Spectrofluorimetric Determination of Bile Acid Using a Europium-Doxycycline Probe

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A new spectrofluorimetric method was developed for the determination of trace amounts of bile acid (BA). Using europium ion (Eu^{3+})-doxycycline (DC) as a fluorescent probe, in a buffer solution of pH = 7.0, BA can remarkably reduce the fluorescence intensity of the DC- Eu^{3+} complex at $\lambda = 612$ nm; the reduced fluorescence intensity of the Eu^{3+} is proportional to the concentration of BA. Optimum conditions

for the determination of BA were also investigated. The linear range and detection limit for the determination of BA were 5.0×10^{-8} mol/L to 5.5×10^{-7} mol/L and 1.1×10^{-8} mol/L, respectively. This method is practical and relatively free of interference from coexisting substances, and can be successfully applied to assess BA in serum samples. *J. Clin. Lab. Anal.* 21:207–212, 2007. © 2007 Wiley-Liss, Inc.

Key words: bile acid; doxycycline; europium; spectrofluorometry

INTRODUCTION

Bile acids (BAs) are the major components of bile. They are synthesized in the liver and secreted in the gallbladder or in the intestine, conjugated mainly with taurine and glycine (1). BAs serve many important physiological functions. They have a consanguineous relationship with the metabolism of cholesterol, the dissolution of phospholipid, and the absorption of fatty acid and some liposoluble vitamins (2,3). In health, only small quantities of BA are found and they are normally between 0.48 and 4.8 mmol/L. Any pathologic process causing damage of the hepatic cell, such as hepatitis, hepatocirrhosis, and fatty liver, may induce the decline of biological function and the raise of the concentration of BA (4). Therefore, serum or plasma BA may best reflect abnormalities in BA metabolism, and BA analysis is useful in the evaluation of liver or intestinal functions and in the diagnosis of related diseases.

Estimation of BA in human serum is a sensitive test of liver function (5). However, the analysis of BAs in biological samples has always presented technical difficulty because of their complex structure and low concentration. A reliable procedure for determining the excretion of BA is desirable in studies of cholesterol metabolism in man. The currently available methods include ultraviolet-visible spectrophotometry (UV-VIS) (6), (TLCS) (7), differential refractive detection (8), radioimmunoassay detection (9), high performance

liquid chromatography (HPLC) (10), gas chromatography-mass spectrometry (GC-MS) (11,12), high performance liquid chromatography-mass spectrometry (HPLC-MS) (1,13,14), and enzymatic method (15). The enzymatic method is widely used in clinic because of its good selectivity. However, the reagents of enzymatic method are costly, the experimental conditions are strict, and the procedures are complicated. The sensitivity of fluorescence spectrophotometry is high and the operation is simple. We investigated the determination of BA using DC- Eu^{3+} as a probe. This method has been successfully applied to the determination of BA in serum samples.

To date, there have been some reports on the use of metacycline-europium as a fluorescent probe for the determination of lysozyme (16), doxycycline-europium for determination of human serum albumin (17), europium-doxycycline-derived fluorescent substrate for the determination of catalase activity (18), the highly luminescent europium-doxycycline hydrogen peroxide complex for the determination of hydrogen peroxide in river water (19), and tetracycline-europium for determination of heparin (20). All the Eu^{3+} -based methods

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Fig. 1. Structure of DC.

mentioned above have a high fluorescence quantum yield, a large Stokes' shift, narrow emission bands, and a large fluorescence lifetime; hence, they avoid potential background fluorescent emission interferences from the biological matrix (21). However, there have been no reports of a spectrofluorimetric method for the determination of BA using Eu^{3+} -DC as a fluorescent probe.

DC is an antibiotic of the tetracycline family containing the β -diketonate configuration; its structure is shown in Fig. 1. It is the ideal ligand for Eu^{3+} . In the current work, we chose DC as the ligand of Eu^{3+} and investigated the possibility of enhancing Eu^{3+} fluorescence using BA as coligand. Experimental results showed that the characteristic peak of Eu^{3+} at 612 nm could be greatly reduced, and that the reduction in fluorescence intensity was proportional to the concentration of BA. A new method, with high sensitivity and selectivity, for the spectrofluorimetric determination of BA has therefore been established. This method is simple, relatively free of interference from coexisting substances, and can be successfully applied to the determination of BA in serum samples with satisfactory results.

MATERIALS AND METHODS

Reagents

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

A stock cholic acid (CA) (Beijing Chemical Plant, China) solution (1.0×10^{-5} mol/L) was dissolved in a small quantity of 60% acetic acid and then diluted with DDD water. The working standard solution (1.0×10^{-6} mol/L) was freshly prepared by appropriate dilution with DDD water.

