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Comparative studies on the interaction of tartrazine with bovine serum albumin by fluorescence quenching spectroscopy and synchronous fluorescence spectroscopy

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ABSTRACT

Under simulated physiological conditions, the reaction mechanism between tartrazine (TTZ) and bovine serum albumin (BSA) at different temperatures (293 K, 303 K, 310 K) was investigated by fluorescence quenching method and synchronous fluorescence method, respectively. The results indicated that the fluorescence intensity and synchronous fluorescence intensity of BSA decreased regularly with the addition of TTZ, in addition, the quenching mechanism, binding constants (K_a), the number of binding site (n), type of interaction force and energy transfer parameters of TTZ with BSA obtained from two methods by the same equation were consistent, which indicated synchronous fluorescence spectrometry was a new method of studying the binding mechanism between drug and protein, and it was a useful supplement to the classical method.

KEYWORDS

Fluorescence quenching spectroscopy;
Synchronous fluorescence;
Tartrazine;
Bovine serum albumin;
Interaction.

INTRODUCTION

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 value in life sciences, chemistry, pharmacy and clinical medicine. The classical fluorescence spectroscopy studies the reaction mechanism of small molecule drugs and proteins, mainly by studying the change of fluorescence intensity of protein in the maximum emission wavelength before and after adding the drugs, and derives the binding constants, binding sites and the donor-to-acceptor distance with other information between proteins and drugs[1-3]. Synchronous fluorescence spectrometry technology was first proposed by Lloyd[4], and the biggest difference between this and fluorescence measurement method is that the excitation and emission monochromators were scanned simultaneously. Comparing with classical fluorescence spectroscopy, synchronous fluorescence method has some advantages such as good selectivity, high sensitivity, less interference etc[5-6], and it can be used for the simultaneous determination of multi-com-

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ponent mixture. However, studies on the binding constant and binding sites between the protein and TTZ with this method have not been reported.

Food colorants are a very important class of food additives and may be natural or synthetic. Synthetic food colorants are widely used due to their low price, high effectiveness and excellent stability[7]. Tartrazine (TTZ) (the structure shown in Figure 1) is common synthetic food colorants,which is used in a wide variety of food such as beverages, dairy products, cereals, snack foods and ice creams. In this study, we study the binding mechanism of drugs and proteins by utilizing classical fluorescence quenching method and synchronous fluorescence method with the research object being TTZ. The data were obtained and analyzed. It shows that the research of combination mechanism of proteins and drugs has become more abundant with the new method and it is a useful supplement to the application of fluorescence spectroscopy in the field.

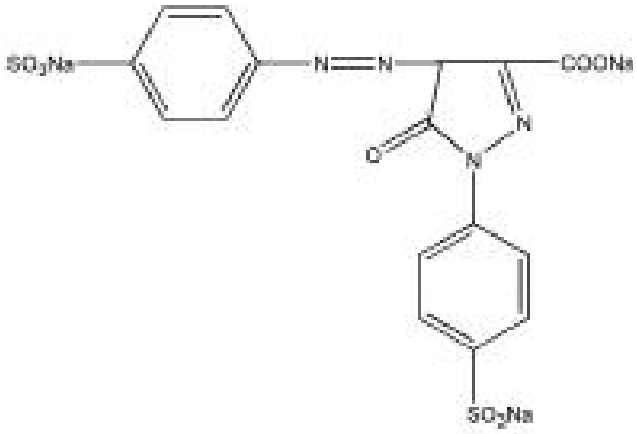

Figure 1 : Chemical structure of tartrazine

EXPERIMENTAL

Apparatus

All fluorescence spectra were recorded with a Shimadzu RF-5301PC spectro- fluorophotometer. 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). All temperatures were controlled by a CS501 super-heated water bath (Nantong Science Instrument Factory).

