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Determination of vitamin B_{12} in multivitamin tablets and fermentation medium by high-performance liquid chromatography with fluorescence detection

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Abstract

A novel method for the determination of vitamin B_{12} by high-performance liquid chromatography with fluorescence detection is reported. The method was simple and highly sensitive with good precision. Vitamin B_{12} was analyzed by HPLC on a μBondapak C_{18} column (300×3.9 mm, 10 μm) with methanol–water (30:70) as mobile phase and fluorescence detection at 305 nm (with excitation at 275 nm). The calibration graph was linear from 1.000 to 100.0 ng ml^{-1} for vitamin B_{12} with a correlation coefficient of 0.998 ($n=6$). The detection limit was 0.1 ng ml^{-1}. The method was successfully applied to the determination of vitamin B_{12} in vitamin B_{12} tablets, multivitamin tablets and fermentation medium. The recovery was from 94 to 102% and the relative standard deviation was in the range of 1.8 to 4.1%.

Keywords: Pharmaceutical analysis; Vitamins

1. Introduction

Vitamin B_{12} (cyanocobalamin) has an important function in human physiology. Vitamin B_{12} deficiency in humans is manifested by an anaemia and a neuropathy. Naturally occurring vitamin B_{12} originates solely from synthesis by bacteria and other microorganisms growing in soil or water, in sewage, and in the rumen and intestinal tract of animals. Seaweed can release or uptake vitamin B_{12} [1,2]. In the literature, several methods including microbiological assay [3], spectrophotometry [4], chemiluminescence [5], atomic absorption spectrometry [6], capillary electrophoresis [7] and high-performance liquid chromatography (HPLC) [8–12] have been proposed for the determination of vitamin B_{12}. Microbiological method has been used for the routine analysis of vitamin B_{12}. However, this method is tedious and time-consuming because it requires that the tissue is cultured and preserved. Spectrophotometry is not suitable for a complex sample matrix and has low sensitivity. Vitamin B_{12} could be determined indirectly by chemiluminescence and atomic absorption spectrometry by determining the cobalt, but the interference could be arisen from cobalt out of vitamin B_{12}. Barbera Saez et al. [13] compared the determination of vitamin B_{12} in milk formulae for infants by microbiological

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method and atomic absorption spectrometry, and concluded that no correlation was found between cobalt and vitamin B_{12} content. Capillary electrophoresis had low sensitivity for the determination of vitamin B_{12}. The most common method for the determination of vitamin B_{12} was reversed-phase HPLC with isocratic or gradient elution. However, the sensitivity of HPLC with UV detection was low because vitamin B_{12} lacks an adequate chromophore. When using HPLC with flame atomic absorption spectrometric or inductively-coupled plasma mass spectrometric detection [14,15] for the determination of vitamin B_{12}, the sensitivity of the methods was not high. In this paper, we report a novel method for the determination of vitamin B_{12} by HPLC with fluorescence detection. The proposed method was simple and highly sensitive. The method was applied to determine vitamin B_{12} in vitamin B_{12} tablets, multivitamin tablets and fermentation medium.

2. Experimental

2.1. Apparatus

The HPLC system used throughout this study consisted of a Waters 510 pump (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, and a Waters 474 scanning fluorescence detector. Evaluation and quantification were made on a Millenium chromatography data system (Waters). The column used was a reversed-phase μBondapak C_{18} column (300×3.9 mm I.D., 10 μm, Waters).

2.2. Reagents

All solutions were prepared with analytical-reagent grade compounds. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. The phosphate buffer solution was prepared by the addition of 0.100 mol l^{-1} potassium dihydrogen phosphate (Riedel-de Haën, Germany) and 0.100 mol l^{-1} potassium hydroxide (BDH, UK) for pH 7.0. The vitamin B_{12} stock solution was 1.000 mg ml^{-1} which was prepared by dissolving 0.1000 g of vitamin B_{12} (Sigma, USA) in 100 ml of the phosphate buffer solution and stored in the dark at 4°C. The working solutions were prepared by suitable dilution of the stock solutions with the phosphate buffer solution. All other solutions were prepared by dissolving appropriate amounts of commercially available chemicals in water.

The vitamin B_{12} tablets and multivitamin tablets were purchased from a local drug store. Each vitamin B_{12} tablet contains 25 μg of vitamin B_{12}, and each multivitamin tablet contains 2.0 mg of vitamin B_1, 1.0 mg of vitamin B_2, 1.0 mg of vitamin B_6 and 10.0 μg of vitamin B_{12}.

