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Full Paper

Study of the Interaction of Cephalosporin Class Medicine with Albumin by Fluorescence Enhancement and Fluorescence Quenching Theories

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The interaction of 7 kinds of cephalosporin-medicine with HSA and BSA was studied and compared using fluorescence enhancement and fluorescence quenching method, then deeply analyzed. The binding characteristics of medicine with albumin and usual characteristic constants such as dissociation constant, quenching constant, quenching efficiency, energy-transfer efficiency and the distance between donor and accepter were also deeply analyzed.

Keywords fluorescence enhancement and quenching, human serum albumin, bovine serum albumin, cephalosporin

Introduction

The cephalosporin class medicine, such as the first generation cefazolin sodium, is an effective broad-spectrum antibiotics to gelanmasculincocci, pneumococcus, anthrabacilli, *etc*. and has been used in clinic broadly, from which the second generation cefuroxime sodium and the third generation cefoperazone sodium, ceftriaxone sodium, *etc*. were developed.

Because albumin is the most abundant protein in blood plasma, which can save and transfer many medicine, and act with medicine to control the release and metabolization of medicine, the study of such an interaction of albumin with medicine is very active.[1-8] With the development of this new class of medicine, its interaction mechanism is worth studying more deeply. Until now spectrum analysis is still the most common method. There are many differences among different author's results, especially in the mechanism, binding type and binding site numbers.[2-4] For example, the binding number between medicine molecule and albumin obtained from Ref 2 was 0.5, but the biggest binding number, which from another article of the same author was 30.[4] Hence we try to use fluorescence enhancement[9] and fluorescence quenching[10] theories deduced a few years ago to study the interaction rules of 7 cephalosporin class medicines with albumin, and verify each other at the same time. Accordingly more definite results about binding type, binding site number and fundamental information for explaining the interaction mechanism of the medicine and the relation between albumin structure and function were obtained.

Experimental

Reagent

Human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Shanghai Chemical Reagent Providing Station. Cefoperazone sodium and cefazolin sodium from Zhongnuo Medical Limited Corporation, cefuroxime sodium from Lizhu Pharmaceutical Factory, ceftriaxone sodium from Shanxi Pude Pharmaceutical Factory, ceflazidime and cefotaxime sodium from Xiangbei Weierman Limited Corporation and cefadroxil from Jiangxi Huiren Corporation are all powder for injection. Other chemicals used are of analytic grade and made in China.

Instrument and method

HSA and BSA were dissolved with 0.05 mol/L Tris-HCl buffer solution to concentration of 1×10^{-4} mol•L^{-1}, and the other 7 medicines were dissolved with 0.05 mol/L Tris-HCl buffer solution to concentration of 1×10^{-2} mol•L^{-1} respectively. Fluorescence quenching of the medicine with BSA was measured on an RF-540 fluorospectrophotometer (Shimadzu, Japan) equipped with a 1 cm quartz cell, and Tris-HCl buffer solution was used as reference. Fluorescence emission spectra of BSA were measured when exitation wavelength was fixed at 280 nm and scan range of emission spectrum was 290—500 nm, while the absorption spectra were measured on a UV-265 spectrophotometer (Shimadzu, Japan) when molar ratio of medicine to albumin was 1 in 290—500 nm.

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Theories and equations of fluorescence enhancement and fluorescence quenching

When biomacromolecules co-exist with some ligands, obvious change of fluorescence intensity can be observed, which can be used to study the binding situation of biomacromolecules and ligands. Supposing when biomacromolecule (P) binds with ligand (M), the number of same and separating binding sites is *n*. For the reaction $P+nM=M_nP$, we can obtain the fluorescence enhancement equations:[9]

$$\frac{[M_t]}{[M_b]}=\frac{1}{K_A(n[P_t]-[M_b])}+1 \quad (1)$$

$$\frac{1}{\Delta F-F_A}=\frac{1}{F_b-F_A}\left(1+\frac{1}{K_A(n[P_t]-[M_b])}\right) \quad (2)$$

where $[M_t]$ is the total concentration of ligand, $[M_b]$ is the concentration of ligand binding with biomacromolecule, $[P_t]$ is the total concentration of biomacromolecules, the concentration of resultant M_nP can be expressed as $[M_b]/n$, and $[M]=[M_t]-[M_b]$, $[P]=[Pt][M_b]/n$. $\Delta F=F-F_D$ [F is the fluorescence intensity of the system after ligand were added to the solution of biomacromolecule, F_D is the fluorescence intensity of free biomacromolecule (donor), F_A is the fluorescence intensity of free ligand (acceptor), F_b is the contribution of ligands to fluorescence which were bond to the biomacromolecules]. According to Eq. (2), the double reciprocal curve of $(\Delta F-F_A)^{-1}$ against $(n[P_t]-[M_b])^{-1}$ was made, which was a straight line, and from the curve formation constant K_A and binding site number n were obtained.

