Analytical Determination of Vitamin B\textsubscript{12} Content in Infant and Toddler Milk Formulas by Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS)

Jung-Hoon Lee\textsuperscript{1}, Jin-Ho Shin\textsuperscript{1}, Jung-Min Park\textsuperscript{1}, Ha-Jung Kim\textsuperscript{1}, Jang-Hyuk Ahn\textsuperscript{2}, Byung-Man Kwak\textsuperscript{3}, and Jin-Man Kim\textsuperscript{1*}

\textsuperscript{1}Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea
\textsuperscript{2}KOTITI Testing & Research Institute, Seongnam 13202, Korea
\textsuperscript{3}Food Safety Center, Research and Development Institute, Namyang Dairy Co., Ltd., Gongju 30053, Korea

Abstract

The development of a sample preparation method and optimization of the analytical instrumentation conditions were performed for the determination of the vitamin B\textsubscript{12} content in emulsified baby foods sold on the Korea market. After removal of the milk protein and fats by chloroform extraction and centrifugation, the vitamin B\textsubscript{12} was water extracted from the sample. Following filtration of the solution through a nylon filter, the water-soluble extract was purified by solid-phase extraction using a Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The solution eluted from the cartridge was dried under a stream of nitrogen gas and reconstituted with 1 mL of water. The sample solution was injected into an LC-MS/MS system after optimizing the mobile phase for vitamin B\textsubscript{12} detection. The calibration curve showed good linearity with the coefficient of correlation ($r^2$) value of 0.9999. The limit of detection was 0.03 µg/L and the limit of quantitation was 0.1 µg/L. The method of detection limit was 0.02 µg/kg. The vitamin B\textsubscript{12} recovery from a spiking test was 99.62% for infant formula and 99.46% for cereal-based baby food. The sample preparation method developed in this study would be appropriate for the rapid determination of the vitamin B\textsubscript{12} content in infant formula and baby foods with emulsified milk characteristics. The ability to obtain stable results more quickly and efficiently would also allow governments to exercise a more extensive quality control inspection and monitoring of products expected to contain vitamin B\textsubscript{12}. This method could be implemented in laboratories that require time and labor saving.

Keywords: Vitamin B\textsubscript{12}, Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), analytical method, infant formula, toddler formula

Introduction

Vitamin B\textsubscript{12}-containing coenzymes play an important role in the folate-dependent methylation of homocysteine to methionine and in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A (Herbert, 1987). Vitamin B\textsubscript{12} is an essential nutrient for the process of homocysteine methylation (Min and Kim, 2009), as it is the direct cofactor for methionine synthetase, the enzyme that recycles homocysteine back to methionine (Council for Responsible Nutrition, 2014). Vitamin B\textsubscript{12} is regarded to be safe from toxicity due to an overdose of vitamins, as excess vitamin B\textsubscript{12} is simply discharged from the body through urine (Friedrich, 1988). Vitamin B\textsubscript{12} is present only in animal products (Youn, 2005), of which liver, meat, seafood, fish, eggs, milk, and dairy products are the main food sources (Moon, 2007). The recommended dietary allowance for vitamin B\textsubscript{12} is based on the amount needed for the maintenance of the hematological status and normal serum vitamin B\textsubscript{12} values. An assumed absorption of 50% is included in the recommended daily intake values (Food and Nutrition Board, 1998), which currently stand at 2.4 µg/day for a Korean adult and 2.6 µg/day for pregnant and lactating women (Moon, 2007). A lack of vitamin B\textsubscript{12} intake by pregnant women has been shown to cause severe retardation of myelination in the nervous system of the fetus (Guerra-Shinohara \textit{et al.}, 2002). Furthermore, severe vitamin B\textsubscript{12} deficiency has been shown to affect the neurodevelopment of infants.

*Corresponding author: Jin-Man Kim, Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Korea. Tel: +82-2-450-3688, Fax: +82-2-455-1044, E-mail: jinmkim@konkuk.ac.kr

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(Dror and Allen, 2008). Because of these negative outcomes, vitamin B₁₂ is one of the essential growth nutrients in powdered milk formulas for infants, with the content being in the 2.0-2.8 μg level (The Korean Nutrition Society, 2009).

