Determination of ATP as a Fluorescence Probe with Europium(III)-Doxycycline

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A new spectrofluorimetric method has been developed for the determination of adenosine disodium triphosphate (ATP). We studied the interactions between the doxycycline (DC)-Eu³⁺ complex and adenosine disodium triphosphate (ATP) by using UV-visible absorption and fluorescence spectra. Using doxycycline (DC)-Eu³⁺ as a fluorescence probe, under the optimum conditions, ATP could remarkably enhance the fluorescence intensity of the DC-Eu³⁺ complex at $\lambda = 612$ nm. The enhanced fluorescence intensity of the Eu³⁺ ion was in proportion to the concentration of ATP. The optimum conditions for the determination of ATP were also investigated. The linear ranges for ATP were 1.00×10^{-7} – 2.00×10^{-6} mol L⁻¹ with detection limits of 4.07×10^{-8} mol L⁻¹. This method is simple, practical and relatively free of interference from coexisting substances, and can be successfully applied to the determination of ATP in samples. The mechanism of fluorescence enhancement between the doxycycline (DC)-Eu³⁺ complex and ATP was also studied.

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Introduction

Adenosine disodium triphosphate (ATP) is the mediator of energy exchanges that occur in all living cells, both catabolic, degradative processes and anabolic, biosynthesis processes. Because of its ubiquitous presence in living matter, ATP has been widely used as an index for biomass determinations in clinical microbiology, food quality control and environmental analyses. The most commonly used methods for the determination of ATP include the bioluminescent method,^{1,2} chromatography,³ biosensor using isolated enzyme,⁴⁻⁶ luminescence,^{7,8} HPLC,^{9,10} and enzymatic cycling method.¹¹

In the present work we studied the interactions between the doxycycline (DC)-Eu³⁺ complex and adenosine disodium triphosphate (ATP) by using the UV-visible absorption and fluorescence spectra. The doxycycline is one kind of better antibiotic pharmaceuticals; its antibacterial spectrum is the same as that of tetracycline, but its curative effect is superior to that of chlortetracycline and tetracycline. Doxycycline (DC) containing β -diketonate configuration is a good ligand for Eu³⁺, and can strongly bind with Eu³⁺. In this work, we selected DC as a ligand of the Eu³⁺ ion, and investigated the possibility of enhancing the Eu³⁺ ion sensitized fluorescence by ATP. The experimental results indicate that the characteristic peak of the Eu³⁺ ion at 612 nm can be greatly enhanced in the DC-Eu³⁺ system by ATP, and that the enhancement comes from the intramolecular energy transfer in the DC-ATP-Eu³⁺ ion ternary complex in close proximity. Accordingly, a new fluorescence method with high sensitivity and selectivity has been established for the determination of ATP.

Experimental

Apparatus

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

Solutions

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.

An adenosine disodium triphosphate (ATP, Shanghai Bio Life Science & Technology Co., Ltd.) solution was directly dissolved with doubly distilled demineralized water. The working standard solution was freshly prepared by appropriate dilution with doubly distilled demineralized water.

A stock doxycycline (DC, Biological Product Institution of Chinese Medicine) solution was dissolved with a small amount of hydrochloric acid, and then diluted to the mark with doubly distilled demineralized water. The working standard solution was freshly prepared by appropriate dilution with doubly distilled demineralized water.

A Eu³⁺ ion stock solution was prepared by dissolving Eu₂O₃ (more than 99.99%, Shanghai Yuelong Chemical Plant, China) with a small amount of hydrochloric acid, and then diluted to the mark with hydrochloric acid (0.1 mol L⁻¹). The working solution was obtained by appropriate dilution of the stock solution with doubly distilled demineralized water.

NH₃-NH₄Cl (0.10 mol L⁻¹, pH = 10.00) buffer solution was used. All stocks and working solutions were stored at 0 – 4°C.