The interaction of some biomacromolecules which emit fluorescence with some medicine was able to cause the fluorescence quenching, from which Eqs. (3) and (4) were deduced:[10-12]

$$\frac{F_0}{F}=1+K_A[Q] \quad (3)$$

$$\frac{1}{F_0-F}=\frac{1}{F_0}+\frac{K_D}{F_0[Q]} \quad (4)$$

where [Q] is the concentration of quenching agent. F_0 and F are the fluorescence intensity of free biomacromolecules and that with addition of quenching agent. The double reciprocal curve of $(F_0-F)^{-1}$ against $[Q]^{-1}$ from Eq. (4) was made, from which dissociation constant K_D can be obtained.

Results and discussion

Study of dissociation and quenching constants of the medicine with albumin by fluorescence quenching theory

The measured fluorescence intensity of the medicine was near 0, and free HSA has strong fluorescence at 330 nm. With the titration of the medicine, the fluorescence of HSA was gradually quenched, and the quenching curves of the medicines with HSA are shown in Figure 1.

The interaction of the medicine with BSA was also measured, similar to HSA, free BSA has strong fluorescence at 330 nm. With the titration of the medicine, the fluorescence of BSA was gradually quenched too, the quenching curves are shown in Figure 2.

From Figure 1 it can be seen that there is an obvious turning point when the binding site number $[M_t]/[P_t]$ was 1, 4, 4 and 6 for cefotaxime sodium, cefozolin sodium, cefuroxime sodium and ceflazidime respectively. Accordingly it can be supposed that there are 2 kinds of binding sites for the medicine with HSA. Judging from the slope and the turning point, it can be seen that the quenching effect of the second kind of binding site is stronger. There are also similar turning points in the quenching curves (Figure 2) of the above 4 kinds of medicine with BSA, but the turning points appeared at $[M_t]/[P_t]$ is 1.5, 4, 6 and 4.5 respectively. The quenching curves of ceftriaxone sodium, cefadroxil and cefoperazone sodium with HSA were bent up, while the binding character of their molecular structure with HSA structural domain[11] cannot be thought obvious. The quenching curves (Figure 2) of the 3 kinds of medicine with BSA have similar appearance. In a word, the quenching curves (straight lines or curves) of the 7 medicines with either HSA or BSA were all bent up, showing that the sensitivity of BSA to quenching agents and the quenching effect of the medicine to Trp 214 of BSA were increased with the increase of concentration of the medicine.

Double reciprocal curves were made via the obtained experiment data and Eq. (4), which were good straight lines, and those of the quenching of ceftriaxone sodium with HSA and BSA are shown in Figure 3. From the slope of every straight line their dissociation constants K_D and linear interrelation coefficients C can be calculated, which are listed in Table 1.

Combining static quenching Eq. (4)[10-12] with Stern-Volmer collision quenching equation[11-13] $F_0/F=1+K_q\tau_0[Q]$, an equation $K_A=1/K_D=K_q\tau_0$ can be got, from which the apparent quenching constants K_q of 7 medicines with HSA or BSA can be calculated using K_D listed in Tables 1 and 2, where fluorescence life span of the biological molecule τ_0 is about 10 ns.[11] It has been known that the biggest diffusion-collision-quenching constant[11] of every kind of quenching agent with the biomacromolecule is 2.0×10^{10} L•mol^{-1}•s^{-1}. From above data it can be seen that the apparent quenching constants K_q of the medicine with albumin are much bigger than diffusion-control quenching constants, which shows that the quenching of these medicine with albumin was not caused by dynamic collision.

