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Spectrofluorimetric determination of human serum albumin using a doxycycline–europium probe

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Abstract

A new spectrofluorimetric method is proposed for determination of human serum albumin (HSA) with the limit of detection at ng levels. Using doxycycline (DC)–europium (Eu^{3+}) as a fluorescent probe, in a buffer solution of pH 10.2, HSA can remarkably enhance the fluorescence intensity of the DC–Eu^{3+} complex at 612 nm and the enhanced fluorescence intensity of Eu^{3+} is proportional to the concentration of HSA. Optimum conditions for the determination of HSA are also investigated. The linear ranges for HSA are 0–9.2 and 9.2–34.5 $\mu g\,ml^{-1}$ with limits of detection of 64 and 115 $ng\,ml^{-1}$, respectively. This method is simple, practical and relatively free of interference from coexisting substances, as well as much more sensitive than most of the existing assays. The determination results for human serum and urine samples are identical to those by the AOAO method, with relative standard deviations of five determinations of 1.1–3.6%. By the Rosenthal graphic method, the binding number and association constant of human serum albumin with the probe are 1.8 and $3.71 \times 10^5\,l\,mol^{-1}$, respectively.

Keywords: Spectrofluorimetry; Human serum albumin; Doxycycline; Europium

1. Introduction

There is a connection between the content of human serum albumin (HSA) in urine and some diseases, such as nephropathy, so the determination of HSA is very important in clinical diagnosis. To date, the widely used assays for proteins are the Lowry [1], Coomassie brilliant blue [2,3], bromophenol blue [4] and bromocresol green [5] methods, however these methods have some limitations for determination of protein, such as a narrow linear range or slow reaction; all these limit their practical application. Some new methods have also been tested, such as spectrophotometry [6] spectrofluorimetry [7] and chemiluminescence [8] methods. Recently, spectrofluorimetric methods which can achieve the necessary sensitivity have been applied to HSA assay, such as using as AOAO [9] and the 2,6,7-trihydroxy-9-(4′-chlorophenyl)-3H-xanthen-3-one–molybdenum complex [10] as a fluorescent probe. People pay more attention to synthetic new ligands but ignore some existing drugs, which have a suitable configuration and can be used as ligands. Doxycycline (DC) is one of them, but to date, there has been little investigation of using DC–Eu^{3+} as a fluorescent probe for the determination of HSA.

Doxycycline is one of the kind of antibiotics of the tetracycline family containing the β-diketonate configuration. A literature survey shows that β-diketonate ligands are suitable for efficient energy transfer from ligands to Eu^{3+} ion and for high fluorescence quantum yield, large Stokes shift, narrow emission bands; a long fluorescence lifetime and hence avoid potential background fluorescent emission interferences from the biological matrix [11]. 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 HSA as co-ligand. Experimental results indicate that the characteristic peak of Eu^{3+} at 612 nm can be greatly enhanced in a DC–Eu^{3+} system by HSA and the enhancement comes from the intramolecular energy transfer in DC–HSA–Eu^{3+} ternary complex while the components are in close proximity. Accordingly, a new fluorescence method with high sensitivity and selectivity is established for the determination of HSA using DC–Eu^{3+} as a fluorescent probe. This method is easily carried out, affords good precision and accuracy and has been successfully applied to the determination of HSA in samples of human serum and urine.

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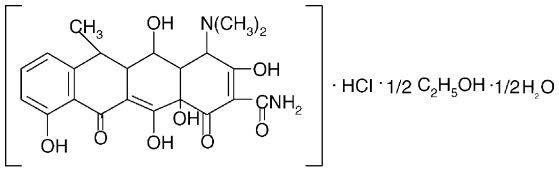

Structure of Doxycycline(DC)

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 human serum albumin (HSA, Huamei Biotechnological, Beijing) solution was directly diluted in water. The working standard solution(345 μg ml^{-1}) was freshly prepared by appropriate dilution with water.

A stock doxycycline (DC, Biological Product Institution of Chinese Medicine) solution was directly dissolved in water. The working standard solution (1.70×10^{-5} mol l^{-1}) was freshly prepared by appropriate dilution with water.

