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Short communication

Enzyme-amplified lanthanide luminescence based on complexation reaction—a new technique for the determination of doxycycline

Chong Qiu Jiang*, Na Zhang

Department of Chemistry, Shandong Normal University, Jinan 250014, China

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Abstract

A new spectrofluorimetric method is described for the determination of doxycycline, based on modified enzyme-amplified lanthanide luminescence. Under the optimum conditions, Eu^{3+}–doxycycline forms a ternary complex with lysozyme in close proximity and lysozyme can remarkably enhance the characteristic fluorescence intensity of Eu^{3+} at 612 nm in doxycycline–Eu^{3+} binary complex. The enhanced fluorescence intensity is in proportion to the concentration of doxycycline. The limit of detection is 1.28×10^{-8} mol l^{-1}, with a linear range from 1.7×10^{-7} to 1.7×10^{-6} mol l^{-1}. Interferences of other coexisting substances were studied. The developed method was successfully applied to the determination of doxycycline in serum, urine and real samples. The mechanism of fluorescence enhancement was also studied.

Keywords: Doxycycline; Europium ion; Lysozyme; Fluorimetry

1. Introduction

Recently, enzyme-amplified lanthanide luminescence (EALL) with high sensitivity and selectivity has been widely used for the determination of horseradish peroxidase (HRP) [1,2], hydrogen peroxide [3], tuberculosis antibody [2], etc. HRP, which can catalyze oxidation–reduction reaction, is of higher importance to the field. However, such unfavorable factors of enzyme as expensive price, free inactivation, and rigorous experimental conditions limit the further application of enzyme catalytic reaction. Furthermore, the determination should be immediately carried out in order to avoid the oxidation of air.

Numerous methods for measuring doxycycline (DC) have been reported, such as HPTLC [4], LC [5], thin-layer chromatography [6], capillary electrophoresis [7], continuous-flow chemiluminometric [8], flow-injection [9], spectrophotometer [10], HPLC [11–14], time-resolved [15]. Up to date, there has been no report for the determination of lysozyme by EALL methods.

Doxycycline is one of the kinds of antibiotics of the tetracycline family containing the β-diketonate configuration. Its curative effect is superior to that of tetracycline. So the determination of doxycycline in hu-

* Corresponding author. Tel.: +86-531-6614486; fax: +86-531-2615258.
E-mail address: jiangchongqiu@sdnu.edu.cn (C.Q. Jiang).

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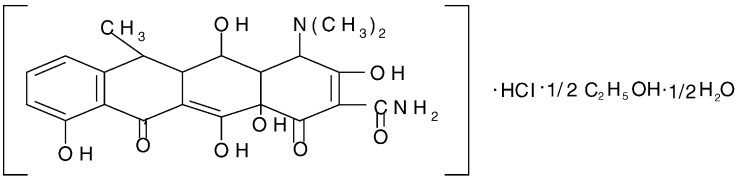
man body fluids is very important. A literature survey shows that β-diketonate ligands are suitable for efficient energy transfer from ligands to Eu^{3+} ion, and hence, avoid potential background fluorescent emission interferences from the biological matrix. In this work, we selected DC as a ligand of Eu^{3+} and investigated the possibility of the enhancement of the Eu^{3+} fluorescence sensitized by DC and using LZ as co-ligand.



Structure of doxycycline (DC)

A wide variety of Eu^{3+}-protein, nucleic acid, and nucleotide complexes have been studied [16]. Protein is composed of amino acids, while lysozyme (LZ) is also a kind of biomacromolecules that is formed by 129-tactic amino residue [17]. In this paper, we selected DC as a ligand of Eu^{3+} ion, and LZ, which replaced HRP as reinforcing agent, as a co-ligand. Under the optimum experimental conditions, the enhanced fluorescence intensity of the system is in proportion to the concentration of DC after addition of LZ. Thus, a new technique based on modified EALL is established for the determination of DC. Comparing to EALL, LZ coordinates to Eu^{3+} directly. This makes the stability of the ternary complex system developed. This method is very easy to perform. What is more, the oxidations of air to the results and the potential background fluorescent emission interferences from the biological matrix are avoided.

The mechanism of the fluorescence enhancement was also studied.

2. Experimental

2.1. Apparatus

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

2.2. Reagents

All used chemicals were of analytical or higher grades. Doubly distilled deionized water was used for the preparation of all solutions.

A stock lysozyme (Biological Product Institution of Chinese Medicine) solution was directly diluted in water. The working standard solution (1.0×10^{-5} mol l^{-1}) was freshly prepared by appropriate dilution with water.

