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Determination of Vitamin B_{12} (Cyanocobalamin) in Fortified Foods by HPLC

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Abstract

This study was conducted to develop an HPLC method for determining vitamin B_{12} in fortified foods which has typically been determined by microbiological assays according to AOAC and Korean Food Code approved methods. Vitamin B_{12} (cyanocobalamin) was determined by reversed-phase HPLC with a triple column and UV/VIS dectector (550 nm) using the column switching technique after extraction with 5 mM potassium phosphate solution by sonication without a clean-up procedure. The recovery of spiked samples and limit of detection (LOD) by HPLC were 78.6~107.5% and 2 ppb (μg/kg), respectively. The LOD of the microbiological assay (MBA) was much lower than that of HPLC. The concentrations of vitamin B_{12} analyzed in all tested samples (n=12) confirmed compliance with declared label claims. The range of recovery ratio by the HPLC method when compared to the microbiological assay was 76.2~140.0%. There was not significant difference between the HPLC and MBA methods ($p<0.01$) with r=0.9791 and linear regression y=0.9923x-0.04. The HPLC method for determining vitamin B_{12} using the column-switching technique appears to be suitable for determining vitamin B_{12} concentrations above 1 μg/100 g in fortified foods.

Key words: vitamin B_{12}, HPLC method, microbiological assay

INTRODUCTION

Vitamin B_{12} is a water-soluble vitamin. Cobalamin is the term used to refer to compounds having vitamin B_{12} activity, as well as to related compounds (1). Vitamin B_{12} is present in animal products, such as meat, poultry, milk and fish, but is not present in plant products or in yeast. All of the vitamin B_{12} in our environment originated from synthesis by bacteria, fungi and algae (1-4).

Cyanocobalamin is the permissive name for vitamin B_{12} (4) and is the form predominantly used in vitamin preparations, supplements, medical foods and fortified foods because of its better stability compared to hydroxycobalamin (1,4).

Strict vegetarian and infants without maternal feeding should be supplied by vitamin B_{12} through nutritional supplements or infant formulas. The Korean food code specifications for vitamin B_{12} in infant and follow-up formulas are 0.1 μg/100 kcal, 0.15 μg/100 kcal, respectively (5).

It is necessary to monitor nutrients in fortified foods for nutrient labeling as well as process and quality control purposes. Hence simple, rapid, sensitive and reproducible analytical methods are required.

Available methods for assay of vitamin B_{12} include polarographic, spectrophotometric, various chromatographic procedures including paper, thin-layer, open column, GC and LC procedures, microbiological and radioligand binding procedures (4). Almost all available data of vitamin B_{12} in food has been obtained by microbiological assays (4-8). However, microbiological assays require the cells to be maintained and preserved and are cumbersome and time consuming. Furthermore, other substances or contaminates may interfere growth of microorganisms, invalidating the assay.

HPLC is used for the analyses for many kinds of vitamins (8-17). But HPLC has not been successfully used for the routine analysis of vitamin B_{12} in foods because proper protocols for sample preparation, such as the concentration of analyte of and removal of interfering substances, caused by the complex sample matrices, have not been developed and confirmed.

Therefore, this study reports the development of an analytical method for the determination vitamin B_{12} by μ-HPLC using the column-switching technique (18-23) and compares the results with a microbiological assay.

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MATERIALS AND METHODS

Reagents and materials

Vitamin B_{12} standard was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade solvents such as acetonitrile and methanol were purchased from Merck (Frankfurter, Germany) and JT Baker (New Jersey, USA).

Vitamin B_{12}-fortified foods were purchased from the department stores in Seoul. Table 1 shows the food group, the product type and the label claim of the fortified foods.

Apparatus

An HPLC (Nanospace SI-2, Shiseido, Tokyo, Japan) equipped with an autosampler, pump, UV detector, PDA detector and valve system was used. Other equipment included a sonicator (Branson 8210, Connecticut, USA), centrifuge (CR21E, Hitacchi, Tokyo, Japan) and UV detector (Biochrom 4060, Pharmacia, Cambridge, USA).

