Comparison of a Microbiological Assay and a Fully Automated Chemiluminescent System for the Determination of Vitamin B_{12} in Food

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A fully automated chemiluminescence analyzer for the determination of vitamin B_{12} in serum has been commercialized and clinically used. To determine the applicability of this apparatus in food analysis, vitamin B_{12} was assayed in foods by the chemiluminescent method, which was compared with a microbiological method. In shellfishes and spirulina, the values determined by the microbiological method were ~6−8-fold greater than the values determined by the chemiluminescence method, although there was good similarity between the values by the two methods in other foods. Except for the shellfishes and spirulina, which contained substantial amounts of vitamin B_{12}-substitutive compounds or inactive vitamin B_{12} analogues (or both), the observed correlation coefficient between the methods in the foods tested was excellent ($r = 0.99$, $y = 1.2x - 1.1$, $n = 9$). The chemiluminescence method was suitable for the determination of vitamin B_{12} in foods as well as in serum and was simpler (fully automated) and more rapid (180 samples analyzed per hour), highly selective (use of intrinsic factor, the most specific vitamin B_{12}-binding protein), and reproducible (coefficients of variation of 1.2−6.7%) than the microbiological method.

Keywords: *Vitamin B_{12}; bioassay; chemiluminescence; intrinsic factor; food*

INTRODUCTION

Historically, vitamin B_{12} (B_{12}) contents of foods have been determined by bioassay with B_{12}-requiring microorganisms; *Lactobacillus leichmannii* ATCC 7830 has been used widely (Schneider, 1987). The microbiological method is tedious, time-consuming, and technically difficult. HPLC is a useful tool for the separation and determination of B_{12}. Numerous HPLC systems for the determination of B_{12} have been reported (Ford et al., 1991; Iwase and Ono, 1997; Jacobsen et al., 1982) but are less sensitive than the microbiological method. The radioisotope dilution assay (RIDA) method with radiolabeled B_{12} and hog intrinsic factor (IF), the most specific B_{12}-binding protein, has been clinically used for the routine assay of human serum B_{12} (Arnaud et al., 1994; Schneider, 1987). The RIDA method has been also used for the determination of B_{12} contents in foods (Bennink and Ono, 1982; Casey et al., 1982; Kralova et al., 1982; Richardson et al., 1978) because several kits for the RIDA method are commercially available. Casey et al. (1982) have reported that the correlation coefficient between the microbiological method and the RIDA method is excellent ($r = 0.983$). The RIDA method, however, needs radioisotope facilities and apparatuses and also raises the problem of the use of a radioisotope.

Recently, a chemiluminescence (acridinium ester) labeled B_{12} derivative has been devised instead of a radioactive label, and a fully automated chemiluminescence B_{12} analyzer (Chiron Diagnostics, East Walpole, MA) with the acridinium ester-labeled B_{12} derivative and IF has been commercialized. As far as we know, B_{12} contents of foods have not been determined with the chemiluminescence B_{12} analyzer.

In this paper we describe comparison of the microbiological and automated chemiluminescence methods for the determination of B_{12} in foods and also discuss the applicability of this apparatus in food B_{12} analysis.

MATERIALS AND METHODS

Materials. Cyano-B_{12} (CN-B_{12}) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A filter paper (type 50, 20 × 400 mm) for chromatography was obtained from Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). A B_{12} assay medium for *L. leichmannii* was obtained from Nissui (Tokyo, Japan). All other reagents used were of the highest purity commercially available.

The foods tested were purchased from a local market in Kochi-city, Japan.

A Hitachi (Tokyo, Japan) spectrophotometer (U-1000) and a Shimadzu (Kyoto, Japan) UV−visible spectrophotometer (UV-1600) were used for measuring the turbidity of *L. leichmannii* test culture in the microbiological method and absorbance of B_{12} and its analogues in paper chromatography, respectively. A fully automated chemiluminescence analyzer ACS 180 PLUS (Chiron Diagnostics) was used for B_{12} assay.

Extraction of Vitamin B_{12} in Foods. Each (10 g) raw food was homogenized in 50 mL of 0.5 mol/L acetate buffer, pH 4.8, using a universal homogenizer (Nihon Seiki Seisakusho Co., Tokyo, Japan). Each (1 g) dried food was powdered by the use of a food mill (MX-X51, National, Osaka, Japan), added to 50 mL of the same acetate buffer, and homogenized with a dispersor (Polytron model PCU 11, Kinematica, Switzerland).