An Eu^{3+} stock solution was prepared by dissolving Eu_2O_3 (Shanghai Yuelong Chemical Plant, China) in a small amount of hydrochloric acid, then diluting to the mark with hydrochloric acid (0.1 mol l^{-1}). The working solution(1.60×10^{-5} mol l^{-1}) was obtained by appropriate dilution of the stock solution with water.

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

An ammonia–ammonium chloride buffer solution (0.10 mol l^{-1}, pH = 10.2) was used.

2.2. Apparatus

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

2.3. General procedure

To 10 ml color comparison tubes, solutions were added in the following order: 2.0 ml of buffer solution, 1.0 ml of 1.70×10^{-5} mol l^{-1} DC, 1.0 ml of 1.60×10^{-5} mol l^{-1} Eu^{3+}, 1.0 ml of 345 μg ml^{-1} HSA solution. The mixture was diluted to the mark with water and stood for 30 min at room temperature. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 385 nm/612 nm. The enhanced fluorescence intensity of DC–Eu^{3+} by HSA is represented as $\Delta F = F - F_0$, where F and F_0 are the fluorescence intensities of the systems with and without HSA, respectively. The standard curve method was used in the quantitation of trace HSA in urine and serum samples.

3. Results and discussion

3.1. Fluorescence spectra

According to the Förster non-radiation energy transfer theory [12], the rate of energy transfer depends upon the extent of overlap J of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the donor and acceptor transition dipoles and the distance between these molecules.

$$E = \frac{R_0^6}{(R_0^6 + r^6)} \tag{1}$$

$$J = \frac{\int_0^\infty F_D(\lambda) \cdot \varepsilon(\lambda) \cdot \lambda^4 \cdot d\lambda}{\int_0^\infty F_D(\lambda) \cdot d\lambda} \tag{2}$$

$$E = 1 - \frac{F}{F_0} \tag{3}$$

$$R_0^6 = 8.8 \times 10^{-25} \cdot k^2 \cdot n^{-4} \cdot \Phi_D \cdot J \tag{4}$$

It can be seen from Fig. 1 that energy transfer easily occurred between DC and HSA for the large spectral overlap between the fluorescence spectrum of the donor (HSA) and the absorption spectrum of the acceptor (DC). So, J can be evaluated by integrating the spectrum in Fig. 1 and Eq. (2) and was 5.25×10^{-15} cm^3 l mol^{-1}. Under these experimental conditions, we found the characteristic distance R_0 = 2.26 nm, using $K^2 = 2/3$, $n = 1.36$, $\Phi_D = 0.118$, and the maximum distance r was 2.12 nm using $E = 0.32$. The data for R_0 and r were also in the academic range, which proved that non-radiative energy transfer occurred between DC and HSA.

Comparing curve 3 with curve 5 in Fig. 2, it can be seen that the characteristic peak of DC at 515 nm can be enhanced remarkably after the addition of HSA, which indicates that

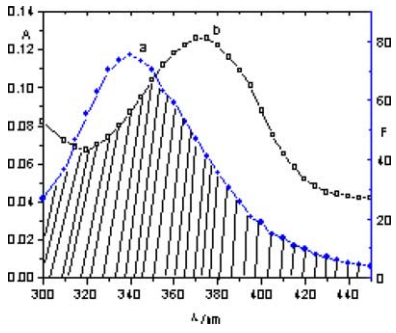

Fig. 1. Overlap of the fluorescence spectra of HSA (a) and absorption spectra of DC (b). Experimental conditions: HSA, 1.70×10^{-6} mol l^{-1}; and DC, 1.70×10^{-6} mol l^{-1}.

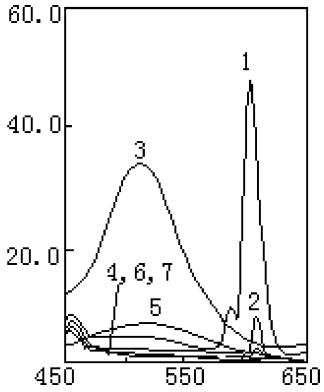

Fig. 2. Fluorescence spectra: (1) DC + Eu^{3+} + HSA, (2) DC + Eu^{3+}, (3) DC + HSA, (4) Eu^{3+} + HSA, (5) DC, (6) HSA, and (7) Eu^{3+}.