Because vitamin B₁₂ is water soluble and nonvolatile, it can normally be analyzed by high-performance liquid chromatography (HPLC) methods. However, because the vitamin B₁₂ amount in food products is usually only of several μg/100 g compared with other vitamins, general HPLC analysis is unable to quantify the content accurately. Because of this limitation, instrumental analysis by the micro-HPLC assay with a test solution concentration system called the switching valve is used instead. The Food Code Test for vitamin B₁₂ is one of the methods used for analyzing the content contained in growth (toddler) and infant formulas. However, in cases where the elements contained in a product are in trace amounts, more sensitive instrumental analysis techniques are required to ensure that the amounts stated on the food label are accurate, and to allow for better quality management and inspection monitoring of foods by governmental offices.

Research on the dietary intake of infants is a fast-growing field of study, because the quantity and quality of foods are the foundation for lifelong health (Ahn and Um, 2003). Because of the complex nature and properties of foods, the limitations of analytical devices have made the testing and development of nutrients contained in foods both difficult and time consuming. The AOAC recently accredited a test for vitamin B₁₂ for the infant formula matrix, developed (Kirchner et al., 2011). This test showed that the biggest difference between imported and domestic infant formulas and infant growth formulas was the degree of proteolytic degradation of the milk product (Om et al., 2007). It is unlikely that the difference found by this matrix was due to substances in the water-soluble layer interfering with the analysis, as centrifugation was used to remove precipitated proteins, so only the water-soluble nutrients such as vitamin B₁₂ were likely to remain.

In this study, we reviewed the HPLC conditions and devices used in existing authorized testing methods. We also developed a fast and accurate method for preparing vitamin B₁₂ samples for testing, with the aim to establish a standardized method for ensuring that the appointed amounts of vitamin B₁₂ in powdered milk products, as required for the needs of infant growth, are met.

Materials and Methods

Samples, standards and reagents

The infant and toddler milk formulas used in this study were purchased from local supermarkets in Korea, and were kept in containers for use in the analyses. In order to verify the experimental results, the vitamin B₁₂ content of 48.2±8.5 μg/kg contained in Infant Formula SRM 1849a (National Institute of Standard and Technology, USA), which is a certified reference material (CRM), was used as the reference standard. Ammonium formate was purchased from Junsei Chemical (Japan). Oasis® HLB 6cc 200 mg (Waters, USA), a solid-phase extraction cartridge, was used for the sample purification process. HPLC-grade water, methanol, and chloroform were purchased from Merck (Germany). Ultrapure water was obtained using a Banstead Diamond TII system (USA). The distilled water had a resistance of 18.0 MΩ.

Standard and sample preparation

Standard preparation

Vitamin B₁₂ (Cyanocobalamin; Cat. No. 1152009) with a purity of 1.04% (10.4 μg/mg) was purchased from the US Pharmacopeial Convention (USP, USA) for use as the reference standard material. Water was used to dissolve 100 mg of the standard material in a 100 mL volumetric flask to make a 10 mg/L (ppm) stock solution. This stock solution was used to make the standard working solutions of 1, 10, 30, 60, and 100 ng/mL, all diluted with water.

Sample preparation

The sample preparation method used was taken from a water-soluble vitamin assay for infant formula that had been developed (Baiyi et al., 2008). A 1 g sample was mixed well with 10 mM chloroform in a 50 mL Falcon tube for 1 min. Then, the mixture was centrifuged at 4°C, 5,000 rpm for 15 min. The supernatant was filtered through a 0.2 μm nylon filter. After flowing the standard 5% methanol in H₂O 5 mL, cartridge thereby removing the residual water. The sample was then purified by solid-phase extraction, using the Oasis® hydrophilic-lipophilic balance (HLB) 6cc 200 mg cartridge. The cartridge was first pre-equilibrated with 5 mL of methanol and 5 mL of H₂O and adjusted to pH 4.2 with acetic acid. Then, 5 mL of the sample was injected onto the cartridge, followed by washing with 5 mL of 5% methanol in H₂O to eliminate the residual moisture. Finally, the vitamin B₁₂ was eluted with 5 mL of methanol. The sample eluate was concen-
trated with nitrogen gas and then redissolved in 1 mL of H$_2$O before analysis by LC-MS/MS.