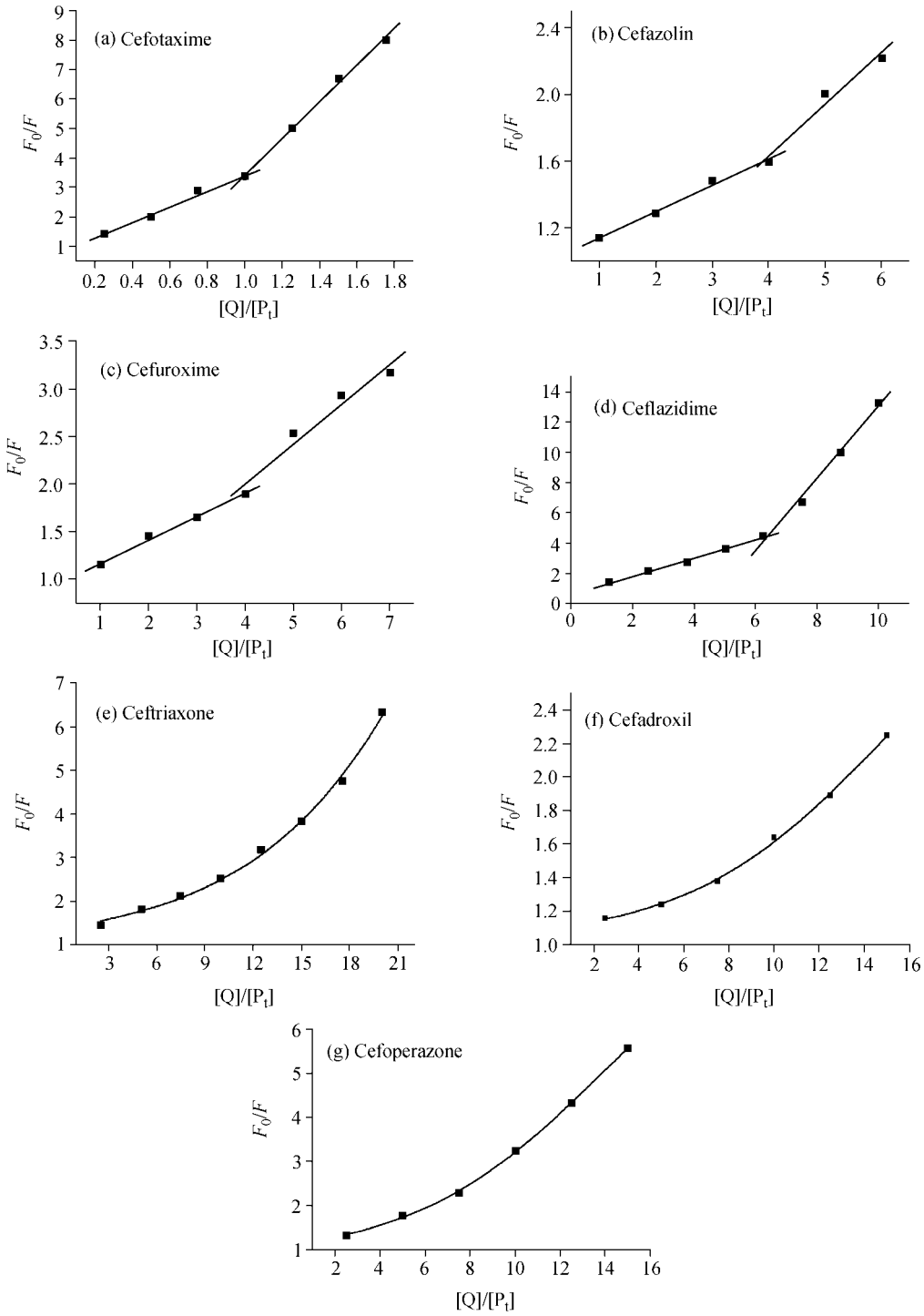

Figure 1 Quenching curves of the 7 kinds of medicine with HSA.