A stock doxycycline (Biological Product Institution of Chinese Medicine) solution was directly dissolved in water. The working standard solution (1.69×10^{-5} mol l^{-1}) was freshly prepared by appropriate dilution with water.

A Eu^{3+} ion stock solution was prepared by dissolving Eu_2O_3 (Shanghai Yuelong Chemical Plant, Shanghai, China) with a small amount of hydrochloric acid, then diluting to mark with hydrochloric acid (0.1 mol l^{-1}). The working solution (1.6×10^{-5} mol l^{-1}) was obtained by appropriate dilution with water.

All stocking solutions and working solutions were stored at 0–4 °C.

An ammonia–ammonium chloride buffer solution (0.10 mol l^{-1}, pH = 9.8) was used.

2.3. Procedure

To 10 ml color comparison tubes, solutions were added in the following order: 1.0 ml 1.69×10^{-5} mol l^{-1} DC solution, 2.0 ml buffer solution, 1.0 ml 1.0×10^{-5} mol l^{-1} LZ solution, 1.0 ml 1.6×10^{-5} mol l^{-1} Eu^{3+} ion solution. The mixture was diluted to the mark with doubly distilled wa-

ter and stood for 70 min at room temperature. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 385/612 nm. The enhanced fluorescence intensity of LZ–Eu^{3+} by DC was represented as $\Delta F = F - F_0$. Here F and F_0 are the fluorescence intensities of the systems with and without DC, respectively. The standard curve method was used in the quantitation of DC in samples of human urine.

3. Results and discussion

3.1. Fluorescence spectra and absorption spectra

The overlap of the absorption spectra of DC and the fluorescence spectra of LZ is shown in Fig. 1.

According to Föster's non-radiative energy transfer theory, the rate of energy transfer depends upon the extent of overlap (J) of the emission spectra of the donor (LZ) with the absorption spectra of the acceptor (DC), the relative orientation of the donor and acceptor transition dipoles, and the distance (r) between these molecules.

The overlap integral J can be evaluated by integrating the spectra in Fig. 1. Under these experimental conditions, we found $J = 1.41 \times 10^{-14}\,cm^3\,l\,mol^{-1}$, the energy transfer efficiency $E = 0.091$, critical distance $R_0 = 2.64$ nm, and the distance (r) between DC and LZ is 3.87 nm. As the distance r is less than 7 nm, this also supports the conclusion of non-radioactive energy transfer.

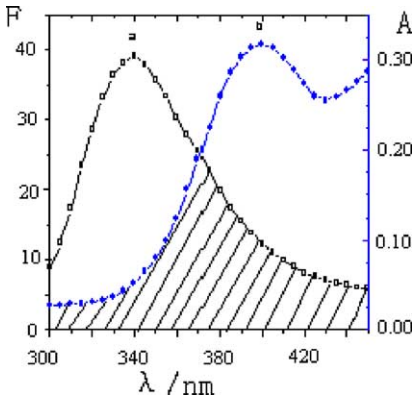


Fig. 1. Fluorescence spectra of LZ (a) and absorption spectra of DC (b).

The fluorescence spectra and absorption spectra of DC–Eu^{3+}–LZ, DC–Eu^{3+}, DC–LZ, DC, Eu^{3+}–LZ, and Eu^{3+} are shown in Fig. 2(a) and (b).

In Fig. 2(a), the characteristic peak Eu^{3+} ion at 612 nm can be enhanced remarkably after the addition of LZ and DC. This is the confirmation that ternary complex is formed. In Fig. 2(b), the absorbance and the wavelength of maximum absorption peak of DC–LZ–Eu^{3+} ternary system differ from unitary and binary system. All of these verify the formation of ternary complex [18].

3.2. Effect of experiment conditions

3.2.1. Effects of pH

The pH of the medium had great effects on both the formation of DC–Eu^{3+} complex and the capability of LZ binding the DC–Eu^{3+} complex. The experimental results showed that ΔF reaches maximum and remains constant over the pH range 9.4–9.8. Therefore, a pH of 9.8 was fixed with the use of 0.1 mol l^{-1} ammonia–ammonium chloride buffer solution. As the volume of buffer solution added from 1.5 to 2.5 ml ΔF reaches maximum and remains constant. So 2.0 ml was selected in the following experiments.

3.2.2. Effect of reaction time

Completing the chelation reaction of DC–Eu^{3+}–LZ system at room temperature needs 70 min at least. The fluorescence intensity then remains constant for at least 2 h. Therefore, all chelation reactions were carried out for 70 min and all measurements were made within 2 h.

3.2.3. Effect of temperature

Temperature had a great effect on the fluorescence intensity of the system. The fluorescence intensity decreases sharply along with the temperature increases. Therefore, we selected room temperature throughout all further work.

