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ORIGINAL PAPER

Hydroxypropyl-β-Cyclodextrin Enhanced Determination for the Vitamin B_{12} by Fluorescence Quenching Method

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Abstract A novel fluorescence quenching method for the determination of Vitamin B_{12} (VB_{12}) had been developed. It was based on that the fluorescence intensity of erythrosine sodium (ES) could be enhanced by Hydroxypropyl-β-cyclodextrin (HP-β-CD) due to the formation of inclusion complex (HP-β-CD-ES), while the fluorescence intensity of HP-β-CD-ES was diminished after adding VB_{12} into the system, and there was a linear relationship between the fluorescence quenching value of the system (ΔF) and the concentration of VB_{12} (c). The mechanism of the determination of VB_{12} was discussed. The results showed that under the optimal conditions, the linear range of calibration curve for the determination of VB_{12} was $0.0 \sim 2.1 \times 10^{-5}$ mol/L, and the detection limit was 1.8×10^{-7} mol/L. It could be satisfactorily applied to the determination of VB_{12} in injections.

Keywords Vitamin B_{12} · Hydroxypropyl-β-cyclodextrin · Fluorescence quenching

Introduction

Vitamin B_{12} (VB_{12}) is one of the Vitamin B family members. It contains cobalt, therefore, it is also named cobalamine. It takes part in the one carbon unit of metabolism of human. When human are lack of VB_{12}, they will have some disease, such as pernicious anemia, and so on. But superfluous VB_{12} would bring some noxious and side effects, too. The determination methods of VB_{12} mainly were spectrophotometry [1], electrochemistry [2], laser resonance Raman spectrometry [3], ICP-AES [4]. Most of those methods need to determine the content of cobalt firstly, then calculate the content of VB12 by the cobalt data. So it was inconvenient. Recently, new methods were developed [5–7]. Liu [8] reported a novel fluorescence quenching method for the determination of VB_{12} based on acridine orange-rhodamine 6G energy transfer system. β-cyclodextrin and its various derivatives (β-CDs) were well known, which structure was that of a truncated cone with the hydrophilic outer surface and a hydrophobic internal cavity. This peculiar molecular structure could include guest molecules to form inclusion complexes. The photochemical and photophysical properties of the guest molecules would be altered due to the formation of a super-molecular complex (guest-β-CDs). Thus, the physical, chemical and biochemical characters of guest molecules were modified, and the application capability of those guest molecules could be improved. So, β-CDs with inclusion properties had been widely used in spectral analysis [9–10]. This paper found that HP-β-CD could form inclusion complex with ES (HP-β-CD-ES), thereby enhancing the fluorescence intensity of ES. Whereas VB_{12} was added into the system of HP-β-CD-ES, the fluorescence intensity of the system was diminished, and there was a linear relationship between the fluorescence quenching value ($\Delta F = F_{HP\text{-}\beta\text{-}CD\text{-}ES} - F_{HP\text{-}\beta\text{-}CD\text{-}ES\text{-}VB_{12}}$) and the concentration of VB_{12} (c). Therefore, based on the ES-HP-β-CD-VB_{12} system a novel fluorescence quenching method for the determination of VB_{12} has been developed. The method features good recurrence and few interfering substance. The detection limit was lower than that of the literature [8]. It has been satisfactorily applied to the determination of VB_{12} in injections.

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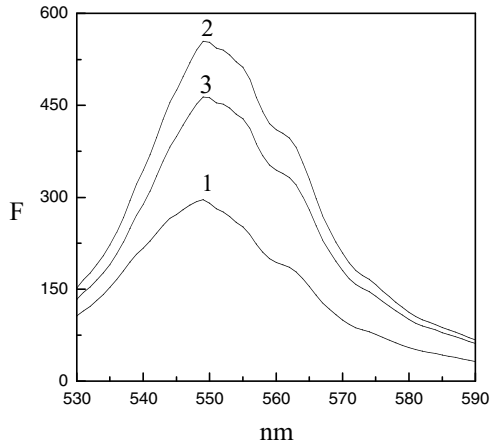

Fig. 1 Fluorescence spectra: 1. ES (1.0×10^{-5} mol/L); 2. ES + HP-β-CD (1.3×10^{-2} mol/L); 3. ES + HP-β-CD + VB_{12} (9.0×10^{-6} mol/L)

