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Study of the interactions between tetracycline analogues and lysozyme

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Abstract—The interactions between tetracycline analogues (oxytetracycline, doxycycline, methacycline) and lysozyme (LYSO) were studied by using UV–visible absorption and spectrofluorimetric method. Tetracycline analogues can make LYSO's fluorescence quenching. The quenching mechanism is static quenching. Absorption spectrum and quenching constant all support this conclusion. The binding constants of tetracycline analogues with LYSO were obtained at various temperatures. According to the Förster nonradiative energy transfer theory, we can get the distances of donators and acceptors. We also determined the main acting force between them is electrostatic gravitation according to the thermodynamic parameter.

1. Introduction

Oxytetracycline (OTC), tetracycline (TC), doxycycline (DC), and methacycline (MC) are all good broad-spectrum antibiotics. The antiviral abilities of DC and MC are better than OTC and TC.[1] Drugs bring the action of medicine via the store and transport of blood plasma and protein can combine with many antibiotics. Therefore, it is important to study the interactions between drugs and protein and the interactions between drugs and human serum albumin have been widely introduced these years[2,3] while the introductions of drugs and LYSO are very infrequent. HSA and LYSO are all formed by amino acid and LYSO is formed by 129 tactic amino residue.[4] LYSO contains six tryptophanes and three tyrosines.[4] LYSO can be used to prepare anticatarrhals. Its favorable cooperative and synergistic actions with antibiotics have a very important practical value in medicine area.

Thereinto, tryptophane or tyrosine residues can cure abscess, stomatitis, rheum, etc. via the binding with antibiotics. However, studies on LYSO presently pay most of their attention on the separation and purification of LYSO, such as crystallization process,[5] ion exchange process,[6] affinity chromatography,[7] etc. So, studies on the interactions between drugs and LYSO in different aspects have a very important meaning on realizing the transport and metabolism process of the drugs, the relation of structure and function of LYSO, and the chemical essence of the interaction between biomacromolecule and small molecule. In this work, we studied the interactions between OTC, TC, DC, MC, and LYSO, determined the binding distance of acceptor and donator, analyzed the binding mechanism of LYSO and the drugs and meanwhile calculated the binding constants and the amount of binding points. It provides useful clinical information to the compatibleness and use of drugs via the reaction mechanism from molecule level.

2. Results and discussion

2.1. Fluorescence quenching spectra

The fluorescence quenching spectra of LYSO with varying concentrations of MC is shown in Figure 1. We found that the endogenic fluorescence intensity reduced well regulated and blue shift occurred to the emission peak ceaselessly. It indicates that both of them exist interactions and have energy transfer. The fluorescence quenching spectra of LYSO and OTC, TC, and DC have the same phenomena and are elliptical here.

Keywords: Lysozyme; Tetracycline analogues; Static quenching; Fluorescence spectrophotometry.
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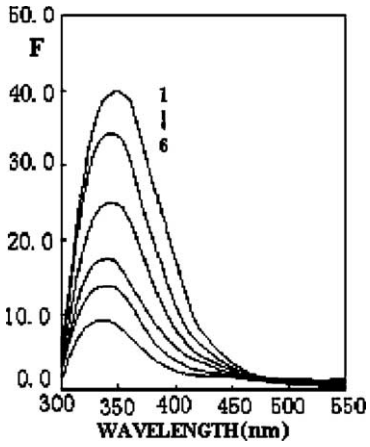

Figure 1. Effect of fluorescence spectra of LYSO in Tris–HCl buffer pH 7.4 at 20 °C ($\lambda_{ex/em} = 285/345$ nm). Concentration of LYSO $= 7.8\times10^{-5}$ M. Concentration of MC $= 7.8\times10^{-5}$ M. The volume of LYSO is 1 mL. Different volume of MC 1–6: 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mL. The total volume of each one is 10 mL.

There are two kinds of fluorescence quenching, which are static quenching and dynamic quenching. For dynamic quenching, it will increase the effective collision number of ions, enhance the transfer of energy, and make the quenching constant of fluorescence substance augment with the increasing of temperature. If it is static quenching, along with the increasing of temperature, it will reduce the stability of the formative compound and the quenching constant. For illustrating its quenching mechanism ulteriorly, The Stern–Volmer[8] graph of LYSO and MC at various temperatures is shown in Figure 2.

Figure 2 shows that the curves have favorable linear relationships and the slopes of the quenching curves decreased with the increasing of temperature. It indicates the static quenching interaction between MC and LYSO.

