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ARTICLE

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

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Abstract

The development of a sample preparation method and optimization of the analytical instrumentation conditions were performed for the determination of the vitamin B_{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_{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_{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_{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_{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_{12}. This method could be implemented in laboratories that require time and labor saving.

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

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Introduction

Vitamin B_{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_{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_{12} is regarded to be safe from toxicity due to an overdose of vitamins, as excess vitamin B_{12} is simply discharged from the body through urine (Friedrich, 1988). Vitamin B_{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_{12} is based on the amount needed for the maintenance of the hematological status and normal serum vitamin B_{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_{12} intake by pregnant women has been shown to cause severe retardation of myelination in the nervous system of the fetus (Guerra-Shinohara *et al.,* 2002). Furthermore, severe vitamin B_{12} deficiency has been shown to affect the neurodevelopment of infants

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(Dror and Allen, 2008). Because of these negative outcomes, vitamin B_{12} 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 B_{12} is water soluble and nonvolatile, it can normally be analyzed by high-performance liquid chromatography (HPLC) methods. However, because the vitamin B_{12} 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} samples for testing, with the aim to establish a standardized method for ensuring that the appointed amounts of vitamin B_{12} in powdered milk products, as required for the needs of infant growth, are met.

Meterials 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 B_{12} 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 B_{12} (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 H_2O 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 H_2O 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 H_2O to eliminate the residual moisture. Finally, the vitamin B_{12} was eluted with 5 mL of methanol. The sample eluate was concen-

trated with nitrogen gas and then redissolved in 1 mL of H_2O 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 ammonuim 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

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

(a) LC condition

Parameter	Condition		
Column	UG120V C18 1.5×250 mm 5 μm, Shiseido		
Detector	MS/MS		
Mobile phase	A: 20 mM ammonuim 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
		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 Ml		

(b) MS/MS condition

Parameter	Condition
Ion source	ESI (Electro spray ionization)
Polarity	Positive
Nebulizer gas	N_2
Nebulizer 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 reation 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
			359	Quantitative	200	158	24

(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

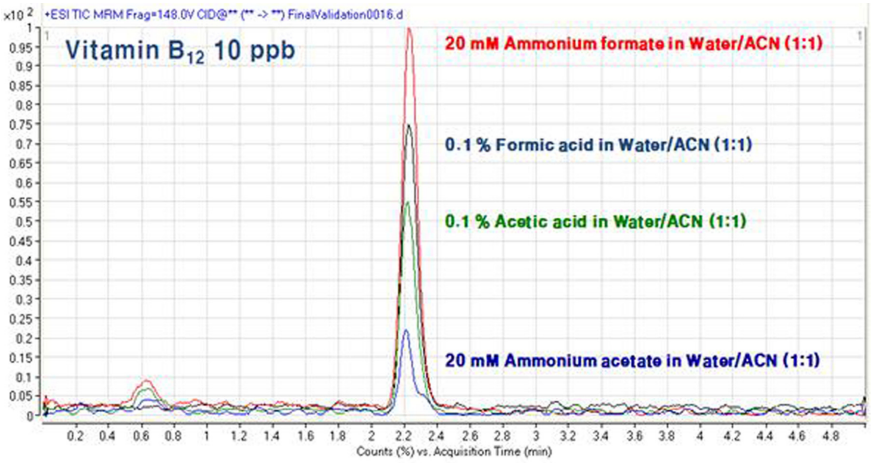

Fig. 1. Result of mobile phase optimization for the detection of vitamin B_{12}.

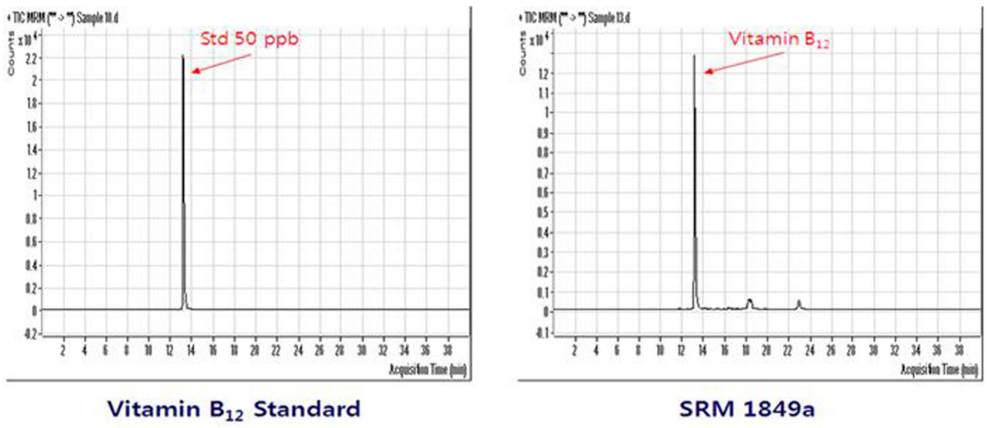

Fig. 2. Separation of vitamin B_{12} standard and sample on the UG120V C_{18} column (5 μm, 1.5 × 250 mm; Shiseido).

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 B_{12} standard solution and the SRM 1849a certified reference material. The result in both cases was a well-separated single peak of vitamin B_{12} 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} 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 B_{12} samples and the internal standard from the HLB cartridge. As shown in Fig. 3, the recovery of the methotrextate internal standard after purification through the HLB cartridge was extremely low, whereas the recovery and concentration of the vitamin B_{12} analyte was unaffected by the purification process. Without application of the internal standard, the vitamin B_{12} 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

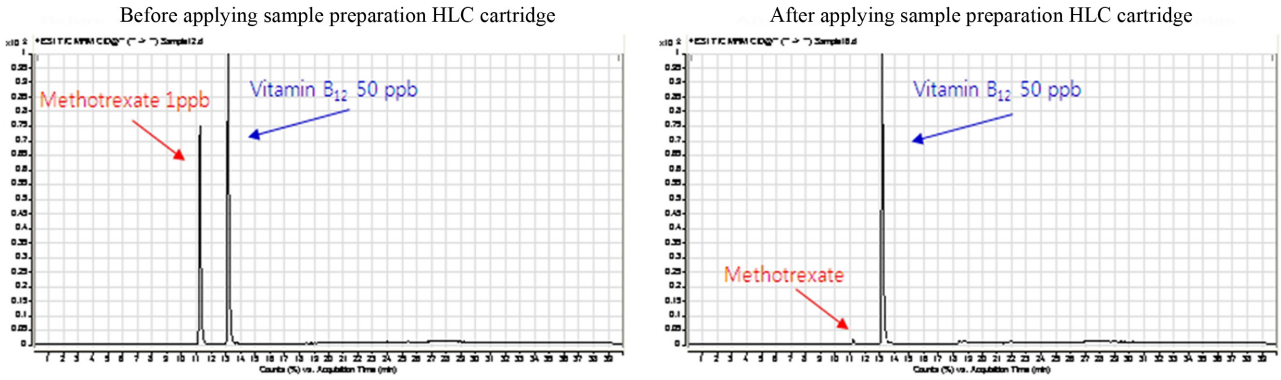

Fig. 3. Effect of hydrophilic-lipophilic balance (HLB) cartridge application on vitamin B_{12} content. Methotrexate was used as the internal standard.

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)	T-1	62.65	59.87	Toddler formula (milk-based, powder)	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[2]		0.03 μg/L		r^2		0.9986	
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.

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 (KS A 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.

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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