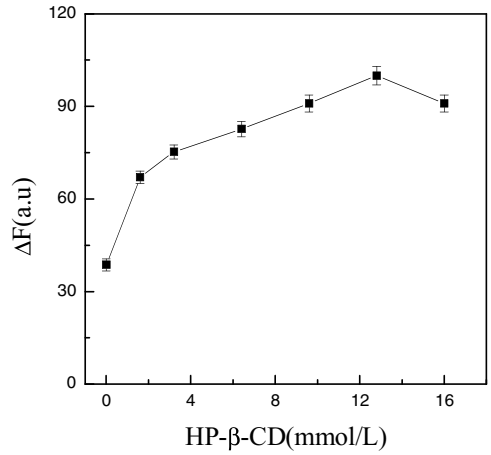

Fig. 3 Effect of HP-β-CD on ΔF. C_{ES}: 1.0×10^{-5} mol/L; $C_{VB_{12}}$: 4.8×10^{-6} mol/L

Experimental

Apparatus and reagents

The fluorescence measurements were performed with a F-4500 spectrofluorimeter (Hitachi, Japan). The absorbance measurements were carried out on a UV-2501 spectrophotometer (Shimadzu, Japan). The pH measurements were made with a model pHS-25 pH meter (Shanghai, China).

HP-β-CD (average MW = 1425, D.S. = 5.0, Yi Ming Fine Chemical Corporation, China) were used without prior purification. A 5.0×10^{-4} mol/L solution of erythrosine sodium (ES) was prepared by directly dissolving the crystal into water. A standard stock solution of Vitamin B_{12} (China Medicine Group Chemical Reagent Corporation) was prepared. HAc-NaAc buffer solution was used to control the pH value. All chemicals were of analytical-reagent grade. All solutions were prepared with distilled water.

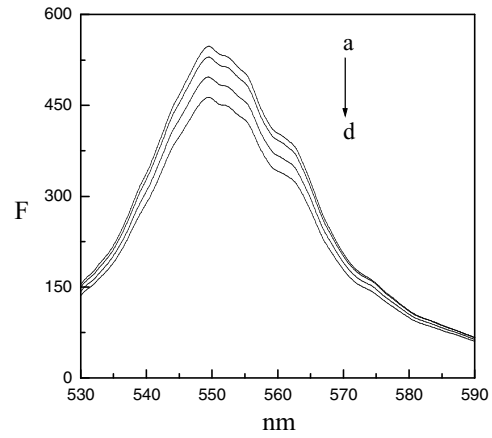

Fig. 2 Fluorescence spectra of ES-HP-β-CD in the presence of VB_{12}. $C_{VB_{12}}$: (a) 3.6×10^{-6} mol/L, (b) 4.8×10^{-6} mol/L, (c) 7.2×10^{-6} mol/L, (d) 9.6×10^{-6} mol/L

Procedure

In a 25 mL volumetric flask, 5.0 mL 5.0×10^{-4} mol/L ES, 4.0 mL 0.08 mol/L HP-β – CD, 3.0 mL HAc-NaAc buffer solution (pH 6.0) and a quantitative standard stock solution of VB_{12} (or the sample of VB_{12}) were added. The mixed solution was diluted to final volume with distilled water and shaken thoroughly. The obtained solution was thermostated at 20.0 ± 1°C for 5 min and the fluorescence intensity of the solution was measured at excitation wavelength 529 nm, emission wavelength 549 nm in a 1.0 cm quartz cell by a F-4500 fluorospectrophotometers, and the blank solution was measured at the same time. The excitation and emission bandwidths were both set at 2.5 nm.

Sample preparation

Took 5 ml of VB_{12} injections (0.5 mg/1 mL) and mixed them in a 25 mL volumetric flask. The mixed solution was

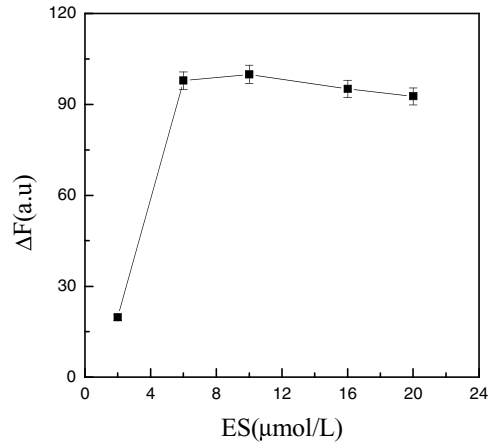

Fig. 4 Effect of the amount of ES on ΔF. $C_{HP\text{-}\beta\text{-}CD}$: 1.3×10^{-2} mol/L; $C_{VB_{12}}$: 4.8×10^{-6} mol/L