DC can form a ground state complex with HSA and energy transfers from HSA to DC.

Comparing curve 1 with curve 2 in Fig. 2, after the addition of Eu^{3+} to the DC–HSA system, the intramolecular energy transfers from the DC–HSA ground state complex to Eu^{3+} can occur more easily than directly from DC to Eu^{3+} and this explain why the fluorescence intensity of HSA–DC–Eu^{3+} at 612 nm is much larger than that of the DC–Eu^{3+} system. The enhancement is in proportion to the concentration of HSA, and according to this a new method for determination of HSA has been established.

3.2. Effect of experimental conditions

3.2.1. Effect of pH

The pH of the medium had a great effect on the fluorescence intensity of the DC–Eu^{3+} and DC–Eu^{3+}–HSA systems, as shown in Fig. 3. The experimental results showed that ΔF reached a maximum at pH 10.2, which was selected, using 0.1 mol l^{-1} ammonia–ammonium chloride buffer solution, for further study. As the volume of buffer solution added was varied from 1.5 to 2.5 ml, ΔF reached a maximum at 2.0 ml, then remained constant. Thus, 2.0 ml was chosen for the subsequent experiments.

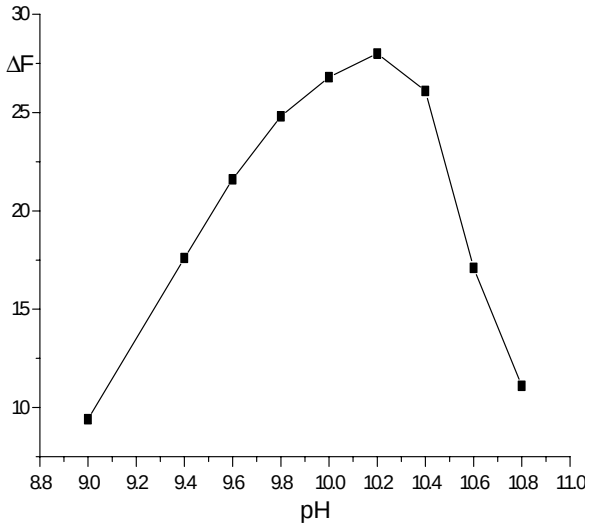

Fig. 3. Effect of acidity.

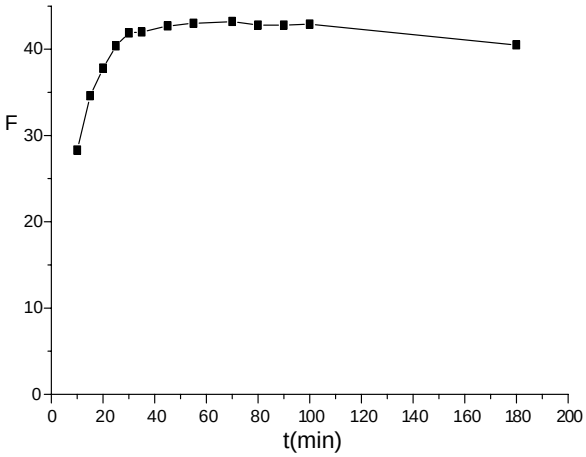

Fig. 4. Effect of reaction time.

3.2.2. Effect of time

Completing the chelation reaction of the DC–Eu^{3+}–HSA system at room temperature needed at least 25 min, then the fluorescence intensity remained constant for at least 3 h (Fig. 4). Therefore, the chelation reaction was carried out for 30 min and all measurements were made within 3 h.

3.2.3. Effect of the order of addition of reagents

Adding the various reagents in different orders had a great influence on F, F_0 and ΔF. The experimental results are shown in Table 1. Considering the enhancement of fluorescence intensity of the system, the no. 7 gave the greatest enhancement.