Sample preparation

Each sample (equivalent to 200 ng of vitamin B_{12}) was suspended in 5 mM potassium phosphate solution. The mixture was extracted by sonication for 10 min and then made to volume (50 mL) with phosphate solution and centrifuged at 23,000×g for 10 min. The middle layer was collected and again centrifuged at 23,000×g for 10 min. The middle layer was transferred to a test tube, chloroform (3 mL) was added to remove lipids and centrifuged at 1,000×g for 10 min. The aqueous layer was then collected and centrifuged at 23,000×g for 10 min and the clear layer was passed through a 0.45 μm membrane filter and analyzed by HPLC.

HPLC analysis

The μ-HPLC system used in this study is illustrated in Fig. 1 and the operating conditions are shown in Table 2. A triple column was used for pretreatment, concentration and separation. Pumps 1 and 2 were used to deliver eluent A at a flow rate of 120 μL/min and eluent B at a flow rate of 500 μL/min, respectively. In the initial step, a sample solution was introduced to the pretreatment col umn via the autosampler using eluent B. By switching the valve as shown in Fig. 1 (B), vitamin B_{12} is eluted from the pretreatment column and introduced to the concentrat ion column. Finally vitamin B_{12} adsorbed in the concentr ation column is introduced to the separation column by switching the valve as shown in Fig. 1 (A) using eluent A. Total analytical time was 40 minutes per a sample.

Microbiological assay

Vitamin B_{12} was also analyzed by the microbiological as

Table 1. Selected samples for analysis

Sample	Food group	Product type	Label claims (μg/100 g)
1	Infant formula	Powder	2.0
2	Infant formula	Powder	2.0
3	Follow up formula	Powder	2.0
4	Follow up formula	Powder	3.7
5	Cereal based infant formula	Powder	2.0
6	Cereal based infant formula	Powder	1.1
7	Cereal based infant formula	Powder	2.0
8	Cereal based infant formula	Powder	2.0
9	Medical food	Powder	2.0
10	Nutritional supplement product	Tablet	50 (0.3 μg/1 tablet)
11	Nutritional supplement product	Tablet	1689 (25 μg/1 tablet)
12	Snack	Flake	1.5

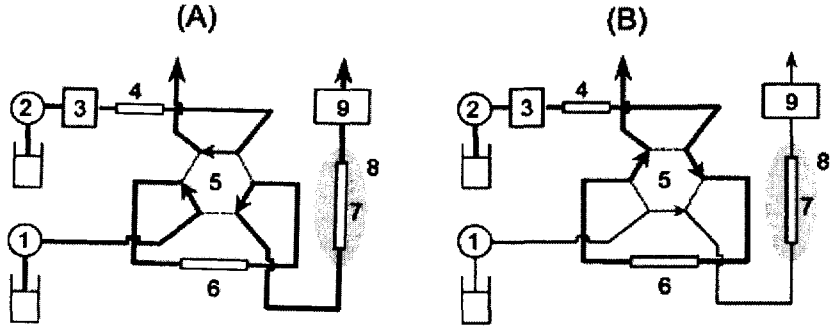

Fig. 1. Schematic diagram of the μ-HPLC system (23). (A) Separation position, (B) Concentration position.
1, pump (for separation); 2, pump (for pretreatment); 3, autosampler; 4, pretreatment column; 5, switching valve; 6, concentration cloumn; 7, analytical column; 8, column oven; 9, UV detector.

Table 2. HPLC operating conditions for vitamin B_{12} (cyanocobalamin) determination

Detector	UV (550 nm)
Mobile phase	A: 5 mM KH_2PO_4/MeOH = 80/20 B: 5 mM KH_2PO_4
Flow rate	120 μL/min for pump 1 500 μL/min for pump 2
Temp.	40°C
Column	Pre-separation column: Capcellpak MF C_8 (4.6 mm×150 mm, 5 μm) Focusing column: Capcellpak MG C_{18} (2.0 mm×35mm, 5 μm) Analytical column: Capcellpak UG C_{18} (1.5 mm×250 mm, 5 μm)
Inj. vol.	400 μL

say as described in the procedures of Korean Food Code (6).

RESULTS AND DISCUSSION

The extraction and analytical conditions

Because vitamin B_{12} (cyanocobalamin) is water soluble and occurs in a free form, potassium phosphate solution at concentration 5 mM was sufficient to extract analyte. A 30 minute extraction time was sufficient to obtain maximum recovery.