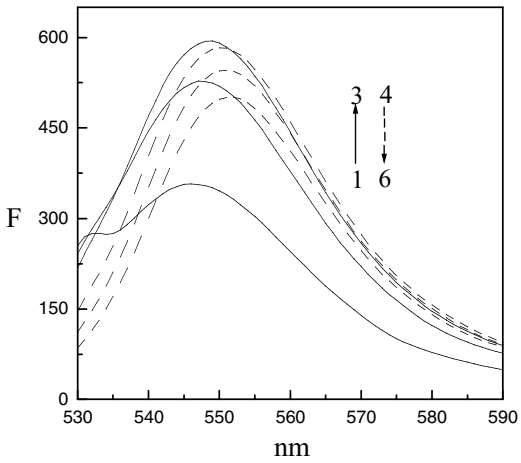

Fig. 5 The fluorescence spectra of different concentration of ES solution. ES concentration was — (1) 4 μmol/L, (2) 8 μmol/L, (3) 12 μmol/L, – – – (4) 20 μmol/L, (5) 24 μmol/L, (6) 28 μmol/L

diluted to final volume with distilled water to be a 0.1 mg/mL solution.

Results and discussion

The interactions of ES-HP-β-CD system and VB_{12}

The fluorescence emission spectra of ES in different media were shown in Fig. 1. Comparing the curves, it was found that the fluorescence intensity of ES was enhanced in presence of HP-β-CD. However, the fluorescence intensity of the system was diminished when VB_{12} was added into the system of HP-β-CD-ES. The fluorescence spectra of HP-β-CD-ES in different concentration of VB_{12} are shown in Fig. 2. From Fig. 2, It was observed that the fluorescence intensity of HP-β-CD-ES gradually decreased with an increase of VB_{12} concentration, It may be the basis of determination for VB_{12} if there was a linear relationship between the fluorescence quenching value ΔF and the concentration of VB_{12}.

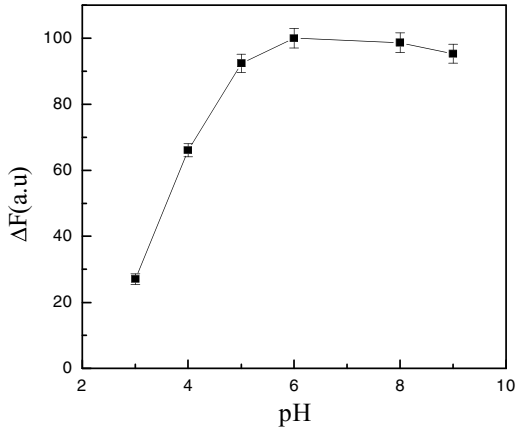

Fig. 6 Effect of pH on ΔF. C_{ES}: 1.0 $\times$ 10^{-5} mol/L; $C_{HP\text{-}\beta\text{-}CD}$: 1.3 $\times$ 10^{-2} mol/L; $C_{VB_{12}}$: 4.8 $\times$ 10^{-6} mol/L

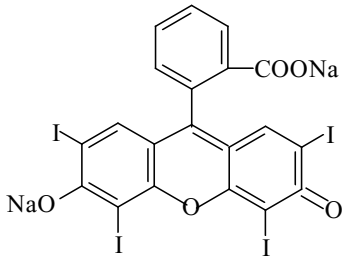

Fig. 7 The structure of ES

Effect of HP-β-CD concentration

HP-β-CD could form inclusion complex with ES and enhance the fluorescence intensity of ES ($F_{HP\text{-}\beta\text{-}CD\text{-}ES} > F_{ES}$). Furthermore, after VB_{12} was added, the ΔF of the system was increased. Namely, HP-β-CD has the sensitizing effect on the determination of VB_{12}. The influence of HP-β-CD concentration on the value of ΔF was studied. It was found that 1.3 $\times$ 10^{-2} mol/L (13 mmol/L) HP-β-CD was appropriate for the maximum ΔF (Fig. 3).