In order to confirm the view, the procedure was assumed to be dynamic quenching. Quenching equation:[8]

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q], \qquad (1)$$

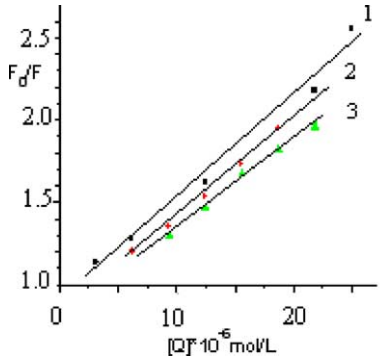

Figure 2. The Stern–Volmer curves of MC and LYSO at different temperatures. 1: 25 °C, 2: 37 °C, 3: 45 °C.

where K_q, K_{sv}, τ_0, and $[Q]$ are quenching rate constant of biomolecule, dynamic quenching constant, average lifetime of molecule without quencher, and concentration of quencher, respectively. $K_{sv} = K_q\tau_0$, $K_q = K_{sv}/\tau_0$. Because fluorescence lifetime of biopolymer is 10^{-8} s,[9] quenching constant (K_q: L mol^{-1} s^{-1}) can be obtained by the slope. Four kinds of drugs' quenching constants and linear correlative coefficients are shown in Table 1.

Table 1 indicates that the quenching constants decrease with the increasing of temperature and are all about 10^{12} level. However, maximum scatter collision quenching constant of various kinds of quenchers to biopolymer is 2.0×10^{10} L mol^{-1} s^{-1}.[10] Obviously, the rate constant of LYSO quenching procedure initiated by drugs is greater than K_q of scatter procedure. So this shows that above quenching is not initiated by dynamic collision but forms compound. It is static quenching. The conclusion is also practicable for TC, OTC, and DC.

2.2. Absorption spectra

The absorption spectra of LYSO and the compound formed of the same amount of LYSO and MC is shown in Figure 3. Figure 3 shows that the addition of MC makes the absorption peak of LYSO increase at the wavelength of 280 and red shift occurs a little of the location of peaks. It indicates that MC and the ground state of LYSO have interactions and form ground state complex. Accordingly, it causes the changes of LYSO's ultra-violet absorption spectra. Dynamic quenching only effects the excitation state of quenching molecule while has no function on the absorption spectrum of quenching substances. So, we can estimate the quenching mechanism of MC and LYSO is static quenching.[11] The absorption spectra of LYSO and OTC, TC, and DC have the same phenomena.

2.3. The determination of binding constants and binding points

Because of the noncovalent bond action between drugs and LYSO, we explain it by the potential point combine model,[12,13] which is

Table 1. MC, OTC, TC, and DC's quenching constants and linear correlative coefficients

Drugs	Temperature (°C)	Quenching rate constant (K_q)	Linear correlative coefficient (r)
MC	20	8.01×10^{12}	0.9952
	37	6.91×10^{12}	0.9992
	45	5.99×10^{12}	0.9992
OTC	20	6.77×10^{12}	0.9984
	37	6.05×10^{12}	0.9997
	45	5.23×10^{12}	0.9976
TC	20	5.65×10^{12}	0.9998
	37	3.60×10^{12}	0.9835
DC	20	7.19×10^{12}	0.9906
	45	5.42×10^{12}	0.9992

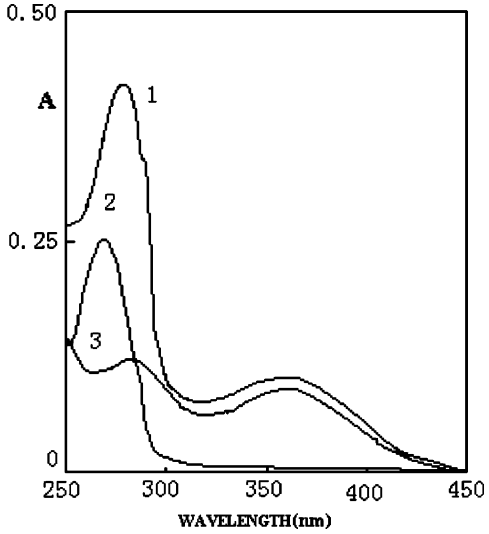

Figure 3. The absorption spectra of LYSO and the compound formed of the same amount of LYSO and MC in Tris–HCl buffer pH 7.4 at 20 °C. Concentration of LYSO = 7.8×10^{-5} M. Concentration of MC = 7.8×10^{-5} M. 1: LYSO+MC (1:1). The volumes of LYSO and MC are all 2 mL. 2: LYSO. The volume of LYSO is 2 mL. 3: MC. The volume of MC is 2 mL. The total volume of each one is 10 mL.

