Analytical procedures

1) 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.
2) 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.
3) 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 2.0×10^{-3} mol L^{-1} SM 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.
4) 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}), 0.5 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.

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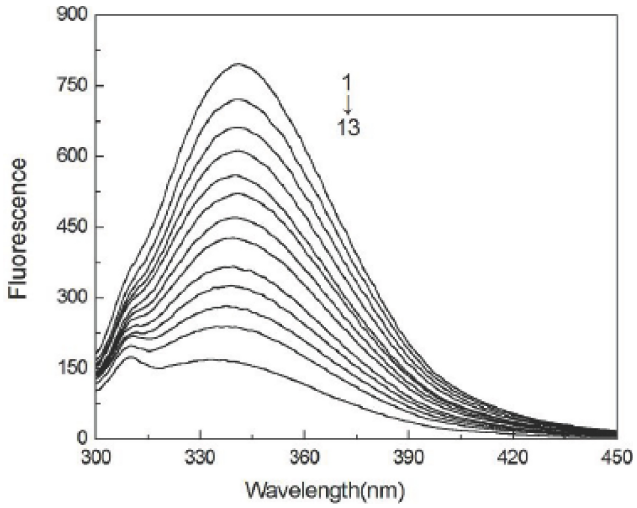
RESULTS AND DISCUSSION

Effect of EY on BSA fluorescence

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample. We measured the fluorescence quenching spectra of BSA at various concentrations of EY. The effect of EY on BSA fluorescence intensity was shown in Figure 1. As it follows from Figure 1 the fluorescence intensity of BSA decreases regularly with the increasing of EY concentration. This indicates that EY could bind to BSA without altering local dielectric environments[21].



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}

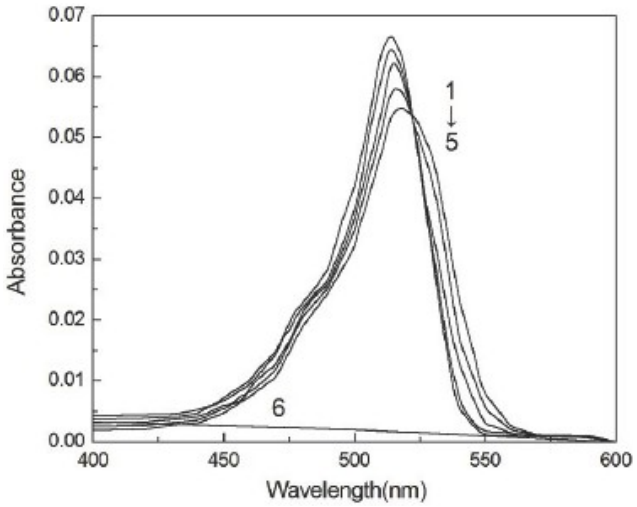
Figure 1 : Fluorescence emission spectra of BSA-EY (T=303K)

Effect of BSA on EY absorption spectra

UV-Vis absorption measurement is a very simple and effective method in exploring the structural change and detecting the complex formation[22]. For confirming the static quenching effect may exist in the system of EY and BSA, the absorption spectra of EY in the presence and absence of BSA were recorded and presented in Figure 2. As can be seen from Figure 2, with the addition of BSA the absorbance decreases and the absorption spectra maximum shifts towards longer wavelength region, which indicate that EY can bind to BSA and form the BSA-EY complex[23].

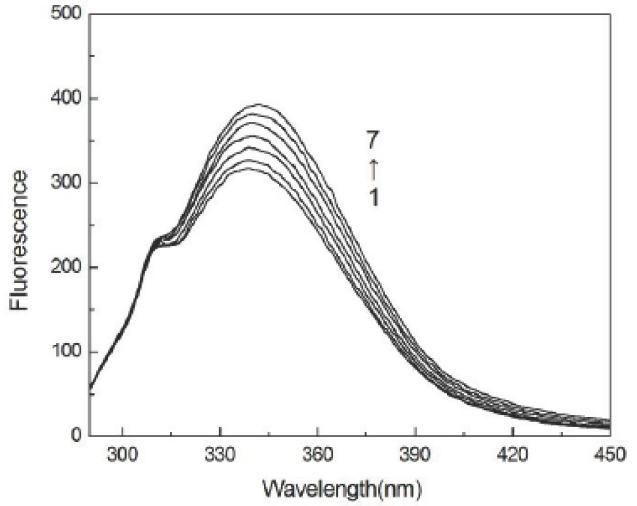
Effect of SM on the fluorescence emission spectra of the BSA-EY system

The emission spectra of BSA-EY in the absence and presence of SM 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 SM. This is indicative of a competitive reaction between SM and BSA for EY.