Distance between fluorescence donor-acceptos

According to Förster-type dipole-dipole non-radiation-energy-transferring mechanism[13-15] the transferring efficiency E is related to the donor-acceptor distance r and the distance R_0 of the critical energy transferring.

$$E=\frac{R_0^6}{(R_0^6+r^6)} \tag{5}$$

where R_0 is the critical distance when the transferring efficiency was 50%.

$$R_0^6=8.8\times10^{-25}K^2n^{-4}J\phi \tag{6}$$

where K^2 is the directional factor of dipole space, n the refraction index of the medium, ϕ the fluorescence quantum yield of the donor and J the spectrum overlap

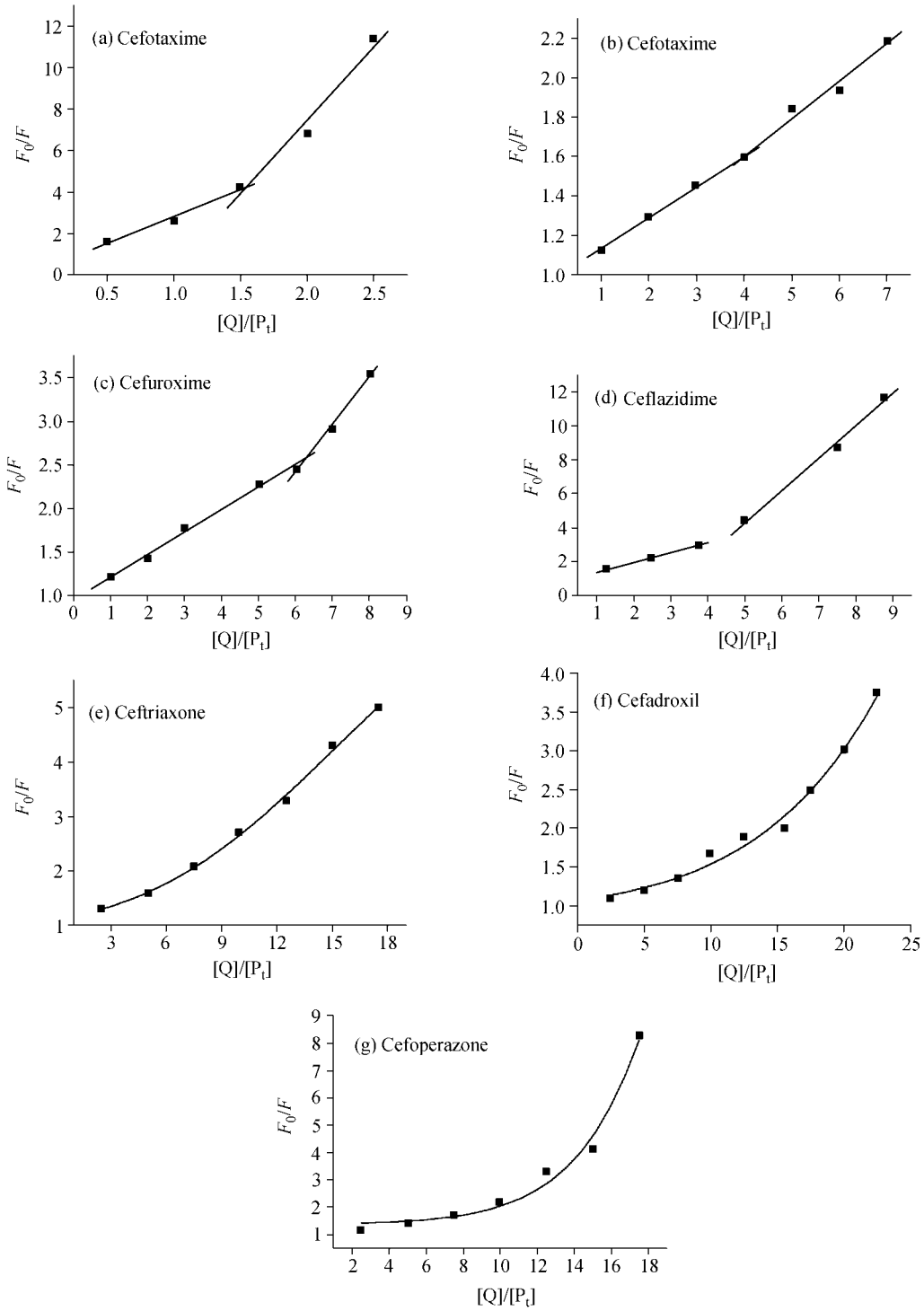

Figure 2 Quenching curves of the 7 kinds of medicine with BSA.