Materials

Bovine serum albumin was purchased from Sigma Company and of the purity grade inferior 99%. Tartrazine (TTZ) was purchased from Aldrich (Wisconsin, USA). Stock solutions of BSA (1.0×10^{-5} mol·L^{-1}), and TTZ (1.0×10^{-4} mol·L^{-1}) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris-HCl buffer solution (0.05 mol·L^{-1} of Tris, 0.15 mol·L^{-1} of NaCl) was used to keep the pH of the solution at 7.40, and NaCl solution was used to maintain the ionic strength of the solution. All other reagents were analytical 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 re-absorption of emitted light to decrease the inner filter effect using the following relationship[8]:

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

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

Procedures

Fluorescence measurements

In a typical fluorescence measurement, 1.0 mL of Tris-HCl buffer, pH 7.4, 1.0 mL of 4.0×10^{-6} mol·L^{-1} BSA solution and different concentrations of TTZ were added into 10 mL colorimetric tube successively. The

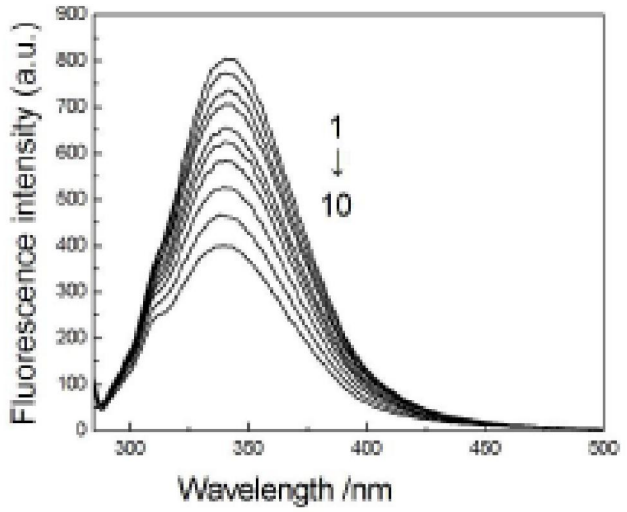

Figure 2 : Fluorescence spectra of TTZ-BSA system (λ_{ex}=280 nm, T = 293 K); C_{BSA} = 4.0×10^{-7} mol·L^{-1}; 1~10 C_{TTZ}= (0, 0.4, 0.7, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0)$\times10^{-6}$ mol·L^{-1}

samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 30 minutes at different temperatures (293, 303 and 310 K). Excitation wavelength for BSA was 280 nm (295 nm), with a 10 mm pathlength cell. The excitation and emission slits were set at 5 nm each. The solution was subsequently scanned on the ûuorophotometer and recorded the fluorescent intensity at 340 nm.

Synchronous fluorescence measurements

The solution preparation was the same as in Section 2.3.1, we recorded the fluorescence spectra of BSA-TTZ system when the value between the excitation and emission wavelengths, $\Delta\lambda$ was 15 and 60 nm.

RESULT AND DISCUSSION

Fluorescence quenching spectra of BSA-TTZ system

Proteins were considered to have intrinsic fluorescence due to the presence of amino acids, mainly tryptophan (Trp), tyrosine (Tyr). When the excitation wavelength was at 280 nm (or 295 nm), BSA has a strong fluorescence emission peak at 340 nm. The fluorescence spectra of BSA-TTZ system was shown in Figure 2. As shown in Figure 2, the fluorescence intensity of BSA decreased regularly with the addition of TTZ when the excitation wavelength is 280 nm (it was similar to 295 nm), This result showed that TTZ could quench the intrinsic fluorescence of BSA and there was an interaction between TTZ and BSA, it could also reveal that a new complex is being formed[9].

