

Discussion on reaction mechanism of enoxacine-terbium complex

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Abstract. In the buffer solution of pH=5.80, bilirubin can remarkably reduce the fluorescence intensity of the enoxacine - terbium (Tb^{3+}) complex at $\lambda=545nm$ and the reduced fluorescence intensity of Tb^{3+} ion is in proportion to the concentration of bilirubin. The influential factors were investigated. The reduction mechanism of the fluorescence intensity in the enoxacine - Tb^{3+} and the enoxacine - Tb^{3+} -bilirubin complex have been discussed. In this experiment, the results showed the binding force between enoxacine and bilirubin was stronger than that of enoxacine and Tb^{3+} , bilirubin can join with enoxacine more easily than Tb^{3+} . Therefore, the enoxacine joined with Tb^{3+} was fewer, the fluorescence intensity of Tb^{3+} at 545nm reduced.

1 Introduction

Bilirubin (BR) is the end product of heme metabolites in humans [1]. BR level in newborns is typically 10 times higher than that of adults. A low bilirubin level may indicate an iron deficiency [3]. So the BR analysis is believed to have considerable medicinal properties. Now the most commonly used methods for bilirubin analysis include capillary electrophoresis[4], diazo reaction [3], high-performance liquid chromatography[4], fiber optic sensor[5], fluoroimmunoassay[6], flow injection[7], spectrophotometer[8-11], But there is no report about spectrofluorimetric method for determination of BR using ENX- Tb^{3+} as a fluorescent probe and the mechanism of the fluorescence intensity in the enoxacine - Tb^{3+} and the enoxacine - Tb^{3+} -bilirubin complex. Enoxacine (ENX) is one kind of bacteriophages containing α -carbonyl carboxylic acid configuration. It is the ideal ligand for Tb^{3+} . In this work, we chose ENX as the ligand of Tb^{3+} and investigated the possibility of the reduction of the Tb^{3+} fluorescence sensitized by it and using BR as co-ligand. Experimental results show that the characteristic peak of Tb^{3+} at 545 nm can be greatly reduced and the reduced fluorescence intensity is proportional to the concentration of BR. Under the optimum condition, the mechanism of fluorescence reduction between Tb^{3+} -ENX complexes and BR was studied.

2 Experimental

2.1 Reagents

All chemicals used were of analytical-reagent or higher grade. Doubly distilled demineralized water was used for the preparation of all solutions and for all determinations.

A stock bilirubin (BR, Weihui Biochemical Co., Shanghai) was dissolved with a small amount of 0.1 mol L⁻¹ NaOH, then diluting to mark with doubly distilled demineralized water and working standard solution (1.0×10⁻⁴ mol L⁻¹) was freshly prepared by appropriate dilution with doubly distilled demineralized water. A Tb^{3+} ion stock solution was prepared by dissolving $TbCl_3 \cdot 4H_2O$ (more than 99.99%, Shanghai Yuelong Chemical Plant, China) with a small amount of hydrochloric acid, then diluting to mark with hydrochloric acid (0.1 mol/L). The working solution (5.0×10⁻⁵ mol/L) was obtained by appropriate dilution of the stock solution with doubly distilled demineralized water. Stock enoxacine (ENX, Biological Product Institution of Chinese Medicine) solution was directly dissolved in doubly distilled demineralized water. The working standard solution (8.8×10⁻⁴ mol/L) was freshly prepared by appropriate dilution with doubly distilled demineralized water. 0.10 mol L⁻¹ NH₄AC-HAC (pH=5.80) buffer solution.

2.2 Apparatus

All fluorescence measurements were carried out on a RF-540 recording spectrofluorimeter (Shimadzu, Kyoto, Japan). A UV-265 recording spectrophotometer (Shimadzu, Kyoto, Japan) was used for UV spectra scanning and the determination experiments. All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device works,China).

