# Method for Quantitation of Total Vitamin $B_{12}$ in Foods Using a Highly Fluorescent Vitamin $B_{12}$ Derivative

Fumio Watanabe,*,† Katsuo Abe,† Shigeo Takenaka,‡ Tomoyuki Fujita,§ and Yoshihisa Nakano§

Department of Food and Nutrition, Kochi Women's University, Kochi 780, Japan, Laboratory of Nutrition and Food Science, Hagoromo-gakuen College, Sakai 592, Japan, and Department of Applied Biological Chemistry, Osaka Prefecture University, Sakai 593, Japan

A highly fluorescent vitamin $B_{12}$ derivative was readily prepared with a fluorescent reagent [6,7-dimethoxy-1-methyl-2(1*H*)-quinoxaline-3-propionylcarboxylic acid hydrazide] specific for carboxylic acids. Hydroxovitamin $B_{12}$ was reduced by $NaBH_4$, and then 4-chlorobutyric acid was added to form carboxypropyl vitamin $B_{12}$, which was labeled with the fluorescent reagent in the presence of carbodiimide. An assay for vitamin $B_{12}$ with the fluorescent vitamin $B_{12}$ derivative and hog intrinsic factor was devised. Vitamin $B_{12}$ could be assayed over a range of 0.2−10 $\mu$g/L by this method, which was useful for vitamin $B_{12}$ assay in foods.

**Keywords:** *Vitamin $B_{12}$; cobalamin; fluorescence assay; intrinsic factor*

## INTRODUCTION

Historically, vitamin $B_{12}$ ($B_{12}$) contents of foods have been determined by bioassay with $B_{12}$-requiring microorganisms, *Escherichia coli*, *Lactobacillus leichmannii*, *Euglena gracilis* Z, or *Ochromonas malhamensis*; the values obtained by the microbiological assay depend on the organism used (Schneider, 1987a). The assay procedures are technically difficult. In addition, radioisotope dilution (RID) assay with radiolabeled $B_{12}$ and hog intrinsic factor (IF), the most specific $B_{12}$-binding protein, has been used in the determination of $B_{12}$ contents of foods because several kits for the RID assay are commercially available (Bennink and Ono, 1982). Recently, a chemiluminescent labeled $B_{12}$ derivative was used instead of a radioactive label for clinical assay of human blood. However, it is costly for workers in nutrition and food sciences to obtain specialized instruments and kits to assay $B_{12}$ in foods.

In this paper we developed a highly fluorescent $B_{12}$ derivative and used it to assay total $B_{12}$ in foods.

## MATERIALS AND METHODS

**Materials.** Hydroxo-$B_{12}$ (OH-$B_{12}$), cyano-$B_{12}$ (CN-$B_{12}$), cobinamide, and IF were obtained from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 for column chromatography was obtained from Merck (Darmstadt, Germany). A reversed-phase HPLC column (Wakosil-II 5C18RS, Ø 4.6 × 150 mm; particle size, 5 $\mu$m), 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxaline-3-propionylcarboxylic acid (DMEQ) hydrazide, $NaBH_4$, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, and HPLC grade methanol and distilled water were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). 4-Chlorobutyric acid and $D_2O$ were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). Amberlite XAD-4 was obtained from Japan Organo Co. (Tokyo, Japan). A prepacked gel filtration column (Econo-pac 10DG) was purchased from Bio-Rad Laboratories (Richmond, CA). All other reagents used were of the highest purity commercially available. Raw beef and pork and pasteurized cow's milk were purchased from a local market in Kochi-city, Japan. A Shimadzu (Kyoto, Japan) UV−visible spectrophotometer (UV-1600) was used for measuring absorbance of $B_{12}$ analogues. A Shimadzu HPLC apparatus (LC6A pump, RE500LCA fluorescence spectrometer, UV-1600 UV−visible spectrophotometer, CTO-6A column oven, C-RA data processor) was used for purification of the DMEQ-labeled $B_{12}$. A Shimadzu spectrofluorophotometer (RF-5000), a Hitachi (Tokyo, Japan) spectrophotometer (200-10), and an automated chemiluminescent $B_{12}$ analyzer ACS-180 (Chiron Diagnostics, East Walpole, MA) were used for $B_{12}$ assay.