Although the PDA spectrum for vitamin B_{12} had a maximum peak at 361 nm, peak separation was very difficult because of matrix interferences. Not only vitamin B_{12} but also other vitamins, amino acids and organic acids absorb in the UV region (9). It was reported that vitamin B_{12} shows UV absorbance at 260 and 360 nm and visible absorbance at 550 nm. However, thiamine, riboflavin, ascorbic acid, nicotinamide, pyridoxine, folic acid, tyrosine, phenylalanine and tryptophan have absorbances at 260 nm and several compounds, such as folic acid and riboflavin, have absorbance at 360 nm (4, 8,9). Hence, monitoring at 550 nm was suitable for the determination of vitamin B_{12}.

The determination of vitamin B_{12} was performed by the column-switching technique using a triple column system as described in earlier reports (18,19), and appears to be a sensitive and effective technique.

Recovery, detection limits, and comparison with a microbiological method

The calibration curve from the vitamin B_{12} standard showed good linearlity (r=0.9999) in the range of 1.0~200 μg/kg. The results in Table 3 demonstrate that the recovery of vitamin B_{12} was 78.6~107.5% by standard addition method. The spiking concentrations were varied with the amount of vitamin B_{12} in the samples; the amounts (20~1000 ng) are shown in Table 3.

The limit of detection (LOD, S/N=3) was 2 ppb (μg/kg) in all tested samples. The HPLC chromatograms of the vitamin B_{12} standard and samples are shown in Fig. 2; the vitamin B_{12} was obtained at 19 min. The limit of quantification, five times of LOD, was 1 μg/100 g, therefore this HPLC method could be applicable for assays of foods fortified with vitamin B_{12} above this level.

The HPLC method was found to have a higher LOD than the microbiological assay and showed large differences among samples in high concentrations of analyte. However, the concentration range with linearity was narrow and errors caused by dilution effects may be problematic in the microbiological assay.

The concentrations of vitamin B_{12} analyzed by both methods in all tested samples confirmed compliance with declared label claims.

The accuracy of HPLC analysis was assessed by com-

Table 3. Recoveries and detection limits for vitamin B_{12} (cyanocobalamine)

Sample	Microbiological assay			HPLC method		
	Added amount (ng)	Recovery (%)	LOD (μg/kg)[1)]	Added Amount (ng)	Recovery (%)	LOD (μg/kg)
1	30	82.2±6.2[2)]	0.0012	200	89.1±6.2	2.0
2	30	106.1±6.3	0.0012	200	84.6±8.5	2.0
3	30	81.9±4.3	0.0012	200	90.1±5.1	2.0
4	30	101.7±9.9	0.0012	400	107.5±6.1	2.0
5	30	87.0±9.7	0.0012	200	78.6±6.5	2.0
6	30	108.5±4.8	0.0012	200	85.0±4.3	2.0
7	30	104.0±1.4	0.0012	200	93.9±4.1	2.0
8	30	106.4±7.6	0.0012	200	86.6±2.6	2.0
9	30	82.8±4.6	0.0012	200	80.0±2.2	2.0
10	30	102.2±7.2	0.0012	1000	104.1±8.5	2.0
11	30	82.3±6.8	0.0012	2000	87.7±7.1	2.0
12	30	105.6±4.9	0.0012	200	88.9±6.5	2.0

[1)]LOD: The limit of detection for S/N=3.
[2)]Mean±SD, results of three replicates.