In order to confirm the quenching mechanism, the fluorescence quenching data are analyzed by the Stern–Volmer equation[10]:

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

Where, F_0 and F represent the fluorescence intensities in the absence and in the presence of quencher, respectively. τ_0 is the average lifetime of fluorescence without quencher, which is about 10^{-8} s. K_{sv} is the Stern-Volmer quenching constant. K_q is the bimolecular quenching constant, and [L] is the concentration of the quencher. According to Eq. (2), based on the linear fit plot of F_0/F versus [L], the K_q values can be obtained. The calculated results were shown in TABLE 1. Different mechanisms of quenching are usually classified as the dynamic quenching and the static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature. The quenching rate constants decrease with the rising temperature for the static quenching, but the reverse effect is observed for the dynamic quenching[11]. In TABLE 1, the values of K_{sv} decreased with the rising temperature in all systems, which indicated that the probable quenching mechanism of the interaction between BSA and TTZ was initiated by complex formation rather than by dynamic collision[12]. In addition, all the values of K_q were much greater than the maximum scatter collision quenching constant of various quenchers (2×10^{10} L·mol^{-1}·s^{-1}), this also suggested that the quenching was a static process[13].

For static quenching process, the relationship between the fluorescence intensity and the concentration of quencher can be usually described by Eq. (3)[14] to obtain the binding constant (K_a) and the number of binging sites (n) in most paper:

$$\log[(F_0 - F)/F] = n \log K_a + n \log\{[D_t] - n[B_t](F_0 - F)/F_0\} \quad (3)$$

Where, [D_t] and [B_t] are the total concentrations of TTZ and BSA, respectively. On the assumption that n in the bracket is equal to 1, the curve of log [(F_0-F)/F] versus log {[D_t]-[B_t](F_0-F)/F_0} is drawn and fitted linearly, then the value of n can be obtained from the slope of the plot. If the n value obtained is not equal to 1, then it is substituted into the bracket and the curve of log [(F_0-F)/F] versus log {[D_t]-n [B_t](F_0-F)/F_0} is drawn again. The above process was repeated again and again till n obtained was only a single value. Based on the n obtained the binding constant K_a can be also obtained. In the work, a calculation program was developed. The calculation process can be finished with calculator based on the above simple program and the calculating result can be obtained by inputting F, [D_t] and [B_t]. The calculated result was shown in TABLE 1. As shown in TABLE 1, the fact that the values of n were all approximately to 1 implied that just one binding site for TTZ existed in BSA. Meanwhile, binding constants K_a were decreased with the rising temperature, further suggested that the quenching was a static process[15].

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TABLE 1 : Quenching reactive parameters of BSA and TTZ at different temperatures

λ_{ex}(nm)	T/(K)	K_q /(L·mol^{-1}·s^{-1})	K_{sv} /(L·mol^{-1})	r_1	K_a /(L·mol^{-1})	n	r_2
280	293	1.47×10^{13}	1.47×10^{5}	0.992 2	1.66×10^{5}	0.89	0.991 8
	303	1.25×10^{13}	1.25×10^{5}	0.998 9	1.33×10^{5}	0.85	0.998 0
	310	1.20×10^{13}	1.20×10^{5}	0.998 1	1.26×10^{5}	0.93	0.998 0
295	293	1.46×10^{13}	1.46×10^{5}	0.990 8	1.20×10^{5}	0.78	0.992 2
	303	1.07×10^{13}	1.07×10^{5}	0.994 2	1.16×10^{5}	0.86	0.996 3
	310	1.03×10^{13}	1.03×10^{5}	0.997 4	1.08×10^{5}	0.86	0.997 6

K_q is the quenching rate constant; K_a is the binding constant; n is the number of binding site. r_1 is the linear relative coefficient of F_0/F~[L]; r_2 is the linear relative coefficient of log[$(F_0-F)/F$]~log{[D_t]-n [B_t]$(F_0-F)/F_0$}.