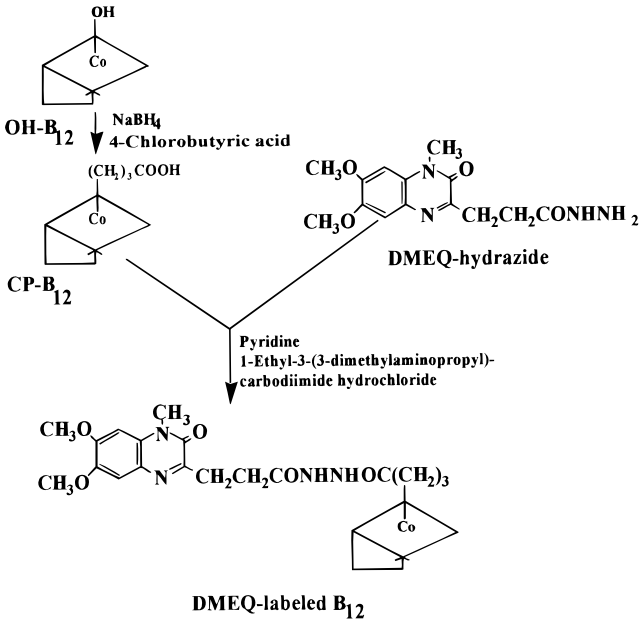


**Figure 1.** Outline of preparation of DMEQ-labeled $B_{12}$.

**Preparation of Fluorescent Vitamin $B_{12}$ Derivative.** The preparation of the fluorescent (DMEQ-labeled) $B_{12}$ derivative is summarized in Figure 1. OH-$B_{12}$ was reduced by $NaBH_4$, and then 4-chlorobutyric acid was added to form carboxypropyl-$B_{12}$ (CP-$B_{12}$) as described by Sato et al. (1978); in brief, OH-$B_{12}$ (0.0138 g) was dissolved in 5 mL of distilled water and bubbled with $N_2$ gas for 20 min. $NaBH_4$ (40 mg) was added to the solution, which was further bubbled with $N_2$ gas for 10 min. 4-Chlorobutyric acid (0.012 g) was added to the solution, which then was neutralized by the addition of 1 mol/L HCl to pH 6−7. The solution was put on a column

* Author to whom correspondence should be addressed (telephone and fax +81-888-31-2876; e-mail watanabe@cc.kochi-wu.ac.jp).
† Kochi Women's University.
‡ Hagoromo-gakuen College.
§ Osaka Prefecture University.

S0021-8561(97)00313-0 CCC: $14.00 

(25 × 50 mm) of Amberlite XAD-4 equilibrated with acetic acid solution (water/acetic acid = 100:1) and washed with 200 mL of the same solution. The reaction products were eluted with 150 mL of 80% (v/v) ethanol, evaporated to dryness, and dissolved in 2 mL of 1-butanol/2-propanol/water (10:7:10). This solution was put on a column (15 × 150 mm) of silica gel 60 equilibrated with 1-butanol/2-propanol/water (10:7:10) and eluted with the same solution to remove OH-$B_{12}$, which binds with the top of the gel. The column eluate was collected at 4 mL with a Bio-Rad Laboratories fraction collector (Model 2110). Red-colored fractions were combined, evaporated to dryness, and dissolved with distilled water. All procedures were done in the dark. The concentration of the CP-$B_{12}$ prepared was calculated by measuring the absorbance of aqua-$B_{12}$ ($AqB_{12}$) at 527 nm [$\epsilon = 8.5 \times 10^3$ (mol/L)$^{-}$·cm$^{-1}$] (Schneider, 1987b) after CP-$B_{12}$ was photolyzed completely to form $AqB_{12}$ by a tungsten lamp (100 W) for 30 min. Aq- and OH-$B_{12}$ are interconvertible in solution depending on pH; below pH 8.0, $AqB_{12}$ tends to predominate over OH-$B_{12}$ at equilibrium (Schneider, 1987b).