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S0021-8561(97)00807-8 CCC: $15.00
Published on Web 02/26/1998

Total B_{12} was extracted from the homogenates by the method of boiling with KCN at acidic pH (Frenkel et al., 1980); specifically, 20 mg of KCN was added to the homogenates, which were boiled for 30 min at 98 °C in the dark. The extraction procedures were done in a Dalton (Tokyo, Japan) draft chamber. 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, 50 mL of 0.2 mol/L acetate buffer, pH 4.8, and 20 mg of KCN were added to 50 mL of the milk, and the mixture was 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.

Paper Chromatography. The extract of short-necked clam was spotted quantitatively (10 pg of B_{12} for the microbiological method and 4000 pg for the chemiluminescence method) on the filter paper (type 50) and developed with 1-butanol/2-propanol/water (10:7:10) as a solvent in the dark at a room temperature. The filter paper was dried, cut into small pieces (5 mm) by scissors, and used as samples for the microbiological method. In the case of the chemiluminescence method, B_{12} was extracted with 1.0 mL of distilled water from the pieces of the filter paper for 24 h at 4 °C in the dark and used as samples. Authentic CN-B_{12} (40 μg) and cyanocobamides (benzimidazolyl, 5-hydroxybenzimidazolyl, *p*-cresolyl, and imidazolyl cyanocobamides and pseudovitamin B_{12}; each ~10 μg) were analyzed under the same conditions. The compounds extracted from the filter papers were determined with a Shimadzu UV−visible spectrophotometer (UV-1600) by measuring absorbance at 361 nm.

Assay of Vitamin B_{12}. B_{12} was assayed by the microbiological method with *L. leichmannii* ATCC 7830 and a B_{12} assay medium (Nissui, Tokyo, Japan) and by the fully automated chemiluminescence B_{12} analyzer ACS 180 PLUS (Chiron Diagnostics) according to the manufacturer's instructions. The above B_{12} extracts were directly applied to the chemiluminescence B_{12} analyzer. They were diluted with distilled water up to B_{12} concentration range of 0.01−0.2 μg/L and used as samples for the microbiological method. The turbidity (% T) of *L. leichmannii* test culture was measured at 600 nm with a Hitachi spectrophotometer (U-1000).

Statistical analysis was performed using GraphPad PRISM 2.0 (GraphPad Software, San Diego, CA). One-way ANOVA was used with the post-hoc Tukey/Kramer procedure. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

B_{12} Contents Determined by the Microbiological and Automated Chemiluminescence Methods in Food. Table 1 shows the B_{12} contents determined by the two methods in some foods. Although the values determined by the microbiological method are similar to those determined by the chemiluminescence method (in experiment 1), there were statistically significant differences between the values determined by the two methods, except for beef muscle and liver. Remarkably, in short-necked clam and spirulina tablet, B_{12} contents determined by the microbiological method were about 5.8- and 8.5-fold greater, respectively, than the values determined by the chemiluminescence method. Herbert and Drivas (1982) have reported that in the spirulina tablet, >80% of B_{12} determined by the microbiological method consists of B_{12} analogues, which cannot be assayed by the RIDA method with IF; the above results support this observation.

In other shellfishes (corb shell, hard clam, and scallop adductor muscle) as well as short-necked clam, B_{12} contents determined by the microbiological method were also ~6−8-fold greater than the values determined by the chemiluminescence method (in experiment 2).

To clarify why there are such differences between the values determined by the two methods in the shellfishes, the extract of short-necked clam was analyzed by paper chromatography. Although authentic CN-B_{12} gave a single red spot with $R_f = 0.48$ (Figure 1 A), in the short-necked clam extract B_{12} was not found in the fraction with R_f of 0.48 by the two methods (Figure 1B,C). The clam B_{12} was determined as six peaks with $R_f = 0.04$, 0.12, 0.27, 0.68, 0.84, and 1.0 by the microbiological method but only as a single peak of $R_f = 0.27$ by the chemiluminescence method. These results indicate that the short-necked clam contains the active B_{12} analogue ($R_f = 0.27$) which can be bound to IF and various B_{12}-substitutive compounds and/or inactive B_{12} analogues unable to be bound to IF ($R_f = 0.04$, 0.12, 0.68, 0.84, and 1.0). About 25% of the sum of B_{12} determined by the microbiological method was recovered in the B_{12} analogue fraction with $R_f = 0.27$ and was identical to the amount of B_{12} assayable by the chemiluminescence method. Although shellfishes are considered to be rich sources of B_{12} because they can siphon large quantities of B_{12}-synthesizing microorganisms from the sea (Herbert, 1990), ~80% of B_{12} determined by the microbiological method consisted of B_{12}-substititive compounds and/or inactive B_{12} analogues. The R_f value (0.27) of the active B_{12} analogue was not identical to the values of any B_{12} analogues that occurred in bacteria [pseudovitamin B_{12} ($R_f = 0.31$) and imidazolyl ($R_f = 0.34$), 5-hydroxybenzimidazolyl ($R_f = 0.36$), benzimidazolyl ($R_f = 0.38$), and *p*-cresolyl ($R_f = 0.62$) cyanocobamides].

