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Determination of water-soluble vitamins (B2, B9 and B12) by immunoassay

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Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) for the detection of water-soluble vitamins (B2, Riboflavin; B9, Folic acid; B12, Cobalamin) was developed. Rabbit polyclonal antibodies were produced after immunization and used for the development of assays. Total assay time was less than 70 minutes. The detection limits for riboflavin, folic acid, cobalamin in PBS buffer are 0.5 ppb, 1.0 ppb and 0.4 ppb, respectively. Matrix effect was observed with tablet samples when extracted with small volume of extraction buffer. Effect of ascorbic acid as an antioxidant on the stability of riboflavin and folic acid were tested. Differences on vitamin content between labeled values and measured values were found from both food products and supplements. Folic acid content from one drink sample was about 12 times higher than its labeled value. However, another drink contained only 2% of folic acid of its labeled value. These high variations indicate that there is possibility of the addition of excessive amount of vitamins due to their degradation during the storage. Vitamins were spiked in three samples (drink, infant formula, cereal) and recovered with high yields (74 – 123%). The results indicate that the immunoassay kit developed in this study can be used as rapid and accurate tools for the determination of water-soluble vitamins in various food products.

Introduction

Vitamins are a group of organic compounds essentially required by an organism. The B vitamins are a group of water-soluble vitamins that are necessary for normal health. Three of these vitamins are B2 (riboflavin), B9 (folic acid), and B12 (cobalamin). A deficiency of any of these vitamins can lead to various illnesses, for example, a deficiency of B12 can lead to anemia and neurological disorders, and a deficiency of B9 during pregnancy can lead to neural tube defects such as spina bifida in newborn infants. The importance of these vitamins has lead to their frequent fortification, sometimes by law, into foods as well as supplements in synthetic forms. Due to poor stability of water-soluble vitamins especially in aqueous product, the fortified vitamin levels are often decreased during processing and storage. This has created a need for the accurate quantification of the vitamins to ensure proper nutritional reporting, compliance, and safety. Since some vitamins are incorporated in very small quantities, such as B12, these methods of analysis must also be sufficiently sensitive as well as accurate. Methods such as MBA (microbiological assay) and HPLC (high performance liquid chromatography) have been successfully used. MBA uses the growth response of various vitamin-dependent Lactobacilli. Although MBA is sensitive and specific, it is time consuming and laborious. Indeed, the results are often susceptible by interference from growth-stimulating artifacts and showing poor precision. HPLC is a more rapid and accurate technique but is often insufficiently sensitive for vitamin B12 and has been shown to require addition of a B12 standard for accurate results. In this study, competitive ELISA were developed and evaluated allowing for simple and accurate determination of vitamins B2, B9, and B12 content in common food products as well as supplements.

Methodology

Sample preparation

Hard and solid sample such as supplement was ground by mortar and pestle. Dry and crunchy sample such as cereal was ground by a kitchen blender. Other powder and liquid samples were directly used for extraction.

- **solid sample (0.2 g) + PBS (20 mL) + vortex (3 min) + centrifuge (3 min at 12,000 rpm) + Dilution of supernatant for assay**
- **liquid sample (1 mL) + PBS (20 mL) + vortex (3 min) + centrifuge (3 min at 12,000 rpm) + Dilution of supernatant for assay**

Polyclonal antibody production:

In the development of these assays, vitamins (B2, B9, B12) were coupled to a protein carrier, which served as an immunogen, and horse radish peroxidase (HRP) which served as the enzyme conjugate. The antibody was produced through the immunization of New Zealand white rabbits. The serum was collected monthly.

Assay protocol:

50 µl of HRP conjugate is added to each well followed by 50 µl of calibrator or sample extract. Lastly 50 µl of antibody solution is added to the wells and mixed gently for 30 seconds and incubated for 30 minutes. The well contents are then decanted and washed 4 times with PBST wash solution. Substrate (100 µl) is added to the wells and incubated for 30 minutes. The reaction is stopped with 100 µl of stop solution and the contents of the wells are read at 450nm-650nm. The concentration is calculated from a calibration curve applying dilution factors.