$$v = \frac{nK[Q]}{(1 + K[Q])}. \tag{2}$$

In the equation, v is the amount of average binding point, that is the amount of substance of drugs bound every molar of macromolecule, K is the binding constant, n is the amount of binding potential point, that is the amount of small molecules a macromolecule can bind, $[Q]$ is the free concentration of drugs in solution. From Eq. 3,[14] we can obtain K and n:

$$\frac{F_0}{F} = \frac{K[Qt]F_0}{F_0 - F} - nK[P_t]. \tag{3}$$

Fix the concentration of LYSO $[P_t]$, change the concentration of drugs $[Q_t]$, and according to Eq. 3, we can get a beeline from the figure of $\frac{F_0}{F}$ to $\frac{[Qt]F_0}{F_0-F}$. From the slope and intercept of this line, we can determine the binding constants K ($L\,mol^{-1}$) and the amount of binding potential points n. The binding constants and the amount of binding potential points of drugs and LYSO at various temperatures are shown in Table 2.

Table 2 indicates that the binding constants decrease with the increasing of temperature and are all about 8×10^4. The binding constants of MC and DC are higher than the constants of TC and OTC. Their bindings obey potential point combine model.

2.4. The determination of acting force between drugs and LYSO

The acting forces between pharmaceutical and biomolecule include hydrogen bond, van der Waals force, electrostatic force, and hydrophobic interaction force, etc. If the temperature changes little, the reaction enthalpy change is regarded as a constant. By the equations:

Table 2. The binding constants and the amount of binding potential points of drugs and LYSO at various temperatures

Drugs	Temperature (°C)	Binding constant (K)	Binding potential point (n)	Linear correlative coefficient (r)
MC	20	11.13×10^4	1.43	0.9911
	37	10.96×10^4	1.32	0.9804
	45	9.47×10^4	1.11	0.9939
OTC	20	7.48×10^4	5.61	0.9868
	37	7.37×10^4	3.41	0.9915
TC	20	5.82×10^4	2.31	0.9882
	37	5.20×10^4	2.30	0.9896
DC	20	9.29×10^4	6.03	0.9887
	45	7.08×10^4	5.27	0.9969

$$\ln\frac{K_2}{K_1} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\frac{\Delta H}{R}, \tag{4}$$

$$\Delta G = \Delta H - T\,\Delta S = -RT\ln K, \tag{5}$$

ΔH, ΔG, and ΔS, which are enthalpy change, free energy change, and entropy change, respectively, can be obtained. ΔH ($kJ\,mol^{-1}$), ΔS ($kJ\,mol^{-1}\,K^{-1}$), and ΔG ($kJ\,mol^{-1}$) in the binding reaction between drugs and LYSO are shown in Table 3.

From Table 3, we can see that $\Delta H < 0$, $\Delta S > 0$, so it can be deduced that the acting force is mainly electrostatic force.[15]

2.5. The determination of drugs' binding location of LYSO

According to Förster dipole–dipole nonradiative energy transfer theory,[16,17] there will be nonradiative energy transfer phenomenon when two compound molecules satisfy the conditions below: (1) the energy donor (d) can emit fluorescence; (2) the energy donor's fluorescence emission spectrum have enough overlap with the absorption spectrum of the energy acceptor (a), (3) the energy donor and the energy acceptor must be near enough and the maximum distance of d–a is 7 nm. The overlap of the fluorescence emission spectra of LYSO (a) with the absorption spectra (b) of MC is shown in Figure 4.

According to Förster dipole–dipole nonradiative energy transfer theory, the energy transfer efficiency (E) can be directly related to the distance using:

Table 3. ΔH, ΔS, and ΔG of LYSO and different drugs

Drugs	ΔH	ΔS	ΔG
MC	−10.09	0.0627	−28.79
OTC	−4.69	0.0752	−27.09
TC	−18.57	0.0304	−27.62
DC	−10.70	0.0592	−28.34