A stock DC (Biological Product Institution of Chinese Medicine) solution was directly dissolved in DDD water. The working standard solution (6.0×10^{-5} mol/L) was freshly prepared by the appropriate dilution with DDD water.

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

A Eu^{3+} ion stock solution was prepared by dissolving Eu_2O_3 (Shanghai Yuelong Chemical Factory, China)

with a small amount of hydrochloric acid and then diluted to the mark with hydrochloric acid (0.1 mol/L). The working solution (5.0×10^{-4} mol/L) was obtained by appropriate dilution of the stock solution with DDD water.

A Tris-HCl buffer solution (0.10 mol/L, pH 7.0) was used for the system.

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 the determination experiments. All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device Works, China).

Experimental Procedure

Solutions were added to 10-mL color comparison tubes in the following order: 1.5 mL of 5.0×10^{-4} mol/L Eu^{3+} solution, 2.5 mL of 1.0×10^{-6} mol/L BA solution, 1.0 mL of buffer solution, and 1.0 mL of 6.0×10^{-5} mol/L DC solution. The mixture was diluted to 10 mL with DDD water. The fluorescence intensity was measured at maximum excitation wavelength (λ_{ex})/maximum emission wavelength (λ_{em}) = 385 nm/612 nm. The decreased fluorescence intensity of DC- Eu^{3+} caused by BA were represented as $\Delta F = F_0 - F$. Here F and F_0 are the fluorescence intensities of the systems with and without BA, respectively. The standard curve method was used to determine trace amounts of BA in serum samples.

RESULTS AND DISCUSSION

Characteristics of Fluorescence and Absorption Spectra

The fluorescence emission spectra of Eu^{3+} , DC- Eu^{3+} , and DC- Eu^{3+} -BA are shown in Fig. 2. Only the Eu^{3+} solution showed little or no peak. Under the same conditions, the characteristic peak of DC- Eu^{3+} was observed, with two characteristic peaks at 590 nm and 612 nm, and they are the $^5\text{D}_0$ - $^7\text{F}_1$ and $^5\text{D}_0$ - $^7\text{F}_2$ transitions of Eu^{3+} , respectively. The fluorescence spectrum of DC- Eu^{3+} -BA was similar to that of DC- Eu^{3+} ; however, the fluorescence intensity of DC- Eu^{3+} was decreased by BA, and the decrease was proportional to the concentration of BA.

The absorption spectra of DC, Eu^{3+} , BA, DC- Eu^{3+} , DC-BA, Eu^{3+} -BA, and DC- Eu^{3+} -BA are shown in Fig. 3. From Fig. 3, it can be seen that curves 1, 2, and 3 have no obvious peak. In comparing curve 3 with curve 5, after the addition of Eu^{3+} into DC solution, a redshift occurs from 350 to 380 nm and the absorbency is also enhanced, which indicates that DC can form a binary

Fig. 2. Fluorescence emission spectra of 1: Eu^{3+} , 2: BA, 3: Eu^{3+} -DC, 4: Eu^{3+} -DC-BA (BA: 2.0×10^{-7} mol/L), 5: Eu^{3+} -DC-BA (BA: 3.0×10^{-7} mol/L), and 6: Eu^{3+} -DC-BA (BA: 4.0×10^{-7} mol/L) were acquired with an RF-540 spectrofluorimeter and show that the characteristic peak of Eu^{3+} is 612 nm. A 10.0-mL solution contains DC (6.0×10^{-6} mol/L), Eu^{3+} (7.5×10^{-5} mol/L), buffer solution (pH 7.0), and BA.

complex with Eu^{3+} . In comparing curve 6 with curve 7, a blueshift occurred and the absorbency is reduced lightly, which indicates that BA reacts on the DC- Eu^{3+} system and the BA-DC- Eu^{3+} ternary complex is formed. The results of the absorption spectra accord well with those of the fluorescence spectra.