integration between the fluorescence emission spectrum of donor and the absorption spectrum of acceptor, which can be expressed as:

$$J=\frac{\sum F_{\mathrm{D}}(\nu)\varepsilon_{\mathrm{A}}(\nu)\nu^{-4}\Delta\nu}{\sum F_{\mathrm{D}}(\nu)\Delta\nu} \tag{7}$$

where $F_{\mathrm{D}}(\nu)$ is the fluorescence strength of donor at the

wavenumber v, $\varepsilon_A(v)$ is the molar extinction coefficient of acceptor at wavenumber v. Energy-transferring efficiency E can be determined by Eq. (8).

$$E=1-\frac{F}{F_0} \tag{8}$$

Table 1 The dissociation constants K_D of the medicine with HSA (upper) and BSA (lower) , the linear interrelation coefficients C, quenching constants K_q, energy-transfer efficiency E, action distance R_0 and r based on fluorescence quenching Eqs. (4)—(8)

Medicine	K_D/(mol•L^{-1})	K_q(L•mol^{-1}•s^{-1})	C	$J\times10^{14}$	R_0/nm	E/%	r/nm	[M$_t$]/[P$_t$]
Cefotaxime	0.64×10^{-5}	1.56×10^{13}	0.9987	4.57	3.16	70	2.740	1.8
	0.95×10^{-5}	1.05×10^{13}	0.9986	3.07	2.96	31	3.388	2.5
Cefazoli	7.41×10^{-5}	1.35×10^{12}	0.9987	2.10	2.77	13	3.840	6.0
	8.01×10^{-5}	1.25×10^{12}	0.9985	1.80	2.70	11	3.810	10.0
Cefuroxime	7.09×10^{-5}	1.41×10^{12}	0.9987	2.03	2.76	13	3.780	7.0
	5.03×10^{-5}	1.99×10^{12}	0.9969	2.60	2.87	13	2.870	9.0
Ceflazidime	0.32×10^{-5}	3.75×10^{12}	0.9984	2.98	2.94	30	3.390	10.0
	2.67×10^{-5}	3.75×10^{12}	0.9999	2.63	2.88	32	3.260	6.3
Ceftriaxone	7.33×10^{-5}	1.36×10^{12}	0.9984	2.71	2.90	15	3.870	20.0
	9.01×10^{-5}	1.11×10^{12}	0.9967	2.53	2.86	6	4.515	17.5
Cefadroxil	25.6×10^{-5}	3.13×10^{13}	0.9997	2.09	2.77	5	4.510	15.0
	30.4×10^{-5}	3.29×10^{12}	0.9987	2.27	2.81	3	4.930	22.5
Cefoperazone	8.15×10^{-5}	1.23×10^{12}	0.9998	2.68	2.89	8	4.350	20.0
	15.9×10^{-5}	0.63×10^{12}	0.9996	2.38	2.83	9	4.183	17.5

Table 2 The dissociation constants K_D of the medicine with HSA (upper) and BSA (lower), the linear interrelation coefficients C, quenching constants K_q, energy-transferring efficiency E, action distance R_0 and r based on fluorescence enhancement Eqs. (2) and (5)—(8)

Medicine	K_D/(mol•L^{-1})	K_q/(L•mol^{-1}•s^{-1})	C	$J\times10^{14}$	R_0/nm	E/%	r/nm	[M$_t$] /[P$_t$]
Cefotaxime	0.20×10^{-5}	5.0×10^{13}	0.9993	4.57	3.16	70	2.740	1.8
	2.11×10^{-5}	4.74×10^{12}	0.9999	3.07	2.96	31	3.388	25.0
Cefazoli	3.62×10^{-5}	2.76×10^{12}	0.9988	2.10	2.77	13	3.840	6.0
	2.90×10^{-5}	3.45×10^{12}	0.9967	1.80	2.70	11	3.810	10.0
Cefuroxime	2.54×10^{-5}	3.94×10^{12}	0.9991	2.03	2.76	13	3.780	7.0
	1.57×10^{-5}	6.37×10^{12}	0.9975	2.60	2.87	13	2.870	9.0
Ceflazidime	0.66×10^{-5}	1.52×10^{13}	0.9995	2.98	2.94	30	3.390	10.0
	0.39×10^{-5}	2.56×10^{13}	0.9965	2.63	2.88	32	3.260	6.3
Ceftriaxone	3.01×10^{-5}	3.32×10^{12}	0.9993	2.71	2.90	15	3.870	20.0
	3.32×10^{-5}	3.01×10^{12}	0.9991	2.53	2.86	6	4.515	17.5
Cefadroxil	0.69×10^{-5}	1.45×10^{13}	0.9954	2.09	2.77	5	4.510	15.0
	6.30×10^{-5}	1.59×10^{12}	0.9989	2.27	2.81	3	4.930	22.5
Cefoperazone	3.51×10^{-5}	2.85×10^{12}	0.9994	2.68	2.89	8	4.350	20.0
	14.8×10^{-5}	0.68×10^{12}	0.9990	2.38	2.83	9	4.183	17.5

Obviously if E, K^2, ϕ, n and J were known, R_0 and r were obtained.