Table 1. Vitamin B_{12} Contents Determined by the Microbiological and Chemiluminescence Methods in Foods[a]

food	B_{12} content (μg/100 g) microbiol method	chemilumin method
Experiment 1		
beef muscle (raw)	1.42 ± 0.04	1.53 ± 0.04
beef liver (raw)	77.38 ± 11.42	62.51 ± 1.28
pork muscle (raw)	2.42 ± 0.26	3.70 ± 0.08[b]
chicken muscle (raw)	1.54 ± 0.04	1.73 ± 0.02[b]
chicken egg yolk (fresh)	2.13 ± 0.06	2.71 ± 0.04[b]
tuna lean meat (raw)	3.07 ± 0.14	3.69 ± 0.08[b]
short-necked clam (raw)	103.30 ± 4.67	17.57 ± 0.19[b]
ordinary liquid milk	0.44 ± 0.03	0.22 ± 0.01[b]
skim milk powder	2.74 ± 0.05	2.01 ± 0.01[b]
nori (dried)	35.16 ± 1.01	35.31 ± 0.88
spirulina (tablet)	147.50 ± 19.30	17.35 ± 0.18[b]
Experiment 2		
corb shell (raw)	211.20 ± 29.83	33.09 ± 0.92[b]
hard clam (raw)	60.96 ± 6.15	10.17 ± 0.23[b]
scallop adductor muscle (raw)	7.31 ± 0.40	0.93 ± 0.01[b]

[a] All values obtained represent mean ± SEM ($n = 4$). [b] Significantly different from the values determined by the microbiological assay ($P < 0.05$). The detailed procedures are described in the text.

Comparison of the Microbiological and Automated Chemiluminescence Methods in Food. Between-run imprecision of the microbiological method with *L. leichimannii* and the chemiluminescence method was significantly different; coefficients of variation varied from 3.9 to 26% using the microbiological method and from 1.2 to 6.7% using the chemiluminescence method. These results indicate that the chemiluminescence method is more precise in the determination of B_{12} of foods than the microbiological method.

For each food tested (except for the shellfishes and spirulina, which contained large amounts of B_{12}-substitutive compounds and/or inactive B_{12} analogues), the mean value of B_{12} contents determined by the