Results

Table 1. Vitamin contents in supplements.

	Riboflavin (B2)			Folic acid (B9)			Cobalamin (B12)		
Sample	ELISA (ppm)	Label (ppm)	Ratio (%)	ELISA (ppm)	Label (ppm)	Ratio (%)	ELISA (ppm)	Label (ppm)	Ratio (%)
Supplement (1)	1211	879	138	140	103	136	16	13	124
Supplement (2)	719	1313	55	299	309	97	5	5	113

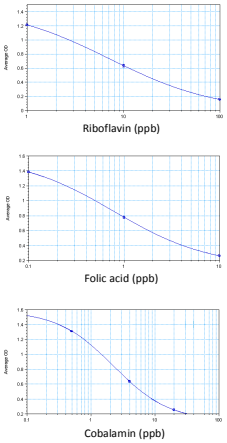
Table 2. Vitamin contents in fortified foods.

	Riboflavin (B2)			Folic acid (B9)			Cobalamin (B12)		
Sample	ELISA (ppb)	Label (ppb)	Ratio (%)	ELISA (ppb)	Label (ppb)	Ratio (%)	ELISA (ppb)	Label (ppb)	Ratio (%)
Drink (1)	753	1395	54	10	465	2	26	14	186
Drink (2)	-	-	-	4462	7000	64	6306	8772	72
Drink (3)	-	-	-	1981	167	1186	2	3	76
Infant formula	8234	6364	129	703	727	97	25	14	187
Flour	2833	4533	62	1319	1333	99	-	-	-
Cereal	14757	15179	97	8903	7143	125	11	54	21

Table 3. Recovery of spiked vitamins.

	Riboflavin (B2)				Folic acid (B9)				Cobalamin (B12)			
Sample	Spike (ppb)	Without spike	With spike	Rec. (%)	Spike (ppb)	Without spike	With spike	Rec. (%)	Spike (ppb)	Without spike	With spike	Rec. (%)
Drink (1)	1395	924	2282	97	465	9	503	106	14	23	37	105
Infant formula	6364	7667	14574	109	727	673	1377	97	14	18	28	74
Cereal	15179	18127	35018	112	7143	8728	12490	123	54	8	54	85

Results



4-P Fit; y = (A – D)/(1 + (x/C)^B) + D

A	B	C	D	R^2
1.48	0.769	7.16	-0.022	1

IC 50: 6.8 ppb

A	B	C	D	R^2
1.60	0.861	0.785	0.108	1

IC 50: 0.9 ppb

A	B	C	D	R^2
1.59	0.99	2.25	0.0992	1

IC 50: 2.6 ppb

Figure. Calibration curves of vitamin assays.

Discussion

Competitive immunoassays were developed for accurate determination of water-soluble vitamins (B2, B9 and B12) in fortified foods and supplements. For good recovery of vitamins, enough amount of extraction solution was added to samples (20 mL into 0.2 g of sample). In order to extend the shelf life of calibrator solution, ascorbic acid was added to prevent oxidative degradation of riboflavin and folic acid. However, addition of ascorbic acid in the calibrator did not improve the stability of each calibrator over the storage time (Data not shown).

Vitamins measured by the assay were compared with the label values. Some of determined values are significantly lower than the label values such as drink (1) which has only 2% of the labeled folic acid value. Cereal also has 21% of the labeled cobalamin value. On the contrary, a drink (3) has 1186% of the labeled folic acid.

Vitamins were spiked with same label value of each vitamin to evaluate the recovery of each vitamin for the assays. Overall recoveries from three food products (drink, formula and cereal) ranged from 74 to 123%. Competitive ELISA assays for determination of water-soluble vitamins (B2, B9 and B12) were successfully developed and evaluated. These immunoassays can provide fast and reliable results for determination of vitamin B2, B9 and B12 in foods as well as supplements.

References

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