Quantification of Vitamin B_{12} in Infant Formulas using UPLC-UV

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Abstract

Vitamin B12 (cobalamin) plays a key role in human biological functions and is vital in the neurological development of infants. Vitamin B12 exists in different chemical forms (i.e., adenosyl-, cyano-, hydroxo- and methyl-cobalamin), but many infant formulas contain cyanocobalamin. This poster presents a study on the quantification of vitamin B12 in infant formulas using ultra-performance liquid chromatography (UPLC) coupled with UV detection according to AOAC Official Method 2014.02. The formula samples were first mixed with a sodium acetate buffer and heated in the presence of cyanide to convert all forms of cobalamin to a more stable cyanocobalamin. The supernatant was passed through an immunoaffinity column for cleanup and analyte preconcentration. The analyte was then eluted from the column with methanol, reconstituted in a small volume of water, and analyzed. Separation was under gradient elution on a reversed-phase column with mobile phases consisting of water and acetonitrile with trifluoroacetic acid. Samples of different matrices, including milk-based, soy-based, partially hydrolyzed milk and soy, amino acid-based, plant-based, milk-based ready-to-feed formulas and formulas containing added starch were analyzed for verification of method performance. Standard reference materials and fortified analytical portions were used for validation. Results from AOAC 2014.02 are compared to a method being developed at the FDA using high performance liquid chromatography (HPLC) interfaced with inductively coupled plasma-mass spectrometry (ICP-MS).

Introduction

Vitamin B12 is used in the formation of red blood cells, and is important in the proper development of infants. Vitamin B12 is added to infant formulas to ensure infants, whose sole source of nutrition is formula, are receiving the proper amount of the nutrient to meet their nutritional needs. Traditional methods including microbiological assays, spectrophotometric, and various chromatographic techniques are used for vitamin B12 determination. The AOAC Official Method 2014.02 (hereafter called AOAC Method) utilizes ultra-performance liquid chromatography interfaced with ultraviolet detection (UPLC-UV) to determine vitamin B12 in infant and adult/pediatric formula after preconcentrating the nutrient on an immunoaffinity sorbent. In the present study, a variety of infant formula products were analyzed using the AOAC method as well as with a method under development and validation at the FDA, which uses high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

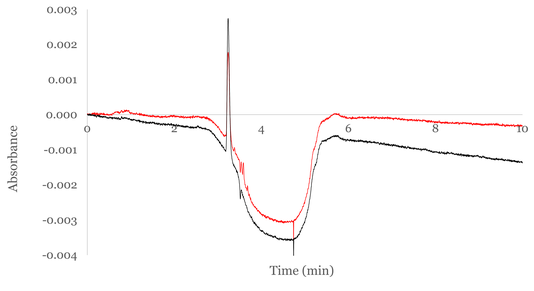

Figure 1. *Chromatograms showing separation of CNCbl from milk based infant formula (red) and a 0.1 ppm CNCbl standard (black) using the AOAC Method.*

Materials and Methods

AOAC Method

- Waters Acquity UPLC with PDA eλ detector was used. The column was a Waters Acquity UPLC BEH C18 (2.1 x 100mm, 1.7µm). EASI-EXTRACT Vitamin B12 LGE immunoaffinity column from r-biopharm was used for cleanup and preconcentration.

FDA Draft Method

- Agilent 1260 HPLC coupled with an Agilent 7900 ICP-MS and an Agilent Zobrax Eclipse XDB-C8 (3 x 150mm, 3.5µm) with XDB-C8 (2.1X12.5mm, 5µm) guard were used. Analyte was preconcentrated on Oasis HLB cartridge from Waters.

Standard and Reference Materials

- Cyanocobalamin (CNCbl) standards were obtained from Sigma Aldrich. Infant formula standard reference materials (SRM) 1846 and 1869 from NIST were used.

UPLC analysis

- The mobile phases contained 0.025% trifluoroacetic acid in (A) water and (B) acetonitrile; Table 1 shows the gradient program. The flow rate was 0.4 mL/min, and 50 µL of a sample was injected. The analysis time was 10 min and cyanocobalamin was eluted at 3.2 minutes (see Fig. 1). Analyte was detected at 361nm.
- Representative chromatograms in Fig. 1 show there was no significant effect of the matrices on the separation of CNCbl using this method.