3.2.4. Effect of Eu^{3+} concentration

The influence of the Eu^{3+} concentration on the fluorescence intensities of the solutions containing 1.70×10^{-6} mol l^{-1} DC and 34.5 μg ml^{-1} HSA was studied under the conditions established above. The enhanced fluorescent intensity ΔF increased with increasing Eu^{3+} concentrations up to 1.60×10^{-6} mol l^{-1} and then decreased. When the concentration of Eu^{3+} ion was 1.60×10^{-6} mol l^{-1}, the

Table 1
Effect of the order of addition reagents ($n = 2$)

Order	F	F_0	ΔF
DC, HSA, Eu^{3+}, buffer	24.0	8.6	15.4
DC, Eu^{3+}, HSA, buffer	26.5	8.6	17.9
DC, buffer, HSA, Eu^{3+}	46.2	13.6	32.6
HSA, $Eu3^{+}$, buffer, DC	49.8	15.3	35.5
Eu^{3+}, buffer, DC, HSA	51.8	15.3	36.5
Buffer, DC, HSA, Eu^{3+}	43.1	11.2	31.9
Buffer, DC, Eu^{3+}, HSA	50.3	11.2	39.1

Table 2
Effect of coexisting substances

Coexisting substance	Concentration (mol l^{-1})	ΔF (%)
Co^{2+}	1.72×10^{-6}	2.0
Cr^{3+}	1.47×10^{-5}	1.6
Mg^{2+}	4.07×10^{-5}	6.2
Mn^{2+}	1.89×10^{-6}	−5.7
Ca^{2+}	4.18×10^{-6}	3.0
Fe^{3+}	1.78×10^{-10}	−6.2
Cu^{2+}	2.03×10^{-7}	5.6
Zn^{2+}	5.50×10^{-6}	2.8
Cytosine	1.00×10^{-6}	7.1
Thymine	1.00×10^{-5}	1.7
Guanine	1.60×10^{-6}	8.9
Adenine	1.00×10^{-7}	5.7

mole ratio for DC to Eu^{3+} in the DC–Eu^{3+} –HSA system was 1:1. Thus, 1.60×10^{-6} mol l^{-1} Eu^{3+} was selected for further study.

3.2.5. Effect of temperature

Temperature had a great influence on the fluorescence intensity of the system. The fluorescence intensity decreased sharply with increasing temperature. Therefore, we selected room temperature for further study.

3.2.6. Influence of coexisting substances

Under the optimum conditions, a systematic study of various non-protein substances on the determination of HSA (34.5 μg ml^{-1}) was carried out. The criterion for interference was fixed at a ±10% variation of the average fluorescence intensity calculated for the established level of HSA. The experimental results are shown in Table 2. From Table 2, it can be seen that the coexisting substances tested showed little or no influence.

4. Analytical application

4.1. Linear range and limit of detection

The linear calibration graph for determination of HSA was constructed from results obtained under the optimal condition; the limit of detection was determined from a standard deviation of 0.090 obtained from a series of 11 reagent blanks. The figures of merit are shown in Table 3.

Table 3
Linear range and limit of detection of HSA

Linear range (μg ml^{-1})	Detection limit (ng ml^{-1})	Correlation coefficient (r) ($n = 6$)	Standard deviation (s) ($n = 11$)
HSA: 0–9.2	64	0.9948	0.08997
HSA: 9.2–34.5	115	0.9987	0.08997

By comparison with some existing methods, as shown in Table 4, the present method has the advantages in the following terms: high sensitivity and selectivity, good stability and wide linear response range.

4.2. Determination of HSA in samples of serum and urine

The developed method was applied to the determination of HSA in samples of urine and serum. The results are shown in Table 5. For the assay of HSA in urine and serum, the fresh samples must be diluted appropriately to be within the linear range of determination of HSA. A portion (1.0 ml) of this sample solution was analyzed by the method developed above, using the standard calibration method. From Table 5 it can be seen that the developed method can be easily performed and affords good precision and accuracy when applied to real samples.

4.3. Measurement of association constant and binding numbers

The Rosethal graphic method [16], regarded as a modification of the Scatchard method, was used to estimate the association constant (K) and the binding number (n) of HSA to the DC–Eu^{3+} probe. Briefly, when p is the HSA concentration in the system, and C_b, C_f and C are correspondingly the HSA-bound, free and total concentrations of the complex, the Rosenthal plot follows Eq. (1) [15].