Experiment procedure

To 10 ml color comparison tubes, solutions were added in the

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Fig. 1 (a) Fluorescence excitation spectra. (b) Fluorescence emission spectra. 1, Eu^{3+} ; 2, DC; 3, ATP; 4, DC- Eu^{3+} ; 5, DC-ATP; 6, Eu^{3+} -ATP; 7, DC- Eu^{3+} -ATP. Experiment conditions: DC, $3.58 \times 10^{-6} \text{ mol L}^{-1}$; Eu^{3+} , $6.0 \times 10^{-6} \text{ mol L}^{-1}$; ATP, $2.0 \times 10^{-6} \text{ mol L}^{-1}$; pH, 10.00; λ_{ex} , 385 nm; λ_{em} , 612 nm.

Fig. 2 Absorption spectra. 1, Eu^{3+} ; 2, DC; 3, ATP; 4, DC- Eu^{3+} ; 5, DC-ATP; 6, Eu^{3+} -ATP; 7, DC- Eu^{3+} -ATP. Experiment conditions: DC, $1.43 \times 10^{-5} \text{ mol L}^{-1}$; Eu^{3+} , $2.5 \times 10^{-5} \text{ mol L}^{-1}$; ATP, $1.0 \times 10^{-5} \text{ mol L}^{-1}$.

following order: DC, Eu^{3+} , ATP and buffer solution. Each mixture was diluted to the mark with doubly distilled demineralized water, and left standing for 20 min at room temperature. The fluorescence intensity was measured at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 385 \text{ nm}/612 \text{ nm}$. The enhanced fluorescence intensity of DC- Eu^{3+} by ATP was represented as $\Delta F = F - F_0$. Here, F and F_0 are the fluorescence intensities of the systems with and without ATP, respectively.

Results and Discussion

Excitation spectra and emission spectra

Fluorescence excitation spectra and fluorescence emission spectra of Eu^{3+} , DC, ATP, ATP + Eu^{3+} , DC + ATP, DC + Eu^{3+} , DC + ATP + Eu^{3+} are shown in the Fig. 1(a) and Fig. 1(b). Pure Eu^{3+} does not show the characteristic spectrum of Eu^{3+} , and the characteristic spectrum of ATP- Eu^{3+} is not apparent. The characteristic spectrum of DC- Eu^{3+} is shown in Fig. 1 with $\lambda_{\text{ex}} = 385 \text{ nm}$ and $\lambda_{\text{em}} = 590.0$ and 612.0 nm , corresponding to the $^5\text{D}_0\text{-}^7\text{F}_1$ and $^5\text{D}_0\text{-}^7\text{F}_2$ transition of Eu^{3+} , respectively. After the addition of ATP into the DC- Eu^{3+} system, a light blue shift occurred to the maximal excitation wavelength, and the

Fig. 3 Influence of the pH on ΔF .

Fig. 4 Influence of the DC concentration on ΔF .

Fig. 5 Influence of the Eu^{3+} concentration on ΔF .

fluorescence intensity of the Eu^{3+} ion at 612 nm could be enhanced remarkably. Therefore, 612 nm was chosen for the determination of DC.

The absorption spectra of Eu^{3+} , DC, ATP, ATP + Eu^{3+} , DC + ATP, DC + Eu^{3+} , and DC + ATP + Eu^{3+} are shown in Fig. 2. It can be seen that after the addition of ATP into the DC- Eu^{3+} system, a little blue shift occurred to the maximal absorption peak. This resulted from the formation of the DC + ATP + Eu^{3+} complex.

Optimization of reaction conditions

Effect of the acidity. The effects of the pH on the F are shown in Fig. 3. The experimental results showed that F reached the maximum value in the pH range 9.40 – 10.40. We thus selected pH = 10.00 of a $0.10 \text{ mol L}^{-1} \text{ NH}_3\text{-NH}_4\text{Cl}$ buffer solution for