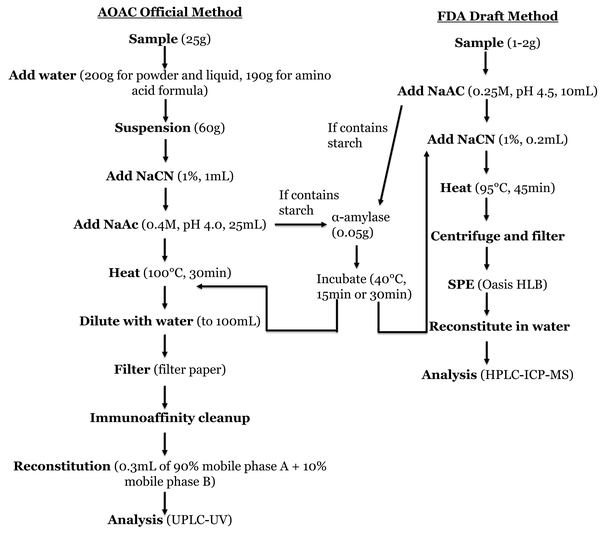

Figure 2. *Flow charts showing sample preparation for vitamin B_{12} determination in infant formula according to AOAC Method 2014.02 and FDA draft method.*

Results and Discussions

Sample analysis

- Milk-based powder, soy-based powder, partially hydrolyzed milk powder, partially hydrolyzed soy powder, milk-based ready-to-feed liquid, amino acid-based powder, plant-based powder, and starch-containing infant formula powders, as well as toddler beverage samples, were analyzed using the AOAC Method.
- Fig. 3 compares the measured concentrations of vitamin B12 in the samples against the claimed values. The experimental values are greater than the claimed concentrations for all the formulas tested
- Milk-based ready to feed formulas contain the least concentration of vitamin B12 for both claimed and experimental values.
- Table 2 shows CNCbl recoveries from spiked formula samples; the values were in the range of 75%–125% except one outlier at 146%.

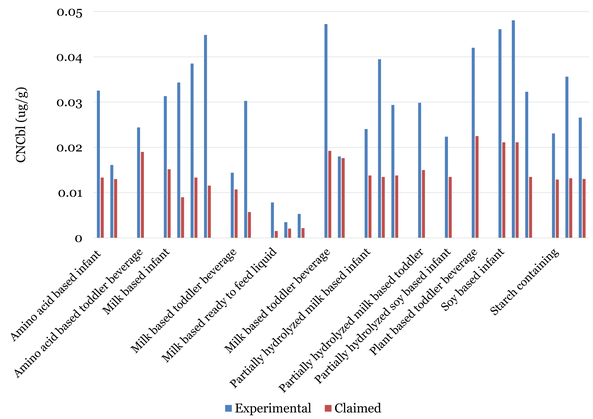

Figure 3. *Comparison of claimed and experimental vitamin B12 concentration in infant formula and toddler beverage samples. All samples excluding milk-based ready to feed liquids are powder samples.*

Accuracy

- The accuracy of the AOAC method was evaluated by analyzing SRMs 1846 and 1869. The recovery values were 85% and 109%, respectively.

Limits of detection and quantitation

- Vitamin B12 can be detected and quantified using the AOAC method at levels as low as 0.9 ng/g and 5.8 ng/g, respectively, after preconcentration on an EASI-EXTRACT immunoaffinity sorbent.

Table 1. *UPLC gradient elution program.*

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	90	10
1.7	90	10
2.5	75	25
2.9	10	90
3.9	10	90
4.0	90	10
8.0	90	10

Table 2. *Spike recovery (%) of CNCbl from infant formula and toddler beverage samples using the AOAC method.*

Sample	Infant formula	Toddler beverage
Milk based	106	99
Soy based	126	N/A
Partially hydrolyzed milk	90	146
Partially hydrolyzed soy	107	N/A
Milk based ready to feed	76	N/A
Amino acid based	124	84
Starch containing	123	N/A
Plant based	N/A	111

Comparison with FDA draft method

- The AOAC Method was compared against the draft FDA Method by analyzing all the samples listed in Fig. 3.
- Fig. 2 shows flowcharts summarizing the procedures of the two methods.
- The slope of 0.78 and R^2 of 0.94 of Fig. 4 indicates a good agreement between the concentrations determined using the AOAC and FDA method.

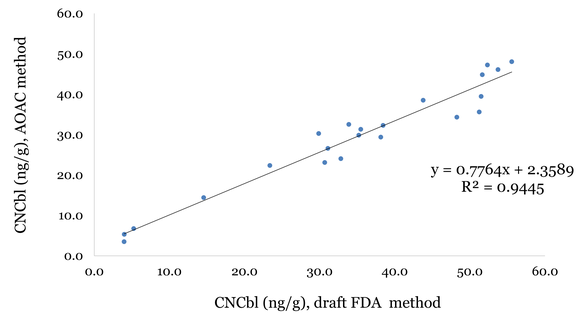

Figure 4. *Comparison of AOAC method to the draft FDA method.*

Conclusion

- The vitamin B12 concentrations determined using AOAC Official Method 2014.2 are within regulations and are also in good agreement with those determined by the draft FDA method.

Reference

1. AOAC Official Method 2014.02 Vitamin B12 (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula Liquid Chromatography-Ultraviolet Detection, AOAC INTERNATIONAL, 2018.

*[*]**Infant**: a person not more than 12 months of age, **Infant Formula**: a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk, **Toddler beverage**: Products intended for children >12 months of age and are regulated as food, not as infant formula.*