According to the method in Ref. 1, the overlap integration J of the 7 medicines and the two kinds of albumin, the critical energy-transferring distance R_0, energy-transferring efficiency E and the distance between donor-acceptor can be calculated respectively, which were listed in Table 1 already.

It can be seen that E was changed inversely with the value of [M$_t$]/[P$_t$] from Table 1. For example, energy-

transferring efficiency of cefotaxime sodium with HSA was as high as 70% and the donor-acceptor distance *r* was the least, and $[M_t]/[P_t]$ was 1.8, while the *E* of cefotaxime sodium with BSA was 31%, and $[M_t]/[P_t]$ was 2.5; the *E* of cefoperazone sodium with HSA was 8% and $[M_t]/[P_t]$ was 20, while the *E* with BSA was 9% and $[M_t]/[P_t]$ was 17.5. Namely 1.8 cefotaxime sodium molecules, the *E* of which is 70%, can make 1 molecule of HSA quenched, but 20 molecules of cefoperazone sodium, the *E* of which is 8%, can make only 1 molecule of HSA quenched. This result shows that there was dipole-dipole non radiation-energy transition in the interaction of the medicine with albumin in different degrees.

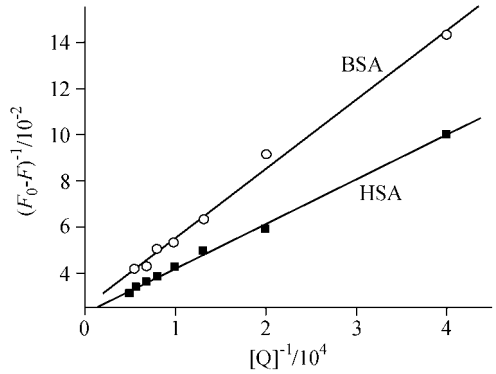

Figure 3 The reciprocal curves of the interaction of ceftriaxone with HSA and BSA based on fluorescence quenching Eq. (4).

From the molar ratio of the effective quenching medicine to albumin ($[M_t]/[P_t]$) in Table 1, it can be seen that the order of the quenching efficiency of the medicine with HSA was cefotaxime > cefazolin > cefuroxime > ceflazidime > cefadroxil > ceftriaxone = cefoperazone, that of BSA was cefotaxime > ceflazidime > cefuroxime > cefazolin > ceftriaxone = cefoperazone > cefadroxil, which was almost the same as that of HSA. From such an order it has been found that the K_q of cefotaxime with the 2 kinds of albumin among the 7 medicines was the biggest while the corresponding $[M_t]/[P_t]$ was the least. But the order of the K_q of the other 6 kinds of medicine shows no regularity. This is because K_q is deduced from Stern-Volmer collision-quenching formula, but the interaction of this kind of medicine with albumin was not caused by dynamic collision, and the K_q should not be considered as the only basis of the quenching efficiency.

Study of the dissociation and quenching constants of the medicine with albumin by fluorescence enhancement theory

With the obtained experiment data of interaction of the medicines with HSA and BSA the double reciprocal curves of the interaction of the ceftriaxone with HSA or BSA were made from enhancement Eq. (2), which were good straight lines as shown in Figures 4 and 5 respectively.

Based on Eq. (2) and the slope of every straight line in Figures 4 and 5, their dissociation constants K_D and linear interrelation coefficients *C* can be calculated, which were listed in Table 2, along with the overlap integration *J*, the critical energy-transferring-distance R_0, energy-transferring efficiency *E*, the distance between donor-acceptor *r* and $[M_t]/[P_t]$ of the 7 kinds of medicine with the two kinds of albumin calculated according to the method in Ref. 11 and 14.