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 ET-BSA (T=303K)



1~7 BSA (4.0×10^{-7}mol/L)+EY (4.0×10^{-7}mol/L)+SM (0,0.1,0.2,0.4,0.8,1.2,1.6) $\times10^{-3}$mol/L

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

Quenching mechanism

Fluorescence quenching can proceed by different mechanisms, which are usually classified as either dynamic quenching or static quenching. For dynamic quenching, the Stern-Volmer equation can be used to

describe the mechanism[24]:

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

Where F_0 and F represent the fluorescence intensities in the absence of and in the presence of the quencher, K_q is the bimolecular quenching rate constant and it is equal to K_{SV}/τ_0, τ_0 is the average lifetime of the molecule without the quencher and is taken as 10^{-8}s, *[Q]* is the concentration of the quencher and K_{SV} is the Stern-Volmer quenching constant. TABLE 1 presents the calculated K_q and linear relative coefficient r_1 at each temperature studied. The results show that the K_q decreased with the increase of temperature and the values of K_q were much larger than 2.0×10^{10} L mol^{-1} s^{-1}. So, the mechanism between the BSA interacted with EY is static quenching and resulted from the formation of ground state complex between them[25].

Binding constant and binding sites

When small molecules bind independently to a set of equivalent sites on a macromolecule, Eq. (3) can be used to describe the relationship between fluorescence intensity and the concentration of the quencher[26,27]:

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

Where K_a is the binding constant and n is the number of binding sites per BSA. Based on Eq. (3), the values of K_a and n can be obtained using the double–logarithm algorithm curve and the corresponding calculated results are 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 EY-BSA complex[28].

Thermodynamic properties and type of interactions between EY and BSA

In general, the interaction forces between proteins and ligands include hydrophobic, hydrogen bonds, van der Waals, and electrostatic interactions, etc[29]. Supposing that there was no significant change in the enthalpy (ΔH) value over the temperature range, the entropy change (ΔS) and the free energy change (ΔG) of binding can be estimated from the following equations:[30]

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

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

where the associative binding constant K is analogous to the effective quenching constant K_a at the corresponding temperature, R is the correlation coefficient. If ΔH<0 and ΔS>0 electrostatic force plays an important role in the reaction. If ΔH<0 and ΔS<0, van der Waals forces and hydrogen bonds are dominant in the reaction. If ΔH>0 and ΔS>0 typical hydrophobic interactions are dominant in the reaction. The values of the thermodynamic parameters (ΔH, ΔS, ΔG) were obtained by Eqs (4), (5) and they were -11.9 KJ mol^{-1}, 89.4 KJ mol^{-1}, -39.0 KJ mol^{-1} (T=303 K), respectively. The ΔG<0 indicated a spontaneous reaction occurred between EY and BSA. And, electrostatic interaction can play a major role in the binding process[31].

Binding distance between BSA and EY

According to the Förster non-radioactive resonance energy transfer theory[32], the effective energy transfer from donor to acceptor will occur when two molecules meet the following preconditions: (1) donor is a fluorophore; (2) the overlap is enough between the fluorescence emission spectrum of the donor and UV-vis absorption spectrum of the acceptor; (3) the distance between the donor and the acceptor is within 2-7 nm. The energy transfer effect is not only related to the distance between the donor (tryptophan residue) and acceptor, but also influenced by the critical energy trans-

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

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fer distance R_0. It is described by the following equations[33]:

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

$$R_0^6 = 8.78\times10^{-25} K^2 \Phi N^{-4} J \quad (7)$$

where R_0 is the critical energy transfer distance when the transfer efficiency is 50%, r is the distance between the acceptor and the donor, and E is the energy transfer efficiency. K is the spatial orientation factor of the dipole, N is refractive index of the medium, Φ is the fluorescence quantum yield of the donor, and the overlap integral (J) between the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor can be calculated by the equation[34]:

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

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at some wavelength, $\varepsilon(\lambda)$ is the molar absorbance of the acceptor at the wavelength λ. Figure 4 presents the overlap integral of the fluorescence emission spectrum of BSA and the absorption spectrum of EY. In the present case, K^2=2/3, N=1.336, and Φ=0.118[35]. Hence, from the above equations we could calculate the following parameters: J, R_0, E, and r, and they were summarized in TABLE 2, implying that the energy transfer from BSA to EY with high possibility.