Operating conditions and validation of the method

LC-MS/MS conditions

The instrumental analysis conditions used to test for water-soluble vitamins in infant formula were as described previously (Baiyi et al., 2008). The gradients for the mobile phase consisted of 20 mM ammonium formate in water (solution A) and acetonitrile (solution B). The HPLC was operated at a flow rate of 0.2 mL/min and column temperature of 35°C, with a sample injection volume of 10 μL. The HPLC solvents used were filtered with a 0.45 μm membrane and degassed by ultrasonic agitation. The analysis conditions are described in Table 1.

Validation of the method

The chromatography analysis was performed to establish the column equipment, mobile phase, UV length, and MS/MS multiple reaction monitoring conditions established for HPLC-UVD or LC-MS/MS. The developed method was validated by comparing the results with criteria set by the Ministry of Food and Drug Safety (MFDS, 2012) and AOAC Guidelines for Single Laboratory Validation of Chemical Methods (AOAC, 2002). For the method validation, the following eight parameters were evaluated: specificity, accuracy, precision, limit of detection, detection limit, quantitation limit, linearity, and recovery.

Table 1. Liquid chromatography (LC) tandem mass spectrometry (MS/MS) conditions for the determination of vitamin B$_{12}$ LC condition

| Parameter          | Condition                                                                 |
|--------------------|---------------------------------------------------------------------------|
| Column             | UG120V C18 1.5x250 mm 5 μm, Shiseido                                     |
| Detector           | MS/MS                                                                    |
| Mobile phase       | A: 20 mM ammonium formate in water                                       |
|                    | B: Acetonitrile                                                          |
| Gradient mode      |                                                                           |
| Time (min)         | Solvent A (%) Solvent B (%)                                              |
| 0                  | 95 5                                                                     |
| 5                  | 95 5                                                                     |
| 10                 | 80 20                                                                   |
| 14                 | 80 20                                                                   |
| 15                 | 20 80                                                                   |
| 16                 | 20 80                                                                   |
| 35                 | 20 80                                                                   |
| 36                 | 95 5                                                                     |
| 40                 | 95 5                                                                     |

| Flow rate          | 0.2 mL/min                                                               |
| Column temperature | 35°C                                                                     |
| Run time           | 40 min                                                                   |
| Injection volume   | 10 μL                                                                    |

(b) MS/MS condition

| Parameter           | Condition                                                                 |
|---------------------|---------------------------------------------------------------------------|
| Ion source          | ESI (Electro spray ionization)                                            |
| Polarity            | Positive                                                                  |
| Nebulizer gas       | N$_2$                                                                     |
| Pressure            | 50 psi                                                                    |
| Gas flow            | 10 L/min                                                                  |
| Ion spray voltage   | 5000 V                                                                    |
| Source temp.        | 350°C                                                                     |
| Resolution          | Q1(unit) Q3(unit)                                                         |
| Scan mode           | MRM (Multiple reaction monitoring)                                        |

MRM condition

| Retention time (min) | Compound     | Precursor ion (m/z) | Product ion (m/z) | Dwell (ms) | Fragmentor (V) | Collision energy (V) |
|----------------------|--------------|---------------------|-------------------|------------|----------------|---------------------|
| 13.34                | Cyanocobalamin | 678                 | 147               | Quantitative | 200            | 158                 | 40                  |
(LOD), limit of quantitation (LOQ), method of detection limit (MDL), linearity, and range.

Result and Discussion

The infant and infant growth (toddler) milk formulas in domestic circulation are almost all manufactured by one of the spray-drying methods used in microencapsulation technology, which is already widely used in the food and pharmaceutical sectors. Powder fats are used to wrap various carbohydrates or proteins to improve the product’s storage stability, and other flavoring or food additives have coating characteristics that help to protect the core component of the foods. There are two types of powder matrices prepared by the microencapsulation technique. One has an independent pitch form, and the other type is grape shaped. Therefore, there is a need to develop sample preparation methods suitable for each type of food matrix. The assay developed (Kirchner et al., 2011) for determining vitamin B$_{12}$ in infant formula and adult nutrition is listed in the AOAC official journal an accredited sample preparation method. The authors purified the samples using an immune affinity column and then used a liquid chromatography-ultraviolet detector (LC-UVD) to quantitate the vitamin B$_{12}$ in the sample (Kirchner et al., 2011).