Effect of ES concentration

In this work, the fluorescent reagent ES was an important influence factor for determination of VB_{12}. Different concentration of ES was added to investigate the effects on ΔF of the system. The results were shown in Fig. 4. From Fig. 4, firstly, the ΔF was gradually increased with the increase of ES concentration, then, the ΔF was gradually decreased with the increase of ES concentration when the concentration of

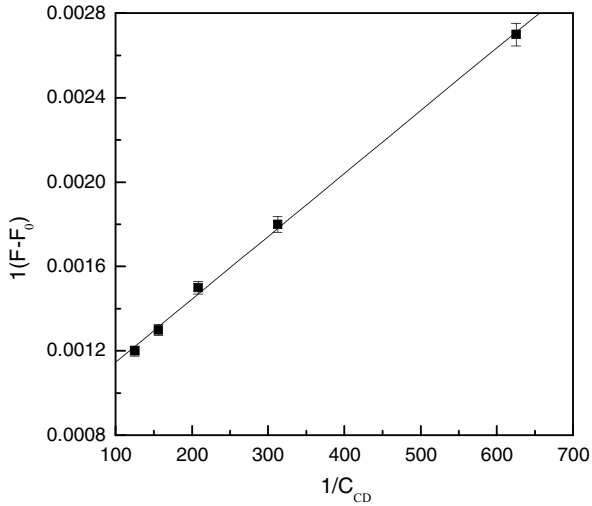

Fig. 8 Double reciprocal plot of ES in HP-β-CD

Table 1 The inclusion constant K of HP-β-CD-ES at different pH

pH	3.2	5.0	6.0	11.3
K	141 ± 10	340 ± 22	286 ± 15	189 ± 13

ES was relatively higher. This may be caused by the self-quenching of ES because of the formation of the aggregate of ES at higher concentration.

As to prove this conclusion, the change of fluorescence intensity of ES with the concentration of ES was investigated (Fig. 5). It was found that the fluorescence intensity of ES was becoming self quenching when the range of concentration of ES was higher than 12 μmol/L, This was with the result of Fig. 4 consistent. Thus, 1.0×10^{-5} mol/L (10 μmol/L) ES was chosen for the test.

Effect of pH

The influence of pH on the relative fluorescence intensity of system (ΔF) was investigated. As could be seen in Fig. 6, in acidic medium, the ΔF gradually increased with the increase of pH; in subacid, neutral and basic media, the ΔF was reached maximum and was relatively stable. The effect of pH on ΔF mostly influenced the speciation of ES. From the structural formula (Fig. 7), ES is a positive ion or neutral molecule in strong acidic medium; but in subacid, neutral and basic media, it is a negative ion and could produce more fluorescence. On the other hand, the inclusion interaction of ES and HP-β-CD was influenced by the pH of the system (discuss in 3.5.1). Thus, HAc–NaAc buffer solution of pH = 6.0 was chosen for the determination, and the amount of the buffer solution was selected 2.0 mL as suitable for the optimized method.

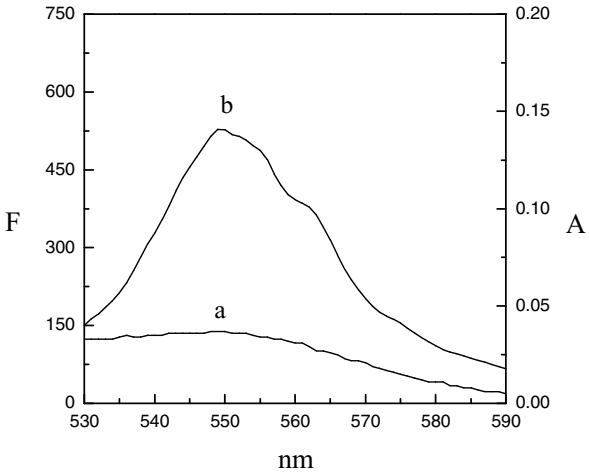

Fig. 9 The spectrum. (a) The absorbance spectrum of VB_{12}; (b) The fluorescence spectrum of ES-HP-β-CD

Discussion of mechanism

The inclusion interaction of ES and HP-β-CD

HP-β-CD is a derivative of β-CD, which structure is that of a truncated cone with the hydrophilic outer surface and a hydrophobic internal cavity [11]. This peculiar molecular structure of HP-β-CD could include guest molecule which had suitable polarity, shape and property into their hydrophobic cavity to form inclusion complexes. The formation of a super-molecular complex with β-CD or HP-β-CD could alter the photochemical and photophysical properties of the guest molecules. The chemical reactivity and the spectroscopic properties of the guest molecules are modified as a result of the inclusion, and the application capability of those guest molecules could be improved [12].