The CP-$B_{12}$ was labeled with the fluorescence reagent (DMEQ-hydrazide) specific for carboxylic acids according to the method of Yamaguchi et al. (1990); in brief, to 100 $\mu$L of 5 mmol/L CP-$B_{12}$ solution were added 50 $\mu$L each of 2 mol/L 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride and 10% (v/v) pyridine and 100 $\mu$L of 4.9 mmol/L DMEQ-hydrazide/*N*,*N*-dimethylformamide solution. The mixture was gently stirred for 30 min at 30 °C. The mixture was put on a column (25 × 50 mm) of Amberlite XAD-4 equilibrated with acetic acid solution (water/acetic acid = 100:1) and washed with 200 mL of the same solution. The DMEQ-labeled $B_{12}$ was eluted with 150 mL of 80% (v/v) ethanol, evaporated to dryness, and dissolved in 1.0 mL of distilled water. All procedures were done in the dark.

The DMEQ-labeled $B_{12}$ formed was isolated with a Shimadzu HPLC apparatus (LC6A pump, RE500LCA fluorescence spectrometer, UV-1600 UV−visible spectrophotometer, CTO-6A column oven, C-RA data processor). The solution was put on a reversed-phase HPLC column (Wakosil-II 5C18RS, ∅ 4.6 × 150 mm; particle size, 5 $\mu$m) equilibrated with 20% (v/v) methanol solution containing 1% (v/v) acetic acid at 35 °C at a flow rate of 1 mL/min. The DMEQ-labeled $B_{12}$ was eluted with a linear gradient (40 mL) of 20−70% (v/v) methanol in the same 1% (v/v) acetic acid solution.

Fluorescence intensities of DMEQ-labeled compounds were monitored at an extinction wavelength of 365 nm and an emission wavelength of 447 nm. Other $B_{12}$ analogues were monitored at an absorbance of 527 nm. The fluorescent compound and $B_{12}$ were coeluted in a peak with retention time of 24 min. Retention times of authentic DMEQ-hydrazide and prepared CP-$B_{12}$ were 16 and 19 min, respectively. The peak fractions (1 mL) with retention time of 24 min were combined, evaporated to dryness, and dissolved in 1.0 mL of distilled water. Concentration of the DMEQ-labeled $B_{12}$ prepared was calculated by measuring the absorbance of $AqB_{12}$ at 527 nm [$\epsilon = 8.5 \times 10^3$ (mol/L)$^{-1}$·cm$^{-1}$] after the DMEQ-labeled $B_{12}$ was photolyzed completely to form $AqB_{12}$ by the tungsten lamp (100 W) for 30 min.

**$^1$H-NMR Spectra.** The spectra were measured on a JEOL (Tokyo, Japan) JNM-A500 at 500 MHz in $D_2O$; HDO was the internal reference: chemical shifts with $\delta$ (HDO) = 4.71 ppm. $^1$H-NMR spectrum of the DMEQ-labeled $B_{12}$ in $D_2O$: $\delta$ 7.33 (1H, s), 7.03 (2H, s), 6.80 (1H, s), 6.12 (1H, d, $J$ = 2.4 Hz), 6.06 (1H, s), 5.83 (1H, s), 3.95 (3H, s), 3.88 (3H, s), 3.73 (3H, s), 3.06 (2H, t, $J$ = 6.7 Hz), 2.77 (2H, t, $J$ = 6.7 Hz), 2.16 (3H, s), 2.10 (3H, s), 2.08 (3H, s), 2.07 (3H, s), 1.48 (3H, s), 1.29 (3H, s), 1.28 (3H, s), 1.09 (3H, d, $J$ = 6.1 Hz), 0.94 (3H, s), 0.86 (3H, s), and 0.37 (3H, s).