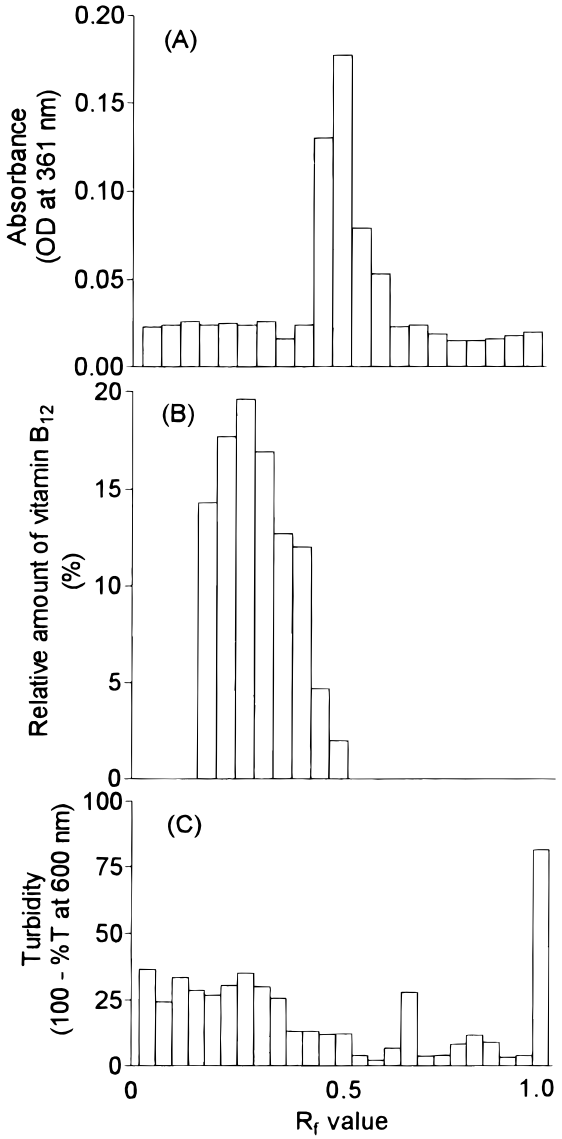

Figure 1. Paper chromatographic analysis of B_{12} of short-necked clam. Migration patterns of authentic CN-B_{12} (A) and clam B_{12} were determined by the chemiluminescence (B) and microbiological methods (C). Data present a typical migration pattern of B_{12} on paper chromatography from three experiments.

microbiological method was plotted on the *y*-axis and that determined by the chemiluminescent method on the *x*-axis and a linear regression curve was drawn (Figure 2). The observed correlation coefficient is 0.99 ($y = 1.2x - 1.1$, $n = 9$), indicating that the two methods were well-correlated.

The microbiological method is time-consuming (2 or 3 days) and requires a sterile technique and a well-trained full-time technician. On the contrary, the chemiluminescence method is rapid (180 samples analyzed per hour) and easy to run (fully automated). Although the sensitivity of the microbiological method (~0.01−0.2 μg/L) is much higher than that of the chemiluminescence method (~0.05−2 μg/L), samples containing large quantities of B_{12} must be significantly diluted for the microbiological method because of the higher sensitivity. In the case of the chemiluminescence method, such samples can be automatically diluted and assayed. It has been reported that *L. leichmannii* can utilize the B_{12} analogues inactive for humans as well as intact B_{12} and that both deoxyribosides and deoxy-

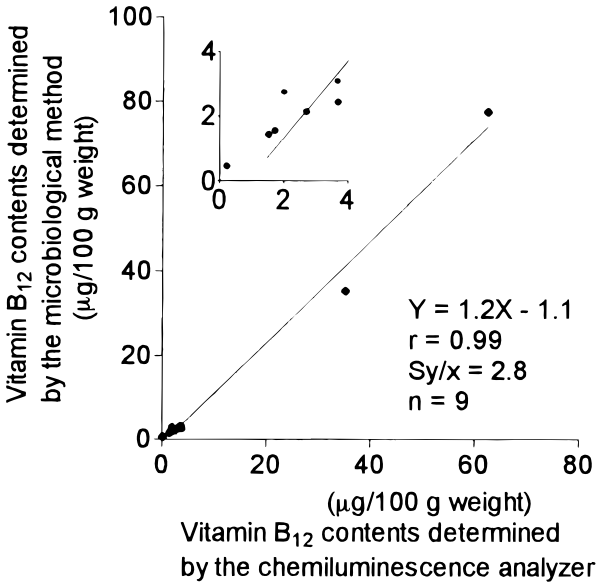

Figure 2. Comparison of B_{12} contents obtained by the microbiological and automated chemiluminescence methods in foods.

nucleotides may substitute B_{12} (Schneider, 1987); the results of the clam B_{12} analysis in Figure 1 also support the observations. Thus, the chemiluminescence method is more selective than the microbiological method because the most specific B_{12}-binding protein (IF) used in this system cannot bind the inactive B_{12} analogues.

These results indicate that the chemiluminescence method is suitable for the determination of B_{12} in foods as well as in serum and that it is simpler, quicker, and more highly selective and reproducible than the microbiological method.

ACKNOWLEDGMENT

We thank Chiron Diagnostics Co., Japan, for technical support, and Dr. K. Sato, Hiroshima University, Japan, for a kind gift of *Lactobacillus leichmannii* ATCC7830. We are grateful to Dr. E. Stupperich, Ulm University, Germany, for a kind gift of the cyanocobamides isolated from bacteria.

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Received for review September 15, 1997. Revised manuscript received January 2, 1998. Accepted January 13, 1998.

JF970807J