3.2.4. Effect of the addition order of reagents

The experimental results showed that adding various reagents in different order had influence on F and ΔF. Considering the enhancement of fluorescence intensity of the system, we chose the following order for further study: DC, buffer, LZ, and Eu^{3+}.

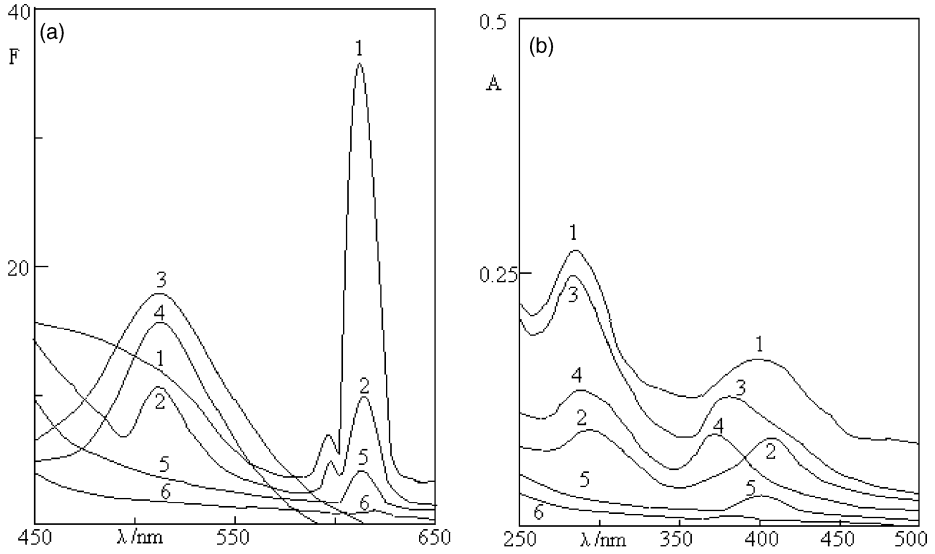


Fig. 2. (a) Fluorescence spectra, (b) absorption spectra. (1) DC, Eu^{3+}, LZ, (2) DC, Eu^{3+}, (3) DC, LZ, (4) DC, (5) Eu^{3+}, LZ, (6) Eu^{3+}. Experimental conditions: DC: 1.69×10^{-6} mol l^{-1}, Eu^{3+}: 1.6×10^{-6} mol l^{-1}, LZ: 2×10^{-6} mol l^{-1}, pH = 9.8, λ_{ex} = 385 nm (for the system of DC and DC–LZ, λ_{ex} = 375 nm).

3.2.5. *Effect of LZ concentration*

The influence of the amount of LZ on the fluorescence intensity of the solutions containing DC (1.69×10^{-6} mol l^{-1}) and Eu^{3+} (1.6×10^{-6} mol l^{-1}) was studied under the conditions established above. The enhanced fluorescent intensity (ΔF) increases with the increasing amount of LZ up to 0.8 ml (i.e. 2.0×10^{-6} mol l^{-1}) and then remains constant. When the concentration of LZ is 2.0×10^{-6} mol l^{-1}, the composition ratio for the LZ to Eu^{3+} in the DC–Eu^{3+}–LZ system is 1.25:1. Thus, 2.0×10^{-6} mol l^{-1} of LZ was selected for further study.

3.3. *Influence of coexisting substances*

Under the optimum conditions, a systematic study of various coexisting substances in the determination of DC (7.0×10^{-7} mol l^{-1}) was carried out. The criterion for interference is fixed at a ±10% variation of the average fluorescence intensity calculated for the established level of DC, the experimental results are shown in Table 1. From Table 1 it can be seen that most of the coexisting substances, which are in correspondence with the concentration in human body fluids, are found to show no influence. However, HSA interferes with determination under the experimental conditions and the interferences can be effectively removed by the dilution process and blank deduction (using the standard calibration method). The addition of trichloroacetic acid can also remove the interferences.

Table 1
Effect of coexisting substances in the determination of DC (7.0×10^{-7} mol l^{-1}); tolerance error ±10%

Coexisting substances	C (mol l^{-1})	ΔF (%)
Co^{2+} (Cl^-)	1.70×10^{-7}	−6.2
Cu^{2+} (SO_4^{2-})	2.03×10^{-7}	−9.81
Mg^{2+} (SO_4^{2-})	4.11×10^{-6}	−7.13
Fe^{3+} (Cl^-)	1.79×10^{-7}	−0.97
Mn^{2+} (SO_4^{2-})	1.90×10^{-6}	1.03
Cr^{3+} (Cl^-)	1.47×10^{-7}	−3.57
Zn^{2+} (SO_4^{2-})	5.50×10^{-5}	5.3
Ca^{2+} (Cl^-)	4.19×10^{-6}	8.69
Guanine	1.6×10^{-7}	6.92
Cytosine	1.0×10^{-7}	−3.42
Thymine	1.0×10^{-7}	−2.38
Adenine	1.04×10^{-5}	−5.1