**Extraction of Vitamin $B_{12}$.** Ten grans each of raw beef and pork was homogenized in 50 mL of distilled water using a universal homogenizer (Nihon Seiki Seisakusho Co., Tokyo, Japan). Total $B_{12}$ was extracted from the homogenates by the method of boiling with KCN at acid pH (Frenkel et al., 1980); specifically, 10 mL of 0.5 mol/L acetate buffer, pH 4.8, and 20 mg of KCN were added to the beef and pork homogenates, which were boiled for 30 min at 98 °C in the dark. These homogenates were centrifuged at 10000*g* for 10 min. The supernatant was used for the $B_{12}$ assay. In the case of cow's milk, 100 mL of 0.2 mol/L acetate buffer, pH 4.8, and 20 mg of KCN were added to 100 mL of the milk, boiled for 30 min at 98 °C in the dark, and then centrifuged at 10000*g* for 10 min. The supernatant was used for the $B_{12}$ assay.

**Assay of Vitamin $B_{12}$.** The assay mixture (1.0 mL) contained 10 mmol/L potassium phosphate buffer, pH 7.0, 1.4 ng of the DMEQ-labeled $B_{12}$, authentic CN-$B_{12}$ or sample (0−12 ng), and 1 unit of IF. The IF used was preincubated with 30 ng of cobinamide for 30 min at room temperature to remove the $B_{12}$ binding by non-IF binder contaminants in the reagent. The mixture was incubated for 10 min at room temperature in the dark, immediately put on a prepacked gel filtration column (Econo-pac 10DG, Bio-Rad Laboratories), which was equilibrated with 10 mmol/L potassium phosphate buffer, pH 7.0, containing 0.1 mol/L KCl, and eluted with 4.0 mL of the same buffer. The column eluant was collected, and its fluorescence intensity was measured at an excitation wavelength of 365 nm and an emission wavelength of 447 nm with a Shimadzu spectrofluorophotometer (RF-5000). The amount of the DMEQ-labeled $B_{12}$ bound to IF was calculated by subtracting fluorescence intensity in the absence of IF (control) from that in the presence of IF. A best fit standard curve for the $B_{12}$ assay was drawn with GraphPad PRISM 2.0 (GraphPad Software, San Diego, CA).

$B_{12}$ was also assayed by the bioassay with *Lactobacillus leichmannii* ATCC 7830 and a $B_{12}$ assay medium (Nissui, Tokyo, Japan) according to the manufacturer's instructions, and by an automated chemiluminescent $B_{12}$ analyzer ACS-180 (Chiron Diagnostics). The above $B_{12}$ extracts were diluted with distilled water up to $B_{12}$ concentration ranges of 0.01−0.2 and 0.05−2 $\mu$g/L for the microbiological and chemiluminescent assay methods, respectively.

Statistical analysis was performed using GB-STAT 5.4 (Dynamic Microsystems, Inc., Silver Spring, MD). One-way ANOVA was used with post-hoc Tukey/Kramer procedure. Differences were considered significant if $P < 0.01$.

## RESULTS AND DISCUSSION

**Preparation of a Fluorescent $B_{12}$ Derivative.** The mammalian $B_{12}$-binding protein, IF, specifically recognizes the structure of the $B_{12}$ molecule but is less selective with regard to substitution of the $\beta$-ligand of $B_{12}$ (Gräsbeck, 1967). Thus, a $B_{12}$ derivative with the fluorescent (DMEQ) moiety as the $\beta$-ligand was prepared and applied to total $B_{12}$ assay in foods.