2.3 Experiment procedure

According to the experiment results, the optimum condition is that: to 10ml color comparison tubes, solutions were added in the following order: 1.5 mL pH=5.80 0.10 mol L⁻¹ NH₄AC-HAC buffer solution, 1.5 mL 8.8×10⁻⁶ mol L⁻¹ ENX, 1.5mL 5.0×10⁻⁴mol/L Tb³⁺ and 0.5mL 1.0×10⁻⁴ mol L⁻¹ BR. The mixture was diluted to 10ml with deionized distilled water and stand for 15min at room temperature. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em} = 335\text{nm}/545\text{nm}$. The decreased fluorescence intensity of ENX-Tb³⁺ by BR was represented as $\Delta F = F_0 - F$. Here F and F₀ are the intensities of the systems with and without BR, respectively.

3 Results and discussion

3.1 Apparatus

Fluorescence Spectra. Fluorescence spectra of ENX, Tb³⁺, BR, ENX-Tb³⁺, ENX-BR, Tb³⁺-BR and ENX-Tb³⁺-BR are shown in the Fig.1. It is known that it is hardly to excite the characteristic peak of Tb³⁺, and the peak is also unclear in the system of BR- Tb³⁺. But at the same condition, characteristic peak of ENX-Tb³⁺ is observed and the two emission peaks are at 490nm and 545nm, which correspond to the 5D₀-7F₁ and 5D₀-7F₂ transitions of Tb³⁺ respectively. The fluorescence spectra of ENX- Tb³⁺-BR system is similar to that of ENX- Tb³⁺, but the fluorescence intensity of ENX- Tb³⁺ is decreased by BR, and the decreased fluorescence intensity is proportional to the concentration of BR.

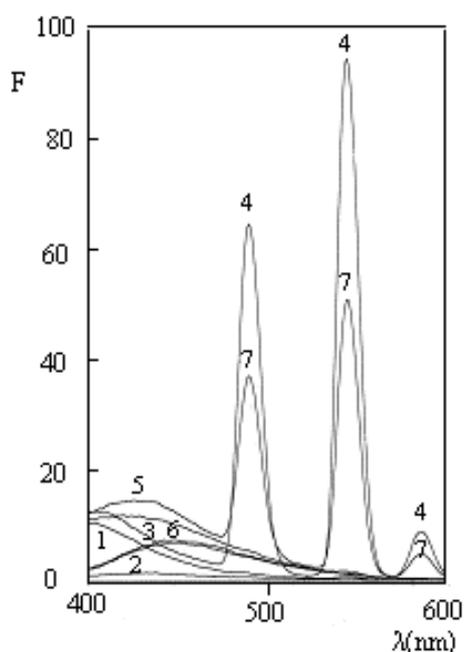


Fig.1 Fluorescence spectra

3.2 Effect of the addition order of reagents.

Adding various reagents in different order had an influence on the F, F₀ and ΔF . The experimental results are shown in Table 1. Considering both the enhancement of fluorescence intensity and the stability of BR, No.1 was optimum.

3.3 Influence of Coexisting Substance.

Series experiments of coexist substance had been carried out on the fluorescence of ENX-Tb³⁺-BR complex under the optimum condition. Including metal ions, bases and proteins were also examined for interference. Results are summarized in Table 2. Most of them do not interfere when BR is 2.0×10⁻⁵ mol L⁻¹.

Table 1. Impact of the order of addition of reagents on the fluorescence intensity

NO	Addition order of reagent	F ₀	F	ΔF
1	buffer, ENX, Tb ³⁺ , BR	59.1	33.8	25.3
2	ENX, Tb ³⁺ , BR, buffer	58.7	34.6	24.1
3	ENX, BR, Tb ³⁺ , buffer	58.7	33.6	25.1
4	BR, buffer, ENX, Tb ³⁺	59.1	33.1	26.0
5	buffer, BR, Tb ³⁺ , ENX	61.3	35.6	25.7
6	BR, Tb ³⁺ , ENX, buffer	58.6	34.6	24.0
7	Tb ³⁺ , buffer, BR, ENX	58.3	33.7	24.6
8	BR, ENX, buffer, Tb ³⁺	60.3	35.8	24.5