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

Effect of site markers on binding of EY to BSA

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[36,37]. 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 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[38]. 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 SS 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 SS specifically binds to BSA.

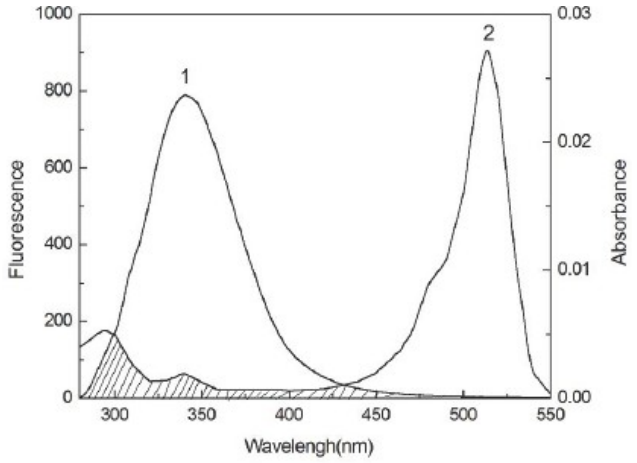
Fluorescence recovery mechanism

When SM 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 SM and EY for BSA. Eqs. (9) and (10) show the competitive reaction:

BSA-EY+SM→BSA-SM+EY (9)

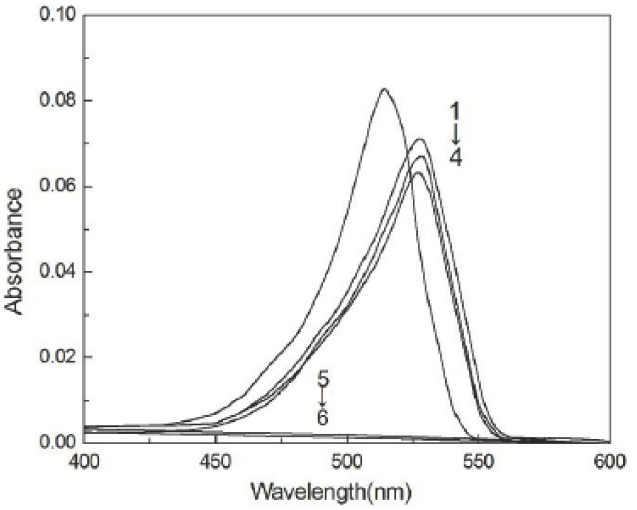
Or BSA-EY+SM→EY-SM+BSA-SM (10)

The absorption spectra of BSA-EY in the absence and presence of SM were recorded to confirm the competitive reaction according to Eqs. (9) or (10) (Figure 5). As it can be seen from Figure 5, SM and BSA had no absorption in the range 450–600 nm and λ_{max} of EY was at 514 nm. The absorption of EY gradually decreased and there was a red shift of the absorption wavelength with increasing concentration of SM. If the competitive reaction was according to Eq. (9), the absorption of EY should be increased with increasing concentration of SM, which is not observed (Figure 5). If



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

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



1, EY (1.0×10^{-6}mol/L); 2~4 BSA (4.0×10^{-7}mol/L)+EY (1.0×10^{-6}mol/L)+SM (0,1.0, 2.0)$\times10^{-4}$mol/L; 5, BSA (4.0×10^{-7}mol/L); 6, SM (2.0×10^{-4}mol/L)

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

the competitive reaction was according to Eq. (10) SM had bound EY and caused the absorption of EY to gradually decrease with increasing concentration of SM, which is consistent with Figure 5. There was no obvious fluorescence intensity change on combining BSA and SM, so the relative fluorescence intensity of BSA gradually recovered with increasing concentration of SM, which is consistent with Figure 3. Therefore, the competitive reaction was according to Eq. (10).

CONCLUSIONS

This paper determines the bonding site (site I) of EY on BSA by the competition reagent, proves the specific binding between EY and BSA, and explains that EY can study the combination of drugs and proteins as a probe instead of competition reagent. Due to After reaction of BSA and SM, the fluorescence intensity of BSA has no obvious change, so there is no way to directly research SM and BSA binding reaction with fluorescence spectrometry.. In this paper using EY as fluorescent probe to study the SM to respond to the combination of the BSA, it proves that there is a reaction between SM 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 drug research.

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