The primary binding site studies

At 280 nm wavelength the Trp and Tyr residues in BSA are excited, whereas the 295 nm wavelength excites only Trp residues[16]. In BSA sub-hydrophobic domain, IIA (containing both Trp and Tyr) and IIIA (containing only Tyr) is the major binding site of small molecule ligands[17]. 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[18]. As seen in Figure 3, in the presence of TTZ, the quenching curves of BSA excited at 280 nm and 295 nm overlap approximately below the molar ratio of TTZ:BSA=10:1, which indicated that only Trp residues played an important role in the interaction of TTZ with BSA. The quenching of BSA fluorescence exited at 280nm above this molar ratio was slightly higher than that excited at 295nm which means that Tyr residues began to take part in the interaction. According to this conclusion, it could be inferred that TTZ molecules only take interaction with Trp residues of BSA at low concentration, whereas both Trp and Tyr residues at high concentration, but the interaction of Tyr with TTZ is weak in terms of Trp. From TABLE 1, in the same temperature the K_a values of BSA at 280 nm was slightly greater than 295 nm, this also suggested that only Trp residues played an important role in the interaction between TTZ and BSA.

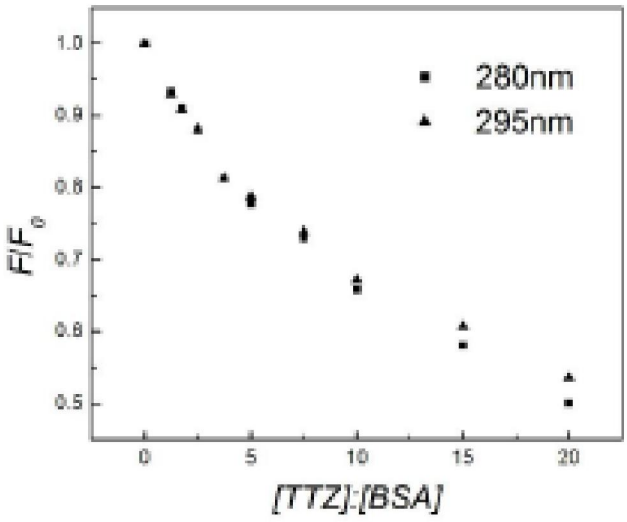

Figure 3 : Fluorescence emission spectra of TTZ-BSA (T =293 K); C_{BSA} = 4.0×10^{-7} mol·L^{-1}, C_{TTZ}=5.0×10^{-7}~ 8.0×10^{-6} mol·L^{-1}.

Synchronous fluorescence spectra studies

Synchronous fluorescence spectra are used to investigate the protein conformational change, as it has been shown to give narrow and simple spectra[19]. When the $\Delta\lambda$ value between the excitation and emission wavelengths were stabilized at either 15 or 60 nm, the synchronous fluorescence gives characteristic information for tyrosine residues or tryptophan residues, respectively[20]. It can be seen from Figure 4, when the $\Delta\lambda$ value was 15 nm, the fluorescence intensities of BSA-TTZ decreased not obviously with no shifts with increasing concentration of TTZ, this indicated that Tyr residues hardly played an important role in the interaction between TTZ and BSA. When the $\Delta\lambda$ value was 60 nm the synchronous fluorescence intensities of TTZ-BSA decreased regularly with some shifts, which indicated that the interaction of BSA with TTZ changed the microenvironment of tryptophan residues[21], which was coincident with the result of Section 3.2. The polarity of hydrophobic environment was enhanced and hydrophobicity was reduced in the BSA cavity due to the changes of tryptophan residues microenvironment with