Table 2
Characteristics of different methods for determination of DC

Method	Linear range (mol l^{-1})	Detection limit (mol l^{-1})	Recovery	Ref.
Flow injection	3.0×10^{-6} to 8.0×10^{-5}	2.0×10^{-6}	95–97	[9]
HPTLC	–	7.09×10^{-3} μg	91.6–100.3	[4]
HPLC	3.90×10^{-8} to 9.76×10^{-6}	1.56×10^{-8}	68.3–78.7	[12]
Chemiluminometril	1.95×10^{-6} to 1.95×10^{-5}	9.76×10^{-7}	100.2–102.4	[8]
EALL	1.7×10^{-7} to 1.7×10^{-6}	1.28×10^{-8}	92.5–96.9	This work

Table 3
Determination of DC in urine, serum, and real samples ($n = 5$)

Sample	Added (mmol)	Found (mmol)	Recovery (%)	R.S.D. (%)
Serum (1 + 500)	0.00	0.00		
	8.45×10^{-6}	7.82×10^{-6}	92.49	0.31
Urine (1 + 1000)	0.00	0.00		
	8.45×10^{-6}	8.19×10^{-6}	96.92	0.27
Urine 1 (1 + 5)[b]		2.07, 2.01, 2.01[a]		1.71
Urine 2 (1 + 5)[c]		17.62, 17.50, 17.74[a]		0.68

[a] mg l^{-1}.
[b] Collect urine sample after 2 h.
[c] Collect urine sample after 4 h.

3.4. Analytical application

3.4.1. Analytical characteristic

Under the experimental conditions, there is a linear relationship between enhanced fluorescence intensity and DC concentration in the range of 1.7×10^{-7} to 1.7×10^{-6} mol l^{-1} with a correlation coefficient (r) of 0.997.

The limit of detection, as defined by IUPAC [19], is determined to be 1.28×10^{-8} mol l^{-1} when the K value is taken as 3, and the standard deviation is 0.09 obtained from a series of 11 reagent blanks.

In Table 2 the characteristics of the method are compared with others for DC determination reported previously. From Table 2, we can obtain that the linear range and detection limit of the proposed method are better than the other methods and the method can be easily carried out.

3.4.2. Determination of DC in samples of serum and urine

The developed method was applied to the determination of DC in samples of serum and urine. For the assay of DC in urine and serum, the fresh samples must be diluted appropriately to be within the linear range of determination of DC (1 + 1000 and 1 + 500, respectively). A portion (2.0 ml) of this sample solution was analyzed by the method developed above, using the standard calibration method. Thus, interferences coming from some organic compounds and albumin in samples were removed by the dilution process and blank deduction. Collect human urine samples (after 2 and 4 h) after an oral administration of 100 mg of DC, add trichloroacetic acid (0.5 g) to remove protein in urine samples [20], and then dilute five-fold (v/v) to make the sample concentrations of DC within the linear range of determination. The concentrations of DC in the urine samples were also determined by the proposed method, respectively. The results obtained are shown in Table 3.

From Table 3 it can be seen that the developed method can be easily performed and affords good precision and accuracy when applied to real samples.

4. Conclusions

Both DC containing β-diketonate configuration and biomacromelecule LZ are ideal ligands for Eu^{3+} ion and it is possible to sensitize the fluorescence intensity

of Eu^{3+} ion via intramolecular energy transfer. DC can form a ground state complex with LZ depending upon non-covalent bond as well as the interaction of ligand to Eu^{3+} ion. The coordination number of Eu^{3+} in its complex is 6–8. The molar ratio of LZ to Eu^{3+} ions in this study is 1.25:1 and the coordination number of Eu^{3+} is not saturation. Therefore, Eu^{3+} will coordinate with H_2O. A very stable ternary complex in close proximity with big degree of molecular conjugation and rigid structure can be formed by the interaction between DC–LZ ground state complex and Eu^{3+} ion after the addition of DC. In ternary complex as a result of effect of packing and cooperation, the energy transfer from DC–LZ complex to Eu^{3+} ion can occur more easily and the non-radiative energy loss through O–H vibration of H_2O in the original Eu^{3+} complex decreases greatly. So the fluorescence intensity of Eu^{3+} ion at 612 nm can be enhanced several times.

The developed method was successfully applied to the determination of DC in serum, urine and real samples. And the applied range of EALL was enlarged.

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