In this work, HP-β-CD formed inclusion complex with ES and The inclusion complex was estimated assuming a 1:1 inclusion model to obtain its inclusion constant by the modified Benesi-Hildebrand equation (double reciprocal plot) [13, 14]. As shown in Fig. 8, a good linear relationship was obtained and supported the formation of a 1:1 complex ($r = 0.9993$). The inclusion constant K of HP-β-CD-ES at different pH was determined (Table 1). It was shown that the value of K in pH 5–6 was bigger than that in strong acidic medium and in alkaline medium. It indicated that the subacid medium was beneficial for the inclusion interaction of ES and HP-β-CD. Namely, the inclusion complex (HP-β-CD-ES) was stable in subacid medium.

As a result of the inclusion effect of HP-β-CD, the excited single state of the fluorescence substance was effectively shielded into a protective microenvironment (the cavity of HP-β-CD). The protective microenvironment decreased the concentration quenching of the fluorescent particles and the quenching effect of external quencher. Therefore, the rate of the non-radiative transition and deactivation process of

Table 2 Efect of interfering substances on fluorescence

Interfering substance	Tolerance ratio in mass	Change in fluorescence intensity/%
Vitamin C	25	−3.9
Vitamin B_2	0.05	0.07
Vitamin B_6	250	−2.6
glucose	1000	−2.2
Serine	50	−2.8
leucine	100	−2.0
L-cysteine chloride	100	−2.0
D-L-tryptophan	40	−2.2
Zn^{2+}	100	−2.8
Cu^{2+}	50	−1.1
Ca^{2+}	50	−0.28
K^+	800	−1.7

Table 3 Recoveries of VB_{12} in the sample

Sample	Content (mg/mL)	Amount added (mg/mL)	Amount found (mg/mL)[a]	Recovery (%)	RSD (%)
Injection of VB_{12}	6.0×10^{-3}	4.0×10^{-3}	1.01×10^{-2}	101.0	1.3
		8.0×10^{-3}	1.41×10^{-2}	101.1	1.5
		1.2×10^{-2}	1.81×10^{-2}	100.9	1.2

[a]Average value of three determination.

the excited single state was decreased obviously and the fluorescence intensity was obvious enhancement.

The interactions of ES-HP-β-CD and VB_{12}

The absorption peak wavelength of VB_{12} was at 550 nm when the pH was at 6.0 (Fig. 9a). The fluorescence emission wavelength of ES-HP-β-CD was at 549 nm (Fig. 9b). Thus, the absorbance spectrum of VB_{12} had a great overlap with the fluorescence spectrum of ES-HP-β-CD. Thereby, VB_{12} could effectively absorb the fluorescence of ES-HP-β-CD and make the fluorescence intensity of the system diminished. Moreover, it was found that there was a linear relationship between the fluorescence quenching value ΔF and the concentration of VB_{12}. Based on a novel fluorescence quenching method for the determination of VB_{12} had been developed.

Interferences

The effects of different foreign substrates were discussed in the determination of the 3.0×10^{-6} μmol/L of VB_{12}. The results are shown in Table 2. It was showed that most of the water-solube vitamin, amino acids and metal ion did not influence the determination of VB_{12}. So the method had a good selectivity.

Analytical performance

The calibration graph for the determination of VB_{12} was obtained under the experimental conditions above described. A good linear relationship was observed over the range of $0.0 \sim 2.1 \times 10^{-5}$ mol/L. The linear equation was $\Delta F = -0.57 + 7.17c$ (μg/mL), $r = 0.9996$. The detection limit estimated (S/N = 3) was $1.8 \times 10^{-}7$ mol/L. The recoveries are presented in Table 3. The recoveries were between 100.9% and 101.1%.

Table 4 The results of determination of the sample

Sample	Marked content	Proposed method	pharmacopoeial method
Injection (mg/mL)	0.50	0.497 ± 0.005	0.508 ± 0.004

Sample analysis

The proposed method was applied for the determination of VB_{12} in injections. The results were compared with the pharmacopoeial method (UV-vis spectrophotometry) [15]. The results were shown in Table 4. The statistical *t*-test was used to compare the results from both methods, which showed that there were not significantly different between them.

Conclusion

HP-β-CD could form inclusion complex with ES and the fluorescence intensity of ES was enhanced. But the fluorescence intensity of the system was diminished with the VB_{12} added. Based on the ES-HP-β-CD system a novel fluorescence quenching method for the determination of VB_{12} had been developed. The method has been successfully applied to the determination of VB_{12} in injections with satisfactory results.

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