Table 1 Influence of coexisting substances on the DC-Eu³⁺-ATP system

Coexisting substance	Concentration/mol L ⁻¹	ΔF , %
Cu ²⁺ (SO ₄ ²⁻)	1.6×10^{-6}	1.5
Zn ²⁺ (SO ₄ ²⁻)	2.4×10^{-6}	0.1
Fe ²⁺ (SO ₄ ²⁻)	1.1×10^{-6}	-4.6
Al ³⁺ (SO ₄ ²⁻)	3.8×10^{-6}	3.5
Mg ²⁺ (SO ₄ ²⁻)	1.0×10^{-5}	-0.6
Fe ³⁺ (SO ₄ ²⁻)	9.0×10^{-7}	0.8
Hg ²⁺ (Cl ⁻)	2.5×10^{-7}	9.7
Cd ²⁺ (Cl ⁻)	1.8×10^{-6}	3.5
Co ²⁺ (Cl ⁻)	1.0×10^{-6}	-9.4
Mn ²⁺ (Cl ⁻)	1.0×10^{-6}	8.2
Mo ⁶⁺ (Cl ⁻)	1.0×10^{-7}	3.0
Ca ²⁺ (Cl ⁻)	1.0×10^{-6}	7.8
DNA	4.1×10^{-6}	4.6
HSA	2.0×10^{-7}	1.5
BSA	1.0×10^{-7}	2.3
Glucose	1.0×10^{-3}	-4.6
Thymine	1.9×10^{-6}	9.4
Cytosine	1.1×10^{-7}	9.3
Guanine	3.2×10^{-7}	5.3
Adenine	1.1×10^{-8}	3.4
Trichloroacetic acid	1.4×10^{-4}	-6.7
Lactic acid	1.7×10^{-4}	-5.6
Sucrose	1.0×10^{-4}	-3.4
L-Histidine	9.9×10^{-6}	8.8
L-Glutamic acid	2.0×10^{-5}	7.9
L-Lysine	1.0×10^{-5}	-1.8
L-Cystine	2.0×10^{-5}	7.0
Leucine	1.0×10^{-5}	6.7
Tyrosine	1.0×10^{-5}	6.7
Glycin	1.0×10^{-5}	9.4
Methionine	1.0×10^{-5}	9.3
Tryptophane	1.0×10^{-5}	5.3

further study. As the volume of the buffer solution was added from 1.0 ml to 2.5 ml, the fluorescence intensity reached the maximum and remained constant. Thus 2.0 ml was used for the following experiments.

Effect of the DC concentration. The effect of the DC concentration on the proportion of ΔF is shown in Fig. 4. In the system DC-Eu³⁺-ATP, when DC was in the range of 2.2 – 2.8 ml (3.15×10^{-6} mol L⁻¹ – 4.00×10^{-6} mol L⁻¹), ΔF reached the maximum, and remained constant. Thus 2.5 ml was used for further study.

Effect of the Eu³⁺ concentration. The effects of the Eu³⁺ concentration on F and ΔF are shown in Fig. 5. In the system DC-Eu³⁺-ATP, when Eu³⁺ was in the range of 1.0 – 1.6 ml (5.0×10^{-6} mol L⁻¹ – 8.0×10^{-6} mol L⁻¹), the fluorescence intensity reached the maximum, and remained constant. Thus 1.2 ml was used for further study, and $C_{DC}:C_{Eu^{3+}} = 3:5$.

Effect of reaction time. The chelation reaction was completed within 5 – 20 min at room temperature. The fluorescence intensity reached its highest value, remained constant for at least 150 min, and then slowly decreased. Hence, all of the chelation reactions were carried out for 20 min at room temperature. All of the measurements were made at room temperature within 2 h.

Effect of the addition order of the reagents. The experimental results showed that different orders of addition of the components had little impact on both F and ΔF . Considering the enhancement of the fluorescence intensity of the system, we chose the following order for further study: DC, Eu³⁺, ATP, and buffer last.

Influence of the coexisting substance. A series experiments of coexisting substances had been carried out on the fluorescence of the DC-Eu³⁺-ATP system under the optimum condition. The concentrations of the coexisting substances approximate that of the human body fluid. Including mental ions, bases and proteins were also examined for interference. The criterion for interference was fixed at a $\pm 10\%$ variation of the average fluorescence intensity, calculated for the established level of ATP. The experimental results are given in Table 1. From Table 1 it can be seen that most of the coexisting substances were found to show no influence.

Analytical application

Linearity range and detection limit of DC. Under the optimal experimental conditions, there was a good linear relationship between the fluorescence intensity and the concentration of ATP in the range of 1.00×10^{-7} – 2.00×10^{-6} mol L⁻¹ in the DC-Eu³⁺-ATP system with a correlation coefficient (r) of 0.99471. The detection limit for the determination of DC was 4.07×10^{-8} mol L⁻¹. The standard deviation was 0.05, obtained from a series of 11 reagent blanks.