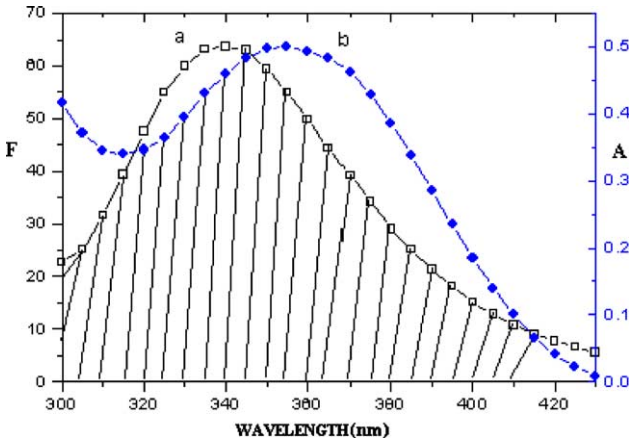

Figure 4. The overlap of the fluorescence emission spectra of LYSO (a) with the absorption spectra (b) of MC.

$$E = \frac{R_0^6}{(R_0^6 + r^6)}, \tag{6}$$

where R_0 is a characteristic distance, called the Förster distance or critical distance, at which the efficiency of transfer is 50%, and r is the distance between the donor and acceptor.

$$R_0^6 = 8.8 \times 10^{-25}\ K^2 N^{-4} \Phi J. \tag{7}$$

In Eq. 7, K^2 is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor in the absence of acceptor and the overlap integral J ($cm^3\,L\,mol^{-1}$) expresses the degree of spectral overlap between the donor emission and the acceptor absorption. J can be given by:

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\sum F(\lambda)\Delta\lambda}, \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor in wavelength λ and is dimensionless, $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor in wavelength λ, and its units are $cm^{-1}\,mol^{-1}\,L$. Then the energy transfer efficiency is frequency calculated from the relative fluorescence yield in the presence (F) and the absence of acceptor (F_0):

$$E = 1 - \frac{F}{F_0}. \tag{9}$$

So J can be evaluated by integrating the spectra in Figure 4. Under these experimental conditions, we found R_0 from Eq. 7 using $K^2 = 2/3$, $N = 1.36$, $\Phi = 0.14$,[18] the energy transfer effect E from Eq. 9, the critical distance R_0 (nm), and the distance between drugs and amino acid residue in LYSO r (nm). The data above are shown in Table 4.

From Table 4, we can see that MC has the highest overlap integral and the overlap integral of different drugs are all 10^{-14} level. TC has the highest energy transfer effect. The critical distances and the distances to amino acid residue are about 2–3.

Table 4. The critical distance and the distance to amino acid residue at different energy transfer effect

Drugs	Overlap integral (J)	Energy transfer effect (E), %	Critical distance (R_0)	The distance to amino acid residue (r)
MC	7.54×10^{-14}	17	2.31	2.27
OTC	1.63×10^{-14}	16	1.84	2.99
TC	3.43×10^{-14}	35	2.95	3.27
DC	1.41×10^{-14}	14	2.64	3.87

3. Summary

Experimental results demonstrate that OTC, TC, DC, and MC can make LYSO's fluorescence quench and the quenching fashion is static quenching. We have used absorption spectrum and binding constants to prove this conclusion. OTC, TC, DC, and MC can form ground state compound and their binding obey potential point combine model. It can be described by Scatchard equation and the binding points of LYSO are obtained accordingly.

4. Materials and methods

4.1. Materials

LYSO ($7.8\times10^{-5}\,mol\,L^{-1}$) (Healthy Department ShangHai Biologic Produce Graduate School), $7.8\times10^{-5}\,mol\,L^{-1}$ MC (Chinese Physic Biologic Produce Critical Graduate School) and OTC, TC, and DC are confected by the same means of MC. Tris–HCl ($0.1\,mol\,L^{-1}$) buffer solution of pH 7.4. All reagents were of analytical reagent grade and double distilled water was used throughout the experiment.

4.2. Methods

All fluorescent measurements were carried out on an RF-540 recording spectrofluorimeter (Shimadzu, Kyoto, Japan) equipped with xenon lamp source and 1.0 cm quartz cells as well as an instrument of constant temperature. A UV-265 recording spectrophotometer (Shimadzu) equipped with 1.0 cm quartz cells was used for the UV spectrum scanning. All pH measurements were made with a pHS-3 digital pH-meter (shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. To a 10 mL comparison tube, 2.0 mL Tris–HCl buffer solution, 1 mL LYSO solution, and 1 mL MC solution were added and were diluted to 10 mL with water, then shaken. Absorption spectra were measured after 10 min. To a 10 mL comparison tube, 2.0 mL Tris–HCl buffer solution, 2 mL LYSO solution and various amount of MC solution were added and

were diluted to 10 mL with water, then shaken. Fluorescence spectra were measured after 10 min. Using the same methods, we can obtain the absorption spectra and fluorescence spectra of OTC, TC, MC, and LYSO.

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