Table 2. Influence of coexisting substance on ENX-Tb³⁺-BR complex

Coexisting substance	concentration (mol L ⁻¹)	ΔF%
Co ²⁺	5.0×10 ⁻⁵	-8.20
Cu ²⁺	2.5×10 ⁻⁷	-1.16
Mg ²⁺	5.0×10 ⁻⁴	-0.61
Fe ³⁺	1.8×10 ⁻⁷	2.12
Hg ²⁺	2.5×10 ⁻⁸	4.76
Cd ²⁺	1.8×10 ⁻⁶	-8.30
Al ³⁺	1.9×10 ⁻⁶	-0.70
Ca ²⁺	1.0×10 ⁻⁴	-7.36
Zn ²⁺	2.4×10 ⁻⁴	-5.37
Mn ²⁺	2.5×10 ⁻⁴	-8.3
Cr ³⁺	3.8×10 ⁻⁶	-1.16
Mo ⁶⁺	1.0×10 ⁻⁶	-7.90
Tb ³⁺	2.0×10 ⁻⁶	-2.58

K ⁺	2.1×10 ⁻⁴	-0.58
Na ⁺	2.2×10 ⁻⁴	2.12
HSA	9.3×10 ⁻⁶	-8.47
C ₂ O ₄ ²⁻	1.3×10 ⁻⁶	2.39
Sr ²⁺	1.0×10 ⁻⁷	-0.79
thymine	1.8×10 ⁻⁶	-1.48
cytosine	1.1×10 ⁻⁴	-0.55
guanine	1.6×10 ⁻⁷	-0.59
adenine	1.0×10 ⁻⁸	0.93
acetocaustin	2.0×10 ⁻⁴	0.39
cane sugar	1.0×10 ⁻³	-1.78
histidine	9.8×10 ⁻⁵	-7.59
lysine	2.0×10 ⁻⁴	-9.00
methionine	1.0×10 ⁻⁴	-4.44
glutamic	2.0×10 ⁻⁴	1.66
cystine	4.0×10 ⁻⁵	4.24
tyrosine	1.0×10 ⁻⁵	1.11
tryptophane	5.0×10 ⁻⁴	3.56
glycine	1.0×10 ⁻⁴	1.30
glucose	1.0×10 ⁻³	-1.29

3.4 Reaction mechanism

Enoxacine is one kind of bacteriophages containing α -carbonyl carboxylic acid configuration. Literature survey showed that α -carbonyl ligands were suitable for efficient energy transfer from ligands to Tb³⁺ and for high fluorescence quantum yield; large stokes shift; narrow emission bands; a large fluorescence lifetime and hence avoid potential background fluorescent emission interferences from the biological background. Therefore, ENX was ideal ligand for Tb³⁺ and it can possibly sensitize the fluorescence intensity of Tb³⁺ via intramolecular energy transfer [10].

In this experiment, the results showed that the characteristic peak of Tb³⁺ at 545 nm can be greatly reduced by BR and the reduced fluorescence intensity is proportional to the concentration of BR. It was assumed that the binding force between ENX and BR was stronger than that of ENX and Tb³⁺, BR can join with ENX more easily than Tb³⁺. Therefore, the ENX joined with Tb³⁺ was fewer, the fluorescence intensity of Tb³⁺ at 545nm reduced.

Next we proved the theory with formation constant. BR can form a complex with ENX, the procedure was dynamic quenching.

Quenching equation [11]:

$$1/(F_0-F) = 1/F_0 + K_D/(F_0 [Q])$$

In the equation, [Q] is the concentration of BR. The plot of $1/(F_0-F)$ vs $1/[Q]$ was showed in figure 2. Figure 2 shows that curves have fine linear relationships. This indicates the static quenching interaction between ENX and BR. The formation constant KA can be obtained from the Fig.2 ($KA=1/KD$). When $t=25.0^\circ\text{C}$, regression equation is $Y=-0.01153+3.98423\times 10^{-7}$, $KA=3.0240\times 10^4$, $r=0.99823$.