Two microalgae, *Crypthecodinium cohnii* and *Nitzschia laevis* grown in our laboratory can accumulate high concentrations of polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively. Vitamin B_{12} as an ingredient in medium is required by the microalgae. The fermentation media (a) and (b) were obtained from *Crypthecodinium cohnii* and *Nitzschia laevis*, respectively.

2.3. Procedure

All chromatographic separations were carried out at ambient temperature. The mobile phase was methanol–water (30:70) and the flow-rate was 0.80 ml min^{-1}. The injection volume was 20 μl. The column eluate was monitored with a fluorescence detector at 305 nm (with excitation at 275 nm). The mobile phase was filtered through a 0.45-μm membrane and degassed by sonication prior to use.

The tablets analyzed were treated individually, dissolving in a 50-ml volume of 0.100 mol l^{-1} phosphate buffer solution (pH 7.0) [16]. Then, the solution was diluted gradually to a 1:500 concentration with the phosphate buffer solution. The fermentation medium was centrifuged to remove solid particles. The sample solution was also filtered through a 0.45-μm membrane filter before injection.

3. Results and discussion

A reversed-phase μBondapak C_{18} column was chosen for the separation of vitamin B_{12} from the other substances in the samples. Vitamin B_{12} was retained on the column quite firmly and it was eluted only with the use of 30% aqueous methanol solution,

and this finding agreed with that reported in the literature [12]. Thus, the 30% aqueous methanol solution was chosen as mobile phase and the flow-rate was set at 0.80 ml min^{-1}. The maximum excitation and emission wavelengths were 275 nm and 305 nm, respectively. The wavelength, 305 nm was chosen as fluorescence detection wavelength with excitation at 275 nm. Under these conditions, the representative chromatograms of vitamin B_{12} in standard solution, multivitamin tablets and fermentation medium (a) are shown in Figs. 1, 2a and b, respectively. The chromatograms of vitamin B_{12} in vitamin B_{12} tablets and fermentation medium (b) were similar to that of the multivitamin tablets (data not shown).

A calibration graph obtained was linear from 1.000 to 100.0 ng ml^{-1} for vitamin B_{12} with a correlation coefficient of 0.998 ($n=6$). The detection limit ($S/N=3$ [17]) was 0.1 ng ml^{-1}. The relative standard deviation (RSD) was 2.1% for determination of 20.00 ng ml^{-1} vitamin B_{12} standard solution ($n=8$).

The interference of a number of different substances was studied by spiking 10.0 ng ml^{-1} of vitamin B_{12} with known quantities of foreign materials and analyzing it by the present method. No

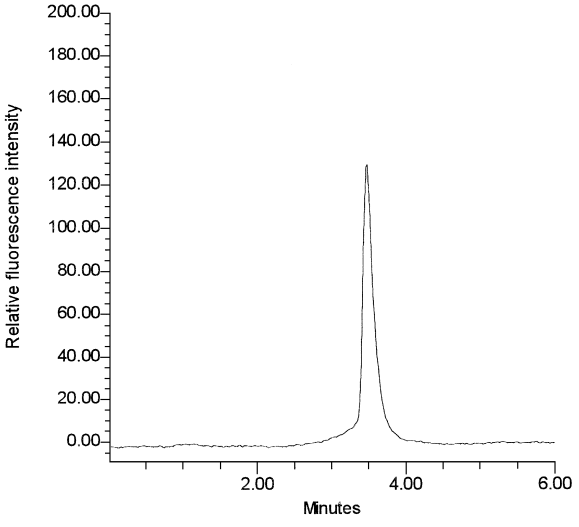

Fig. 1. Chromatogram of vitamin B_{12} standard at 25.00 ng ml^{-1}. Conditions: column, μBondapak C_{18}; eluent, methanol–water (30:70); flow-rate, 0.80 ml min^{-1}; detection, fluorescence at 305 nm (with excitation at 275 nm); injection volume, 20 μl.