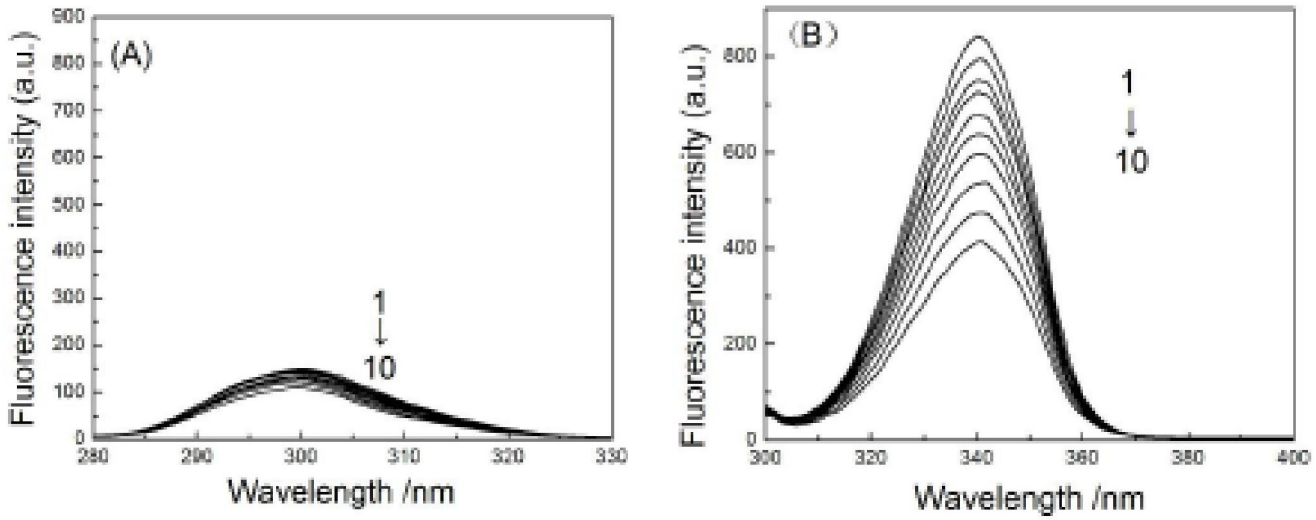

Figure 4 : Fluorescence spectrum of TTZ-BSA system (T = 293 K) (A) $\Delta\lambda$=15nm; (B) $\Delta\lambda$=60nm; C_{BSA} = 4.0×10^{-7} mol·L^{-1}; 1~10 C_{TTZ} = (0, 0.4, 0.7, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0)×10^{-6}mol·L^{-1}

TABLE 2 : Quenching reactive parameters of TTZ and BSA at different temperatures ($\Delta\lambda$ = 60 nm)

$\Delta\lambda$ (nm)	T/(K)	K_q /(L·mol^{-1}·s^{-1})	K_{sv} /(L·mol^{-1})	r_3	K_a /(L·mol^{-1})	n	r_4
15	293	4.59×10^{12}	4.59×10^{4}	0.998 8	4.24×10^{4}	1.01	0.993 7
	303	4.29×10^{12}	4.29×10^{4}	0.998 4	3.33×10^{4}	0.91	0.991 0
	310	4.02×10^{12}	4.02×10^{4}	0.995 4	3.29×10^{4}	0.80	0.992 4
60	293	1.84×10^{13}	1.84×10^{5}	0.992 5	1.39×10^{5}	0.74	0.997 0
	303	1.31×10^{13}	1.31×10^{5}	0.997 7	1.34×10^{5}	0.85	0.995 9
	310	1.25×10^{13}	1.25×10^{5}	0.997 6	1.33×10^{5}	0.86	0.997 8

K_q is the quenching rate constant; K_a is the binding constant; n is the number of binding site. r_3 is the linear relative coefficient of F_0/F~[L]; r_4 is the linear relative coefficient of log[(F_0-F)/F]~log{[D_t]-n[B_t](F_0-F)/F_0}.