Pretreatment methods developed

The existing Food Code Test (Food Code, 2012) requires skilled technicians to prepare the deproteinated sample for analysis using high-level equipment, and there is a wide range of sample reproducibility and safety issues, depending on the food composition being assayed. We therefore attempted to develop a simpler sample preparation method for purification, using solid-phase extraction cartridges, for assay by LC-MS/MS. To assay for water-soluble vitamins in infant formula, we used the sample preparation method described (Baiyi et al., 2008). Chloroform was used to dissolve 1 g of sample in a 50 mL conical tube. After centrifugation, the supernatant was deproteinized and filtered through a nylon filter. Meanwhile, the Oasis® HLB 6cc 200 mg cartridge was first pre-equilibrated with 5 mL of methanol and 5 mL of water and adjusted to pH 4.2 with acetic acid. After loading 5 mL of the sample, the cartridge was washed with 5 mL of 5% methanol in water. The vitamin B$_{12}$ was finally eluted with 5 mL of methanol, concentrated under nitrogen gas, and reconstituted in 1 mL of water before being applied to the LC-MS/MS analysis unit.

Optimization of mobile phase

Four solutions were tested for optimization of the mobile phase for the LC-MS/MS. These included 20 mM ammonium formate in water/acetonitrile (ACN) (1:1), 20 mM ammonium acetate in water/ACN (1:1), 0.1% formic acid in water/ACN (1:1), and 0.1% acetic acid in water/ACN (1:1). A standard vitamin B$_{12}$ solution of 10 μg/L (ppb) was tested with each of the mobile phases. As shown in Fig. 1, the 20 mM ammonium formate in water/ACN gave the highest detection sensitivity of the vitamin B$_{12}$ standard, and was therefore used in all further experiments.

Column selection

Several types of LC-MS/MS columns were tested for their detection sensitivity to vitamin B$_{12}$. These included
the Xterra® RP18 5 μm 4.6 × 250 mm column (Waters), the ACQUITY UPLC® BEH C18 1.7 μm 2.1 × 50 mm column (Waters), the XDB C-18 1.8 μm 4.6 × 50 mm column (Agilent), and the UG120V C18 5 μm 1.5 × 250 mm column (Shiseido). Among these, Shiseido’s UG120V column had the lowest detection limit of 0.0301 μg/kg (ppb). The UG120V column was therefore used to test the vitamin B₁₂ standard solution and the SRM 1849a certified reference material. The result in both cases was a well-separated single peak of vitamin B₁₂ at the same retention time (Fig. 2), and there was no matrix interference effect. Therefore, the UG120V column was used as the analytical column for all further tests.

Validation of the test method
The detection limit test, infant formula recovery test, and quantitative analysis of the certified reference material SRM 1849a were carried out in order to verify the validity of our developed LC-MS/MS vitamin B₁₂ analysis method. The method was validated by comparing the quantitative analysis results with the certified value. The validation tests gave an LOD of 0.03 μg/L, an LOQ of 0.10 μg/L, and an MDL of 0.20 μg/kg. The recovery test showed a recovery range of 110.20-113.00%.

In addition, the amount of vitamin B₁₂ recovered in the SRM 1849a reference was 53.90 μg/kg. Compared with the median value of 48.20 μg/kg, the test exhibited a recovery of the authentication value of 111.83%. Taken together, these results and those of the pre-processing method verify that this analytical unit can be deemed to be valid.

Interlaboratory test results of cross-validation linearity and range
To standardize the vitamin B₁₂ assay developed, the same samples (infant formula SRM 1849a) were analyzed using the same equipment (HPLC-MS/MS, Agilent Model 6410) in different laboratories at Konkuk University Animal Resources Research Center. The assay result was 53.90±0.70 μg/kg from one laboratory and 53.45±3.20 μg/kg from another. Given that the SRM 1849a certified value is 48.20±8.5 μg/kg, the interlaboratory values fell within the certified value range.

Problems using an internal standard
The vitamin B₁₂ assay was developed as a reference standard for the simultaneous analysis of four species of water-soluble vitamins, using methotrexate as the internal standard. However, because this study uses the HLB cartridge for purifying the sample, it was necessary to verify the recoveries of the vitamin B₁₂ samples and the internal standard from the HLB cartridge. As shown in Fig. 3, the recovery of the methotrexate internal standard after purification through the HLB cartridge was extremely low, whereas the recovery and concentration of the vitamin B₁₂ analyte was unaffected by the purification process. Without application of the internal standard, the vitamin B₁₂ recovery after cartridge purification was 90.6%. Thus, it was determined that the assay could be used without an internal standard for evaluations that do not require precise quantitation of the analyte.