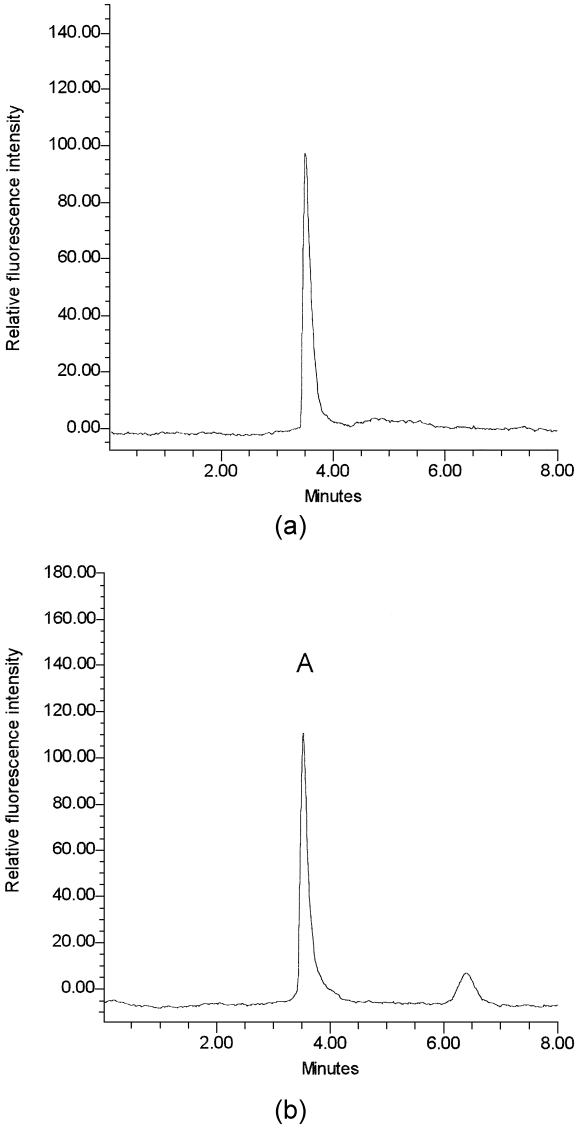

Fig. 2. Chromatogram of vitamin B_{12} in multivitamin tablets (A) and fermentation medium (a) (B). Conditions: column, μBondapak C_{18}; eluent, methanol–water (30:70); flow-rate, 0.80 ml min^{-1}; detection, fluorescence at 305 nm (with excitation at 275 nm); injection volume, 20 μl; peak, A=vitamin B_{12}. The concentrations of vitamin B_{12} in multivitamin tablets solution and fermentation medium (a) were 19.6 ng ml^{-1} and 21.8 ng ml^{-1}, respectively.

interference (relative error less than ±5%) was observed at ratios (m/m) of 1000:1 for K^+, Na^+, NH_4^+, NO_3^-, Cl^-, SO_4^{2-}, HCO_3^-, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, Al^{3+}, vitamin B_1, vitamin B_2,

Table 1
Determination of vitamin B_{12} (VB_{12})[a]

Sample	Labeled VB_{12} (μg)	Found VB_{12} (μg)	RSD[b] (%)	Added VB_{12} (μg)	Recovery (%)
Vitamin B_{12} tablets	25.0	24.7	1.8	25.00	98
Multivitamin tablets	10.0	9.8	3.1	10.00	102
Fermentation medium (a)		21.8[c]	2.7	20.00[c]	97
Fermentation medium (b)		11.5[c]	4.1	10.00[c]	94

[a] Average of five determinations.
[b] $n=8$.
[c] ng ml^{-1}.

vitamin B_6 and vitamin C. No interference was observed which was probably due to the advantage of combining the separation of HPLC and the selectivity of fluorescence detection in the present method.

The present method was applied to determine vitamin B_{12} in vitamin B_{12} tablets, multivitamin tablets and fermentation medium. Results are given in Table 1. As shown as in Table 1, the recovery was from 94 to 102% and the RSD was in the range 1.8 to 4.1%, and the results obtained by the present method agreed with the labeled values for vitamin B_{12} tablets and multivitamin tablets.

Compared with the previous HPLC with UV [8–10] or flame atomic absorption spectrometric [14] or inductively-coupled plasma mass spectrometric detection [15], the present method was more sensitive. The detection limits were 1×10^{-4} μg ml^{-1} (by the present method), 1 μg ml^{-1} [8], 0.2 μg ml^{-1} [9] and 0.81 μg ml^{-1} [10], 4.2 μg ml^{-1} [14] and 0.01 μg ml^{-1} [15], respectively. The present method was also more sensitive than spectrophotometry [4], chemiluminescence [5], atomic absorption spectrometry [6] and capillary electrophoresis [7] for the determination vitamin B_{12}. Their detection limits were 1×10^{-4} μg ml^{-1} (by the present method), 0.22 μg ml^{-1} [4], 0.02 μg ml^{-1} [5], 0.15 μg g^{-1} [6] and 20 μg ml^{-1} [7], respectively.

In conclusion, a new method for the determination of vitamin B_{12} by HPLC with fluorescence detection was described. The fluorescence detection of vitamin B_{12} has not been reported previously, although fluorescence detection is used widely in HPLC. Fluorescence detection is more sensitive and specific than UV detection. The proposed method was simple and highly sensitive with good precision. The present method was successfully applied to the determination of vitamin B_{12} in vitamin B_{12} tablets, multivitamin tablets and fermentation media. The proposed method can be adopted as a routine analytical method for the determination of trace vitamin B_{12}.

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