$$\frac{C_b}{C_f} = -(C - C_f)K + npK \quad (1)$$

Table 4
Comparison of spectrofluorometric methods for determination of HSA

Method	Linear range (μg ml^{-1})	Detection limit (ng ml^{-1})	Reaction time (min)	Stability (h)	Reference
AOAO assay	0.66–39.8	80	2	12	[9]
2,6,7-Trihydroxy-9-(4′-chlorophenyl)-3H-xanthen-3-One-Mo(IV)	0–12	100	3	6	[10]
Bradford (CBB G-250)	2–10	55.6	2	1	[2]
2-(5-Bromo-2-pyridylazo)-5(*N*-phenyl-*N*-sulfopropylamino)phenol-Co(II)	0–7	111.1	10 (50 °C)	2	[13]
o-Sulfophenylfluorone–Ti(IV)	0.5–20	66.7	30 (70 °C)		[14]
This method	0–9.2	64.0	30 (room temperature)	3	–
	9.2–34.5	115.0			

Table 5
Determination of HSA in serum and urine sample

Real sample	This method ($n = 5$)		AOAO assay [9] ($n = 5$)	
	Found (average) ($mg\,ml^{-1}$)	RSD (%)	Found (average) ($mg\,ml^{-1}$)	RSD (%)
Serum1	69.0	2.3	66.8	2.3
Serum 2	81.3	2.7	80.2	2.6
Urine 1	38.2[a]	1.1	36.1[a]	0.9
Urine 2	45.6[a]	3.7	44.6[a]	1.9

[a] $mg\,l^{-1}$.

Since $C = C_b + C_f$, therefore

$$\frac{C}{C_f} = -(C - C_f)K + npK + 1 \quad (2)$$

In the system, if C and C_f are within the dynamic range of the calibration graph for DC–Eu^{3+}–HSA complex, Eq. (3) can be obtained:

$$\frac{F_0}{F} = -\left(1 - \frac{F}{F_0}\right)CK + npK + 1 \quad (3)$$

where F, F_0 are the intensities of the systems with and without HSA respectively. The plot of F_0/F versus $(1-(F/F_0))C$ can be obtained. The values of K and n are $3.71 \times 10^5\,l\,mol^{-1}$ and 1.8, respectively.

5. Conclusion

Both doxycycline, containing a β-diketonate configuration, and the biomacromolecule HSA are ideal ligands for Eu^{3+} and can possibly sensitize the fluorescence intensity of Eu^{3+} ion via intramolecular energy transfer [16], possibly. DC can form a ground state complex with HSA depending upon the non-covalently bound attraction, so a very stable ternary complex in close proximity with a large degree of molecular conjugation and rigid structure can be formed by the interaction between DC–HSA ground state complex and Eu^{3+}. As a result of the effect of packing and cooperation in a ternary complex, the energy transfer from the DC–HSA complex to Eu^{3+} can occur more easily and the non-radiative energy loss through O–H vibration of H_2O in the original Eu^{3+} complex can be decreased greatly. So, the fluorescence intensity of Eu^{3+} at 612 nm can be enhanced several times. The developed method has been successfully applied to the determination of trace amounts of HSA in urine and serum samples.

References

[1] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[2] T. Zor, Z. Selinger, Anal. Biochem. 236 (1996) 302.
[3] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[4] R. Flores, Anal. Biochem. 88 (1978) 605.
[5] F.L. Rodkley, Clim. Chem. 11 (1965) 478.
[6] A.A. Waheed, P.D. Gupta, Anal. Biochem. 233 (1996) 249.
[7] J. Yuan, K. Matsumoto, J. Biomed. Anal. 15 (1997) 1397.
[8] T. Hara, K. Tsukagoshi, Anal. Sci. 6 (1990) 797, and papers cited therein.
[9] Y.J. Luo, H.X. Shen, Anal.Commun. 36 (1999) 135.
[10] Z.X. Guo, H.X. Shen, Analyst 124 (1999) 1093.
[11] J.L. Yuan, G.L. Wang, K. Majima, K. Matsumoto, Anal. Chem. 73 (2001) 1869.
[12] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, 1983, p. 303.
[13] I. Mori, K. Taguchi, Y. Fujita, T. Matsuo, Anal. Lett. 28 (1995) 225.
[14] Y. Fujita, I. Mori, T. Matsuo, Anal. Sci. 13 (1997) 513.
[15] H.R. Rosenthal, Anal. Biochem. 20 (1967) 525.
[16] F.S. Richardson, Chem. Rev. 82 (1982) 541.