More than 96% of OH-$B_{12}$ was converted to CP-$B_{12}$, which was labeled with DMEQ-hydrazide in a yield of about 50%. The DMEQ-labeled $B_{12}$ had a high quantum efficiency; 1 $\mu$g/L of the $B_{12}$ solution showed fluorescence intensity of 0.1. In the $^1$H-NMR spectrum of the DMEQ-labeled $B_{12}$, two aromatic protons ($\delta$ 7.03 and 6.80), two methoxy groups ($\delta$ 3.95 and 3.88), one singlet methyl ($\delta$ 3.73), and two methylene groups [$\delta$ 3.06 (t, $J$ = 6.7 Hz) and 2.77 (t, $J$ = 6.7 Hz)] were observed; these signals show the presence of the DMEQ moiety in the fluorescent $B_{12}$ derivative. The photolytic cleavage of the Co−C bond in alkyl-$B_{12}$ has been reported; under aerobic conditions alkyl-$B_{12}$ is converted to OH-$B_{12}$ or $AqB_{12}$ (Schneider, 1987c). Each absorption peak of CP-$B_{12}$ (510 nm) and DMEQ-labeled $B_{12}$ (498 nm) at visible region was changed to that of $AqB_{12}$ (527 nm) by exposure to a tungsten lamp (100 W) for 30 min (data not shown), suggesting that CP and DMEQ groups are in the $\beta$-ligand of the prepared $B_{12}$ derivatives, respectively. The results indicate that DMEQ-hydrazide is a highly sensitive fluorescence reagent and suitable for preparation of a fluorescent $B_{12}$ derivative.

The DMEQ-labeled $B_{12}$ was slightly unstable in solution even if stored at −40 °C in the dark; it was

**Table 1. Vitamin $B_{12}$ Contents of Foods by the Fluorescence Assay**

| | vitamin $B_{12}$ content[a] ($\mu$g/100 g) | | | |
|---|---|---|---|---|
| | fluorescence assay | chemiluminescence assay | bioassay | reported[b] |
| beef | $4.89 \pm 0.29^a$ (6) | $2.54 \pm 0.01^b$ (4) | $3.15 \pm 0.31^c$ (3) | 2−8 |
| pork | $1.05 \pm 0.17^a$ (6) | $0.70 \pm 0.11^b$ (4) | $1.85 \pm 0.14^c$ (3) | 0.1−5 |
| cow's milk | $0.51 \pm 0.03^a$ (6) | $0.82 \pm 0.02^b$ (4) | $0.86 \pm 0.06^b$ (3) | 0.2−0.6 |

[a] All values obtained represent mean ± SD. Numbers of experiments are given in parentheses. Different letters denote significant differences ($P < 0.01$). Detailed procedures were described in the text. [b] Cited in Schneider (1987a).

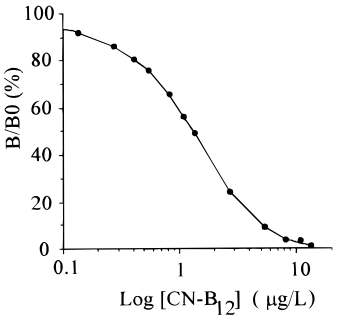


**Figure 2.** Standard curve for the assay. Detailed procedures were described in the text.

decomposed gradually to form $AqB_{12}$. When one needs it for the $B_{12}$ assay, an aliquot of CP-$B_{12}$ stock solution (stable in the dark) can be reacted with DMEQ-hydrazide on a small scale and then purified by HPLC. The fluorescent $B_{12}$ derivative could be readily prepared for a couple of days. The newly prepared DMEQ-labeled $B_{12}$ should be used within 2−3 weeks.

**Assay of $B_{12}$ Contents in Foods Using the Fluorescent $B_{12}$ Derivative.** The principle of the fluorescence dilution assay is that the $B_{12}$ present in the sample or standard $B_{12}$ as a calibrator competes with the fluorescent $B_{12}$ derivative in binding to a $B_{12}$-binding protein. Calibration standards were done with IF by dividing the fluorescence intensity of each of the standards ($B$) by that of the zero standard ($B_0$). The calculated $B/B_0$ (%) for each standard $B_{12}$ was plotted with log [CN-$B_{12}$] on the $x$-axis and $B/B_0$ (%) on the $y$-axis, and then a best-fit curve line between the plotted points was drawn (Figure 2). The level of $B_{12}$ in each of the samples would be determined with the plot, over a range of 0.2−10 $\mu$g/L.