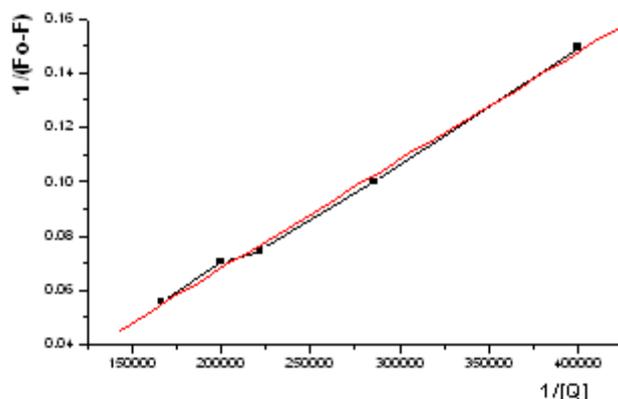


Figure 2

We got the formation constant of ENX and Tb^{3+} with the same method. It was showed in figure 3. When $t=25.0^\circ\text{C}$, regression equation is $Y=-0.00663+1.03927\times 10^{-6}$, $KA=1.1085\times 10^4$, $r=0.99745$.

In conclusion, the formation constant of ENX and BR was bigger than that of ENX and Tb^{3+} , which indicated that the binding force between ENX and BR was stronger than that of ENX and Tb^{3+} , BR can join with ENX more easily than Tb^{3+} . The ENX joined with Tb^{3+} was fewer, so the fluorescence intensity of Tb^{3+} at 545nm reduced. It was ideal proof for the theory above.

Otherwise, according to Förster dipole-dipole nonradiative energy transfer theory [10], there will be nonradiative energy transfer phenomenon when two compound molecules satisfy the conditions below: (1) the energy donor(d) can emits 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 7nm.

The overlap of the fluorescence emission spectra of ENX (a) with the absorption spectra (b) of BR is shown in figure 4, which indicated there was energy transfer between ENX and BR. Figure 4 provided ideal proof for the theory above.

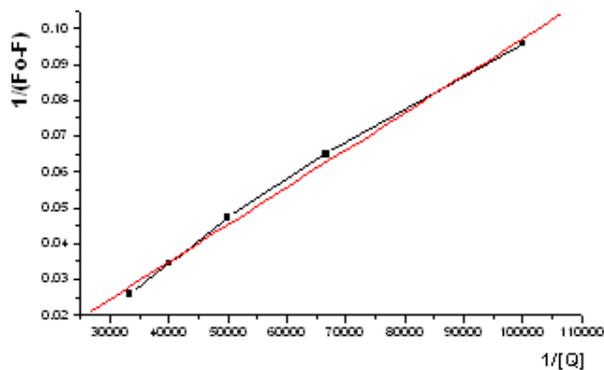


Figure 3

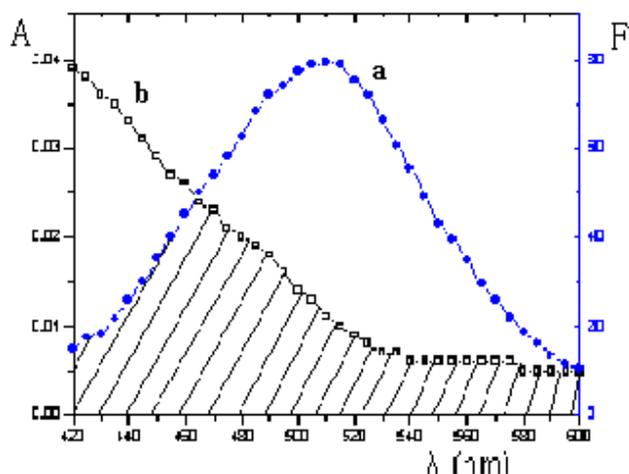


Figure 4. The overlap of the fluorescence emission spectra of ENX(a) with the absorption spectra (b) of BR.

4 Acknowledgements

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