the insertion of TTZ[22], leading to the conformation change of BSA. Protein molecules were extended by high concentrations of drugs, which reduced the energy transfer among the amino acid residues and their fluorescence intensity. For the $\Delta\lambda$ = 15 nm and $\Delta\lambda$ = 60 nm, according to Eqs. (2)(3), the corresponding results are shown in TABLE 2. From TABLE 2, it can be seen the values of K_{sv} decreased with the rising temperature in all systems, which indicated that the probable quenching mechanism of the interaction between BSA and TTZ was a static process. The K_q was the order of 10^{12} L·mol^{-1}·s^{-1}. Obviously, the K_q value of protein quenching procedure initiated by TTZ was greater than 2×10^{10} L·mol^{-1}·s^{-1}, this indicated that the quenching was not initiated from dynamic collision but from the formation of a complex. The n value approaches unity, suggesting that one molecule of TTZ combines with one molecule of BSA; the decreasing trend of K_a with the increasing temperature was in accordance with binding constants dependence on the temperature as mentioned above, which indicated that TTZ-BSA would be partly decomposed when the temperature rising, it also indicated it was a static quenching. The quenching mechanism obtained by synchronous fluorescence method was coincident with the one obtained by fluorescence method. Comparing TABLE 1 and TABLE 2, we can know that the quenching parameters obtained by two methods have the same order of magnitude. However, in TABLE 2 the quenching parameters of $\Delta\lambda$ = 15 nm are significantly smaller than the ones of $\Delta\lambda$ = 60 nm, this also suggested that only Trp residues played an important role in the interaction between TTZ and BSA, which was coincident with the result of Section 3.2.

Type of interaction force of BSA-TTZ systems

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TABLE 3 : The thermodynamic parameters of TTZ-BSA at different temperatures

	T /(K)	K_a /(L·mol^{-1})	ΔH /(KJ·mol^{-1})	ΔS/(J·mol^{-1}·K^{-1})	ΔG /(KJ·mol^{-1})
λ_{ex}= 280nm	293	1.66×10^5	-12.58	57.00	-29.28
	303	1.33×10^5		56.57	-29.72
	310	1.26×10^5		57.06	-30.27
λ_{ex}= 295nm	293	1.20×10^5	-4.51	81.83	-28.49
	303	1.16×10^5		82.10	-29.38
	310	1.08×10^5		81.82	-20.87
$\Delta\lambda$= 60nm	293	1.39×10^5	-2.02	91.57	-28.85
	303	1.34×10^5		91.49	-29.74
	310	1.33×10^5		91.58	-30.41

Generally, the interaction force between the small drug molecule and biological macromolecule include hydrogen bond, Van der Waals force, electrostatic interactions and hydrophobic force, etc. Ross and Subramanian[23] have characterized the sign and magnitude of the thermodynamic parameter, enthalpy change (ΔH), free energy (ΔG) and entropy change (ΔS) of reaction, associated with various individual kinds of interaction. When temperature varies in a small range, the ΔH could be considered as a constant[24]. Negative ΔH and positive ΔS indicate electrostatic interaction plays a major role in the binding reaction. Positive ΔH and ΔS are generally considered as the evidence for typical hydrophobic interactions. In addition, Van der Waals force and hydrogen bonding formation in low dielectric media are characterized by negative ΔH and ΔS. The thermodynamic parameters can be calculated on the basis of the following equation[25]:

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

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

According to the relevant thermodynamic parameters of small molecule drugs and biological macromolecules, the type of interaction force can be simply judged. According to Eq. (4), based on the linear fit plot of ln$K$ versus 1/T, ΔH values can be obtained and then ΔS values can be obtained at each temperature from the K_a values and ΔH value. According to Eq. (5), the ΔG values can be obtained at each temperature from the K_a values. Therefore, the values of ΔH, S and ΔG were listed in TABLE 3. From TABLE 3, it can be seen that the reaction process of TTZ with BSA was a spontaneous molecular interaction procedure in which entropy increased and Gibbs free energy decreased[26]. The positive value of ΔS and ΔH showed that the electrostatic interaction play a major role in the binding process[27]. The conclusions of the synchronous fluorescence method were consistent with fluorescence method, further suggested that synchronous fluorescence spectrometry was a new method of studying the binding mechanism between drug and protein.