Although the sensitivity of this method was slightly lower than that of the RID assay (0.05−2 $\mu$g/L) or *Lactobacillus* bioassay (0.01−0.2 $\mu$g/L) (Schneider, 1987c), this fluorescence method would be suitable for assay of $B_{12}$ in samples (such as foods) that are obtained in large quantities and/or contain a large amount of $B_{12}$ because food extracts must be diluted significantly for $B_{12}$ assay with both microbiological and RID methods.

$B_{12}$ contents of some foods were assayed by this fluorescence method and then compared with the values obtained by the chemiluminescence method and *Lactobacillus* bioassay (Table 1). Although there were significant differences among $B_{12}$ contents of beef, pork, and cow's milk determined according to the three assay methods, the values obtained by the fluorescence method were similar to the values obtained by both the chemiluminescence and microbiological assays and also to the data reported previously (Schneider, 1987a).

Between-run imprecisions of the three methods were similar; coefficients of variation varied from 7.0 to 10.0% using the microbiological method, from 0.7 to 16.9% using the chemiluminescence method, and from 4.2 to 16.3% using the fluorescence method. The observed correlation coefficients ranged from 0.73 to 0.8%. The correlations were slightly weak among these methods, which may be due to the few samples ($n = 9$) tested.

By the use of this fluorescence method, $B_{12}$ contents in foods can be readily assayed without specialized instruments and/or sterile techniques for culture of the $B_{12}$-requring microorganisms. Running cost of this method would be lower than that of the RID or chemiluminescence assay method. These results indicate that this fluorescence method is a useful nonradioisotopic method for assaying total $B_{12}$ of foods in nonspecialized laboratories.

ACKNOWLEDGMENT

We thank Dr. K. Sato, Hiroshima University, Japan, for the kind gift of *Lactobacillus leichmannii* ATCC 7830.

LITERATURE CITED

Bennink, M. R.; Ono, K. Vitamin B12, E and D content of raw and cooked beef. *J. Food Sci.* **1982**, *47*, 1786−1792.

Frenkel, E. P.; Prough, R.; Kitchens, R. L. Measurement of tissue vitamin $B_{12}$ by radioisotopic competitive inhibition assay and quantitation of tissue cobalamin fractions. *Methods Enzymol.* **1980**, *67*, 31−40.

Gräsbeck, R. Intrinsic factor and the transcobalamins with reflections on the general function and evolution of soluble transport proteins. *Scand. J. Clin. Lab. Invest.* **1967**, *Suppl. 95*, 7−18.

Sato, K.; Hiei, E.; Shimizu, S.; Abeles, R. H. Affinity chromatography of $N^5$-methyltetrahydrofolate-homocyteine methyltransferase on a cobalamin-sepharose. *FEBS Lett.* **1978**, *85*, 73−76.

Schneider, Z. The occurrence and distribution of corrinoids. In *Comprehensive $B_{12}$*; Schneider, Z., Stroinski, A., Eds.; de Gruyter: Berlin, 1987a; pp 157−223.

Schneider, Z. Chemistry of cobalamin and related compounds. In *Comprehensive $B_{12}$*; Schneider, Z., Stroinski, A., Eds.; de Gruyter: Berlin, 1987b; pp 17−92.

Schneider, Z. Purification and estimation of vitamin $B_{12}$. In *Comprehensive $B_{12}$*; Schneider, Z., Stroinski, A., Eds.; de Gruyter: Berlin, 1987c; pp 111−155.

Yamaguchi, M.; Iwata, T.; Inoue, K.; Hara, S.; Nakamura, M. 6,7-Dimethoxyl-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide: a highly sensitive fluorescence derivatisation reagent for carboxylic acids in high-performance liquid chromatography. *Analyst* **1990**, *115*, 1363−1366.

Received for review April 14, 1997. Accepted August 19, 1997.[⊗] This study was supported in part by a research fund (Koenkai Gakujyutsu Kenkyu Shuppan Jigyo) of Kochi Women's University (F.W).

JF970313M

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1997.