Monitoring test for infant and toddler formulas
A monitoring test was carried out using 29 samples of infant and toddler formulas, with SRM 1849a as the international certified reference material. The results are shown in Table 2. All products displayed trace nutrient amounts that were more than that displayed on the content labels, as checked by LC-MS/MS and micro (µ)-HPLC. Thus, it
was confirmed that the Nutrition Facts management of domestic products on the market had been carried out well. Analysis of the LC-MS/MS data was done using the Grubbs method, with one-way analysis of variance (ISO 5725, 2002). As a result, there were no significant differences between the values from the Food Code Test and those from the LC-MS/MS assay developed in this study (significance level of \( p < 0.05 \)) (Table 2). Therefore, the improved and novel sample pre-treatment method developed in this study can be concluded to be effective for the quantitative determination of vitamin B\(_{12}\) in infant and infant growth (toddler) milk formulas.

### Table 2. Validation factors and monitoring test for vitamin B\(_{12}\) in certified reference material (SRM 1849a)

| Recovery test | Tested value (mg/kg) | RSD\(^1\) (%) | Recovery (%) |
|---------------|----------------------|--------------|--------------|
| SRM 1849a     | 53.90±0.70           | 1.27         | 111.83±1.46  |

| Samples       | LC-MS/MS results (µg/kg) | µ-HPLC Results (µg/kg) | Samples       | LC-MS/MS results (µg/kg) | µ-HPLC Results (µg/kg) |
|---------------|--------------------------|------------------------|---------------|--------------------------|------------------------|
| Infant formula (milk-based, powder) | Toddler formula (milk-based, powder) |
| T-1           | 62.65                    | 59.87                  | T-1           | 26.18                    | 27.62                  |
| T-2           | 34.89                    | 35.82                  | T-2           | 31.47                    | 34.18                  |
| T-3           | 36.57                    | 35.56                  | T-3           | 28.64                    | 29.85                  |
| T-4           | 28.6                     | 30.15                  | T-4           | 39.97                    | 37.48                  |
| T-5           | 31.83                    | 30.21                  | T-5           | 43.70                    | 41.32                  |
| T-6           | 34.34                    | 33.64                  | T-6           | 37.37                    | 37.85                  |
| T-7           | 34.35                    | 33.82                  | T-7           | 35.35                    | 36.95                  |
| T-8           | 35.94                    | 36.49                  | T-8           | 33.12                    | 33.64                  |
| T-9           | 37.5                     | 38.18                  | T-9           | 34.25                    | 35.29                  |
| T-10          | 31.44                    | 29.87                  | T-10          | 30.99                    | 31.46                  |
| T-11          | 31.71                    | 30.18                  | T-11          | 15.10                    | 14.96                  |
| T-12          | 20.50                    | 21.64                  | T-12          | 17.35                    | 18.49                  |
| T-13          | 42.31                    | 41.54                  | T-13          | 24.31                    | 25.09                  |
| T-14          | 44.83                    | 46.24                  |               |                          |                        |
| T-15          | 17.35                    | 18.49                  |               |                          |                        |
| T-16          | 24.31                    | 25.09                  |               |                          |                        |

| LOD\(^3\)     | 0.03 µg/L                |                          |              |
| LOQ\(^3\)     | 0.10 µg/L                | Linear Regression        | y = 2737.71x − 5508.48 |
| MDL\(^4\)     | 0.20 µg/L                | Range                   | 1~100 µg/L    |

\(^1\)RSD, relative standard deviation; \(^2\)LOD, limit of detection; \(^3\)LOQ, limit of quantitation; \(^4\)MDL, method of detection limit.

### Conclusions

In this study, a vitamin B\(_{12}\) assay based on LC-MS/MS was developed and verified for its use in growth and infant formula component analysis and in cross-validating results obtained between laboratories. Our LC-MS/MS test method can be applied with existing equipment in the typical analytical laboratory, without any major need for changes in the test environment or in the level of skill.
required to conduct the test, and can be done more quickly and easily than currently used methods.

We expect this new test method to be utilized by various analytical organizations as a rapid preprocessing method for the efficient inspection of trace nutrients in food products.

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