Optimization of Reaction Conditions

Effect of acidity

A series of solutions with different pH buffer concentrations but the same concentrations of the other reagents (F), and their corresponding blank solutions (F_0) were measured at $\lambda_{ex}/\lambda_{em} = 385 \text{ nm}/612 \text{ nm}$. The pH of the medium had a significant effect on both the formation of the Eu^{3+} -DC complex and the capability of BA to bind to the Eu^{3+} -DC complex. The variation of fluorescence intensity of the Eu^{3+} -DC-BA system with pH is shown in Fig. 4. After adding BA, the experimental results showed that the ΔF reached a maximum and remained constant from 6.9 to 7.2. Thus, pH 7.0 was

Fig. 3. Absorption spectra of 1: Eu^{3+} , 2: BA, 3: Eu^{3+} -BA, 4: DC, 5: DC-BA, 6: Eu^{3+} -DC, and 7: Eu^{3+} -DC-BA were acquired with an UV-265 spectrophotometer. A 10.0-mL solution contains DC (6.0×10^{-6} mol/L), Eu^{3+} (7.5×10^{-5} mol/L), buffer solution (pH 7.0), and BA (2.5×10^{-7} mol/L).

Fig. 4. Effect of pH on the reduced fluorescence intensity (ΔF). The buffer solutions used were Tris-HCl buffer for pH 6.2 to 7.6.

selected with 0.10 mol/L Tris-HCl buffer for further research. Because the addition of 1.0-mL buffer solution caused the fluorescence intensity ΔF to reach a maximum, this volume was used in the following experiments.

Effect of time

To complete the chelation reaction of the DC- Eu^{3+} -BA at room temperature required at least 40 min. The fluorescence intensity then remained constant for at least 3 hr. Therefore, all chelation reactions were carried out for 40 min, and all measurements were made within 3 hr. The results are shown in Fig. 5.

Effect of the addition order of reagents

A series of solutions with different addition orders of reagent but the same concentrations of other reagent

Fig. 5. Effect of reaction time on the reduced fluorescence intensity (ΔF). A 10.0-mL solution contains DC (6.0×10^{-6} mol/L), Eu^{3+} (7.5×10^{-5} mol/L), buffer solution (pH 7.0), and BA (2.5×10^{-7} mol/L).

(F), and their corresponding blank solutions (F_0) were measured at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 385 \text{ nm}/612 \text{ nm}$. Adding the reagents in different orders had a negligible influence on the F , F_0 , and ΔF . Considering the stability of the system, along with the F , F_0 , and ΔF , the following order was optimal: Eu^{3+} , BA, buffer, and DC.

Effect of the amounts of Eu^{3+}

The influence of the amounts of Eu^{3+} on the fluorescence intensities of the solutions containing 6.0×10^{-6} mol/L DC and 2.5×10^{-7} mol/L BA was studied under the conditions established above. The ΔF reached its maximum and remained constant when the concentration of Eu^{3+} is 7.5×10^{-5} to 1.0×10^{-4} mol/L (the added amount of Eu^{3+} was 1.5 to 2.0 mL). When the concentration of Eu^{3+} was 7.5×10^{-5} mol/L, the composition ratio for the DC to Eu^{3+} in the DC- Eu^{3+} -BA system was 1:12.5. Thus, 7.5×10^{-5} mol/L Eu^{3+} (the added amount of Eu^{3+} was 1.5 mL) was selected for further study. The result are shown in Fig. 6.

Effect of Coexisting Substances

Under the optimal conditions, a systematic study of various substances (including metal ions, amino acids, etc.) in the determination of BA (2.5×10^{-7} mol/L) was carried out. The criterion for interference was fixed at a $\pm 10\%$ variation of the average fluorescence intensity calculated for the established level of BA, and the experimental results are shown in Table 1. Most of the coexisting substances were found to have no influence, so the effects of these possible interferents could be ignored. The interfering substances (lipids, hemoglobin, and bilirubin) present in serum badly interfere with the determination of bile acid. However, we have removed these interfering substances in the

Fig. 6. Effect of the concentration of Eu^{3+} on the reduced fluorescence intensity (ΔF). A 10.0-mL solution contains DC (6.0×10^{-6} mol/L), buffer solution (pH 7.0), BA (2.5×10^{-7} mol/L), and Eu^{3+} (2.5×10^{-5} mol/L to 1.5×10^{-4} mol/L).

pretreatment process before the determination of bile acid in serum samples.

Analytical Application

Linear range and limit of detection

Under the experimental conditions, there was a linear relationship between fluorescence intensity and BA concentration in the range of 5.0×10^{-8} mol/L to 5.5×10^{-7} mol/L with a correlation coefficient of 0.9928 and a standard deviation (SD) of 2.86. The regression equation is $\Delta F = -1.98 + 1.35 \times 10^8 C$ (mol/L). The limit of detection was determined to be 1.1×10^{-8} mol/L when the SDs were 0.48, obtained from a series of 10 reagent blanks with a relative SD of 0.009. The precision of samples ($N = 10$) was also examined with an SD of 0.36 and a relative SD of 0.026.