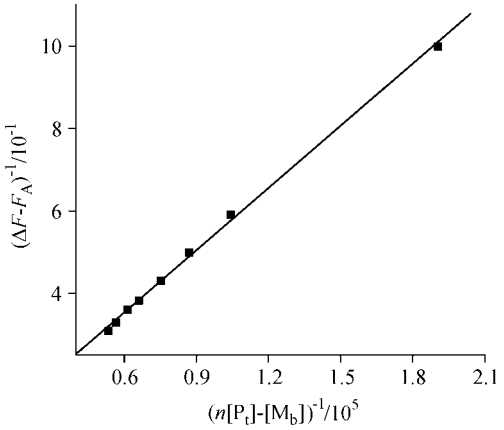

Figure 4 The reciprocal curves of the interaction of the ceftriaxone with HSA based on the fluorescence enhancement Eq. (2).

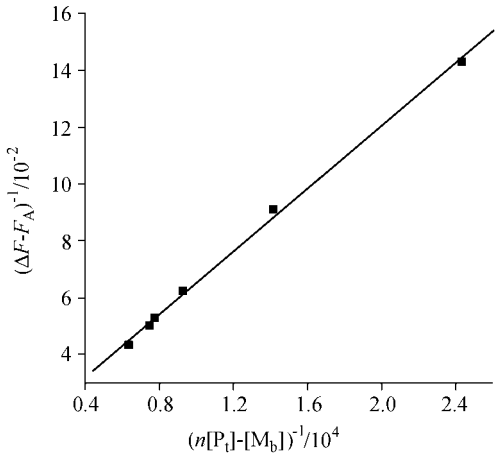

Figure 5 The reciprocal curves of the interaction of the ceftriaxone with BSA based on the fluorescence enhancement Eq. (2).

Discussion and conclusion

Comparing Figure 3 with Figures 4, 5, and data in Tables 1 with 2, it can be seen that the K_D of cefotaxime sodium with HSA was 0.20×10^{-5} mol•L^{-1} based on fluorescence quenching Eq. (4), the K_D was 0.20×10^{-5} mol•L^{-1} based on fluorescence quenching Eq. (2). While the K_D of cefoperazone sodium with BSA was 15.9×10^{-5} mol•L^{-1}, and the K_D was 14.8×10^{-5} mol•L^{-1}. The K_D data of other medicine sample with HSA and BSA based on the two equation were of the same quantity levels. It can be seen that though the apparent forms of the two theories and equations were different,

the calculation data of K_D were almost the same. This result reflects the equal-effect, reasonability and reliability of the two equations from another aspect. From the K_D values calculated, the interactions of cephalosporin class medicine with albumin were all strong. This shows that they can not only be saved and transferred, but also have obvious effect on the conformation and molecular-energy-level of the albumin. Such fact may be one of the possible reasons that the cephalosporin class medicines have obvious antibacterial activity.

The study of the structure of HSA shows that its molecule has at least 3 structural domains.[16] Each structural domain has 2 substructures which form a tube-shaped structure by the mouth of groove in head. Almost all hydrophilic amino acid residues were gathered at the inside of the tube, to form a hydrophilic cavity.[17] In this paper, almost every kind of the cephalosporin class medicine has hydrophilic five membered ring side chain besides a β-lactam ring. To lower the energy of the system, such hydrophilic five membered ring side chain possibly enters the hydrophilic cavity of albumin. The fluorescence chromophores of albumin are Tyr, Phe and Trp, with the fluorescence of Trp 214 to be the strongest. Consequently the distance determined between donor-acceptor is one between the medicine combined in the hydrophilic cavity and the Trp 214.

It is needed to point out that binding substances have been supposed to be formed between donor and acceptor when we deduced the fundamental formula[18] from fluorescence enhancement and quenching theories and belong to static quenching or enhancement at low medicine concentration, but this is not to say that there were no Förster-type dipole-dipole non radiation energy transfer effect. Because the fluorescence spectra were overlapped partially with the absorption spectra when the molar ratio of albumin to medicine were 1∶1 no matter whether for the HSA or BSA. The highest energy transfer efficiencies of HSA and BSA with cefotaxime were 70% and 31% respectiuely. Most of those efficiencies were from 10% to 15%. The efficiencies of cefadroxil with HSA and BSA were the least, which were 5% and 3% respectively. Conclusively there were energy transfer effect in the quenching action of medicine with albumin in different degrees.

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