Energy transfer from BSA to TTZ

According to the Förster nonradioactive resonance energy transfer theory[28] 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 transfer distance R_0. It is described by the following equations:[29-30]

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

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

Where, R_0 is the critical 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[29]:

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

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 5 presents the overlap integral of the fluorescence emission spectrum of BSA and the absorption spectrum of TTZ. In the present case, K^2=2/3, N=1.336 and Φ=0.118[31], according to Eqs. (6)-(8), the corresponding results were shown in TABLE 4. From TABLE 4, it can be seen that the donor-to-acceptor distance r <7 nm indicated that the energy transfer from BSA to TTZ occured with high possibility[32], the distance r increased and the energy efficiency E decreased with increasing temperature (TABLE 4), which resulted in the reduced stability of the binary systems and the values of K_a. Moreover, the value of r was greater than R_0 in this study which suggested that TTZ could quench the intrinsic fluorescence of BSA by a static quenching mechanism[33]. In addition, from TABLE 4, the data obtained by the two methods were basically consistent, and the conclusions also are the same, which indicated that synchronous fluorescence spectrometry was a new method of studying the binding mechanism between drug and protein, and it was a useful supplement to the classical method.

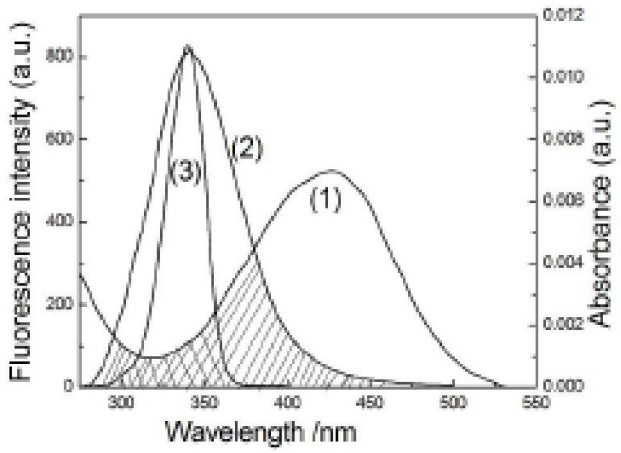

Figure 5 : Overlap of the fluorescence spectrum of BSA (λ_{ex}=280nm) (1) and ($\Delta\lambda$=60nm) (2) with the absorption spectrum of TTZ (3); (T=293K) $C_{TTZ} = C_{BSA}$ =4.0×10^{-7} mol·L^{-1}.

TABLE 4 : Parameters of E, J, r, R_0 between TTZ and BSA at different temperatures

method	T/(K)	E/(%)	J/(cm^3·L·mol^{-1})	R_0/(nm)	r/(nm)
λ_{ex}= 280nm	293	10.37	1.15×10^{-14}	2.510	3.60
	303	9.38	1.14×10^{-14}	2.507	3.66
	310	8.86	1.14×10^{-14}	2.505	3.71
λ_{ex}= 295nm	293	16.57	1.26×10^{-14}	2.548	3.34
	303	7.13	1.25×10^{-14}	2.544	3.90
	310	3.94	1.24×10^{-14}	2.541	4.33
$\Delta\lambda$= 60nm	293	15.09	5.02×10^{-15}	2.185	2.91
	303	7.69	4.98×10^{-15}	2.182	3.30
	310	5.08	4.59×10^{-15}	2.153	3.51

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 donor and the absorption spectrum of the acceptor.

CONCLUSIONS

In this paper, the binding of TTZ to BSA under simulated physiological conditions was studied by classical fluorescence quenching method and synchronous fluorescence method, which were used the same equation for processing data. From all data, we could see data obtained by both methods were in the same order of magnitude and very close, quenching mechanism and type of interaction force were consistent, which indicated synchronous fluorescence spectrometry was a new method of studying the binding mechanism between drug and protein, and it was a useful supplement to the classical method. In addition, synchronous fluorescence method has some advantages such as good selectivity, high sensitivity, less interference etc, which makes synchronous fluorescence method have more advantages than classical fluorescence quenching method to study the reaction mechanism of drugs with proteins.

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