Determination of BA in serum samples

The method developed here was used to determine the BA content of serum samples. To perform an assay on BA from serum samples, they must be pretreated appropriately. Therefore, 1 mL of serum was mixed with 4 mL of methyl cyanide and centrifuged at 4,000 rpm for 15 min to deposit albumen. The purified liquor was collected and then mixed with 5 mL of chloroform and 0.5 mL of glacial acetic acid. Liposoluble substances, such as cholesterol and triglyceride, were dissolved in chloroform while BA was dissolved in glacial acetic acid. The mixture was ultrasonic oscillated for 10 min and then evaporated in a water-bath until the solution was almost dry (22). As the melting points of solvents are different, chloroform, methyl cyanide and water were evaporated with the interference substances

TABLE 1. Effect of coexisting substances

Coexisting substance	Concentration (mol/L)	$\Delta F\%$	Coexisting substance	Concentration (mol/L)	$\Delta F\%$
Co ²⁺	2.50×10^{-6}	-6.03	L-histidine	1.00×10^{-5}	-5.55
Cu ²⁺	2.50×10^{-7}	1.27	L-lysine	2.00×10^{-5}	2.38
Mg ²⁺	5.00×10^{-5}	5.30	Methionine	1.02×10^{-5}	-3.65
Cd ²⁺	1.80×10^{-7}	-0.79	Glutamic	2.00×10^{-5}	0.63
Ca ²⁺	1.00×10^{-5}	1.63	Cystine	4.00×10^{-6}	6.97
Zn ²⁺	1.18×10^{-7}	-5.56	Tyrosine	1.00×10^{-7}	-4.29
Mn ²⁺	1.25×10^{-6}	5.80	Tryptophane	5.00×10^{-6}	-9.35
Cr ³⁺	3.80×10^{-7}	4.92	L-leucine	1.02×10^{-4}	2.37
Mo ⁶⁺	1.04×10^{-7}	8.57	Glycin	1.00×10^{-5}	0.16
Fe ³⁺	1.79×10^{-8}	0.76	Cane sugar	1.00×10^{-3}	-0.40
Hg ²⁺	2.49×10^{-8}	1.33	Glucose	1.03×10^{-3}	0.34
Al ³⁺	3.77×10^{-6}	4.28	Acetocaustin	2.00×10^{-4}	0.88
Thymine	1.85×10^{-6}	1.47	Bilirubin	1.00×10^{-6}	2.15
Cytosine	1.10×10^{-6}	6.11	Creatine	2.00×10^{-6}	6.23
Adenine	1.04×10^{-8}	7.39	Uric acid	3.00×10^{-7}	4.57
HSA*	1.00×10^{-7}	7.08	Lecithin	2.00×10^{-7}	7.41

*HSA = human serum albumin.

TABLE 2. Determination of BA in serum samples

Samples	Initial ($\times 10^{-6}$ mol/L)	Average ($\times 10^{-6}$ mol/L)	SD	Added ($\times 10^{-6}$ mol/L)	Found ($\times 10^{-6}$ mol/L)	Average ($\times 10^{-6}$ mol/L)	SD	Recovery (%)	Concentration in serum ($\times 10^{-4}$ mol/L)
Sample 1	2.72, 2.72, 2.64	2.69	0.05	2.0	4.60, 4.66, 4.56	4.61	0.05	95.7	6.73
Sample 2	2.41, 2.37, 2.37	2.38	0.02	2.0	4.25, 4.21, 4.39	4.28	0.09	95.0	5.95
Sample 3	2.49, 2.41, 2.30	2.40	0.10	2.0	4.49, 4.49, 4.38	4.45	0.06	102.7	6.00

dissolved in them. Through the pretreatment mentioned above, BA was separated from both liposoluble and water-soluble interference substances. Then, well-worked serum samples were obtained. The samples were diluted to a liner range (5.0×10^{-8} mol/L to 5.5×10^{-7} mol/L) and then analyzed using the method described above. The results are shown in Table 2. These findings confirmed that the developed method was easy to perform, and afforded good precision and accuracy when applied to serum samples.

CONCLUSION

A new spectrofluorimetric method was developed for the determination of trace amounts of BA. DC can form a binary complex with Eu³⁺, which emits a peak at 612 nm. BA can combine with this Eu³⁺-DC complex and can remarkably reduce its fluorescence intensity at $\lambda = 612$ nm. The reduction in the fluorescence intensity of the Eu³⁺ ion is proportional to the concentration of BA. This method has been successfully applied to the determination of serum samples.

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