Determination of ATP in injection and tablet forms. The developed method was applied to the determination of ATP in samples of injection and tablet forms. The results were given in Table 2. Injections and tablets were purchased from a drugstore. Two injections (one containing 20 mg) were directly diluted to 100 ml. Two tablets (one containing 20 mg) were added to 0.30 g of trichloroacetic acid, whisked and then filtrated with 0.45 μ m filter paper. The solution was diluted to 100 ml. All were diluted to a linear range, and then analyzed by the method mentioned above, using the standard calibration method. From Table 2, it can be seen that the developed method can be easily performed and affords good precision, and accuracy when applied to real samples.

Conclusion

A literature survey shows that β -diketonate ligands are suitable for efficient energy transfer from them to the Eu³⁺ ion, and for a high fluorescence quantum yield, a large Stoke shift, narrow emission bands, and a long lifetime. They would thus avoid potential background fluorescence emission interference from a biological matrix. Doxycycline (DC) containing the β -diketonate configuration is a good ligand for Eu³⁺, and can strongly bind with Eu³⁺. This can be proved by the fluorescence emission spectra. After the addition of ATP into the DC-Eu³⁺ system, a light blue shift occurred to the maximal excitation wavelength, and the fluorescence intensity of the Eu³⁺ ion at 612 nm could be remarkably enhanced. This was a result from the formation of the DC + ATP + Eu³⁺ complex.

The coordination number of Eu³⁺ in its complex is 8, but the molar ratio of DC to Eu³⁺ ions in this study was DC:Eu³⁺ = 3:5 in aqueous solution without ATP. Therefore, the coordination number of Eu³⁺ cannot be satisfied. An Eu³⁺ will coordinate with H₂O to form the DC-Eu³⁺-(O-H₂)_n complex. DC-Eu³⁺-(O-H₂)_n have an excrescent positive charge, which can easily combine with the electronegative phosphate of ATP by electrostatic binding. When ATP is added into the DC-Eu³⁺-(O-H₂)_n complex, O of electronegative phosphate of ATP can replace (O-H₂)_n and bind to Eu³⁺, forming the DC-Eu³⁺-ATP complex. The energy transfer from DC and ATP to the Eu³⁺ ion can more easily occur, and non-radioactive energy loss through the O-H vibration of H₂O in the original DC-Eu³⁺ complex can be greatly decreased. The fluorescence intensity of the DC-

Table 2 Determination of ATP in injection and tablet samples ($n = 5$)

ATP sample	Sample added (10^{-6} mol L $^{-1}$)	Found			Average recovery, %	RSD, %
		Single found/ 10^{-6} mol L $^{-1}$	Average/ 10^{-6} mol L $^{-1}$	Conversion of mark value/mg unit $^{-1}$		
a	1.16	1.20 1.22 1.26 1.21 1.23	1.22	21.04	105.2	1.9
b	1.16	1.05 1.12 1.19 1.14 1.14	1.13	19.48	97.4	1.6
c	1.16	1.10 1.09 1.14 1.13 1.16	1.12	19.32	96.6	2.6
d	1.16	1.11 1.22 1.20 1.13 1.23	1.18	20.34	101.7	4.4
e	1.16	1.20 1.21 1.17 1.15 1.16	1.18	20.34	101.7	2.2
f	1.06	1.05 0.97 0.96 0.95 1.00	0.99	18.68	93.4	4.1
g	1.06	1.07 1.06 1.05 1.06 1.11	1.07	20.18	100.9	2.7

a, Tianjin jiaozuo pharmaceutical Co., Ltd. (injection); b, Shanghai tongyong pharmaceutical Co., Ltd. (injection); c, Qilu pharmaceutical Co., Ltd. (injection); d, Xuzhou laien pharmaceutical Co., Ltd. (injection); e, Changzhou lanling pharmaceutical Co., Ltd. (injection); f, Shandong laiyang pharmaceutical Co., Ltd. (tablet); g, Fujian gutian pharmaceutical Co., Ltd. (tablet).

Eu $^{3+}$ -ATP system is in proportion to the concentration of ATP. This method has been successfully applied to the determination of ATP in injection and tablet samples.

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