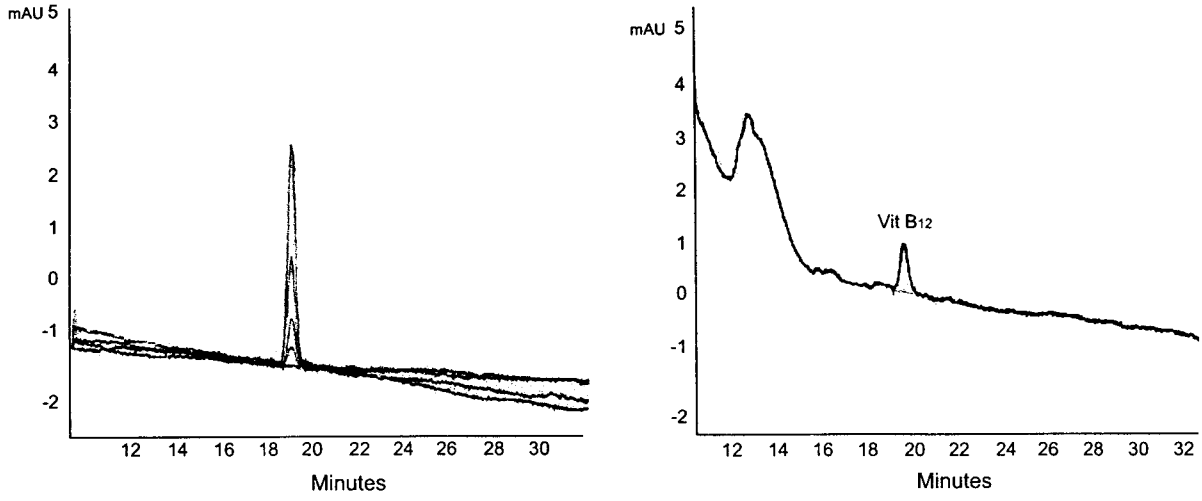

Fig. 2. HPLC chromatograms of vitamin B_{12} using a UV/VIS detector at 550 nm: (a) standard (2~20 ppb) chromatogram, (b) sample No. 6 chromatogram obtained with triple column system.

parison of vitamin B_{12} levels against the microbiological assay for a range of fortified foods and the data are shown in Table 4. The recovery by the HPLC method compared to microbiological method was 76.2~140.0% and there was no significant difference ($p<0.01$), with r=0.9791 and linear regression y=0.9923x-0.04, between the two methods (Fig. 3).

In conclusion, HPLC using the column-switching technique was a useful tool for the separation and determination of vitamin B_{12} in foods because it was simple and rapid with a high degree of recovery. Moreover, this method was shown to have a good correlation with microbiological assays which has been accepted as an official method.

As a result, this method could be used in monitoring and quality control of infant formulas, follow-up formulas, and nutritional supplement products because of its efficiency.

Further studies on concentration and clean-up techniques would be required so that this HPLC method could be used to analyze vitamin B_{12} in all forms of foods.

Table 4. Comparisons for vitamin B_{12} between microbiological assay (MBA) and HPLC method

SA	Label claim (μg/100 g)	MBA[1)] (μg/100 g)	HPLC (μg/100 g)	HPLC/MBA (%)
1	2.0	2.6±0.3[2)]	2.8±1.0	107.7
2	2.0	3.4±0.2	2.9±0.5	85.3
3	2.0	2.4±0.1	2.7±0.1	112.5
4	3.7	5.2±0.4	5.4±1.0	103.8
5	2.0	2.1±0.3	2.1±0.2	100.0
6	1.1	2.1±0.3	1.6±0.7	76.2
7	2.0	2.1±0.1	2.0±0.7	95.2
8	2.0	4.1±0.2	3.8±0.2	92.7
9	2.0	2.1±0.1	2.2±0.7	104.8
10	50.0	98.0±7.7	101.7±56.0	103.8
11	1689	1803±46	2089±330	115.9
12	1.5	4.0±0.2	5.6±0.2	140.0

[1)]Microbiological assay.
[2)]Mean±SD, results of three replicates.

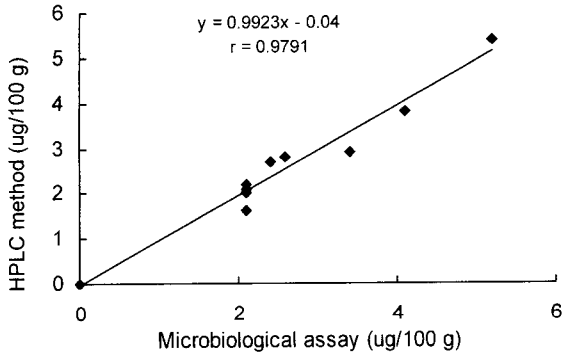

Fig. 3. The correlation between microbiological assay (MBA) and HPLC method. The values of sample No. 10 & 11 were excluded in this regression curve because of their high concentrations.

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