Isotope Dilution LC-MS/MS Analysis of Vitamin B12 in Infant Formula and Nutritional Supplements

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Introduction

Dietary supplements in a variety of forms are widely used to ensure adequate intake of essential nutrients. The determination of vitamin levels in these supplements is important for both regulatory compliance and safety. Vitamin B12 is one commonly supplemented vitamin that is involved in a variety of functions within almost every cell of the human body. There are a number of widely used methods for the quantitation of vitamin B12 in a variety of matrices, including both microbiological and liquid chromatographic approaches. Microbiological methods offer excellent sensitivity, but are not as specific for the analyte of interest as chromatographic methods and they can be very labor intensive with long turnaround times. Achieving sufficient sensitivity for use with a chromatographic approach requires significant effort to concentrate the extract, and interferences with ultraviolet or fluorescence detection require long run times to resolve the peaks of interest. Here, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for the quantitation of vitamin B12 in infant formulas and nutritional supplements. LC-MS/MS provides equivalent sensitivity to microbiological approaches, but also provides excellent specificity for the analyte of interest. No concentration of the extract is required, and many matrix interferences are minimized using an LC-MS/MS approach. An isotope-labeled internal standard was used in the analysis to compensate for matrix effects.

Methods

Several methods for the analysis of cyanocobalamin (vitamin B12) are executed on a regular basis at Covance Laboratories, and this data was used to compare results at Covance. Current methods are those that are in use at this time and run on a routine basis:

- Official Methods of Analysis, Method 2011.10, AOAC INTERNATIONAL

The proposed method for cyanocobalamin utilizes the same initial steps of the AOAC method 2011.10 to prepare and extract the samples. An appropriate amount of sample is weighed into a volumetric flask and 30 mL 0.25M sodium acetate is added to the flask. 1mL of 1% sodium cyanide is added to ensure complete conversion of all cobalamin species to the cyanocobalamin form. The samples are heated for 60 to 120 minutes at 105ºC and then filtered. A solution of $^{13}C_7$ cyanocobalamin was added post-extraction to act as internal standard. Standard curves were prepared by diluting known concentrations of cyanocobalamin with the internal standard. Samples were analyzed using an Agilent® 1290 UHPLC and an Agilent® 6490 LC-MS/MS operating in positive ion electrospray ionization moded (ESI+). A binary gradient of ultra pure water and methanol, each containing 10mM ammonium formate and 0.1% formic acid, was used with a Zorbax® SB-Aq, 3.0 X 100 mm, 1.8µm UHPLC column. The curve range was 0.1 ng/mL to 50.0 ng/mL. Some samples, including several infant formula samples, did require the use of an SPE cleanup to minimize matrix interferences and signal suppression.

Experimental

AOAC Method 2011.10 is a method used in considerable volume at Covance Laboratories. It has also been applied and validated with some modifications to a variety of other matrices. Due to this, there are both a large amount of samples available and data for comparison. The extracts were analyzed using the new LC-MS/MS method intermittently over a period of approximately 3 months. Results obtained were directly compared for a large number of samples and sample types. NIST 1849a is run within each batch, and so there was opportunity to run a large number of that particular sample over others.

Analyte	Type	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy
Cyanocobalamin	Quant	678.6	359.0	25
Cyanocobalamin	Qual	678.6	456.6	30
Cyanocobalamin	Qual	678.6	341.0	25
Cyanocobalamin [$^{13}C_7$]	ISTD	682.1	366.1	25

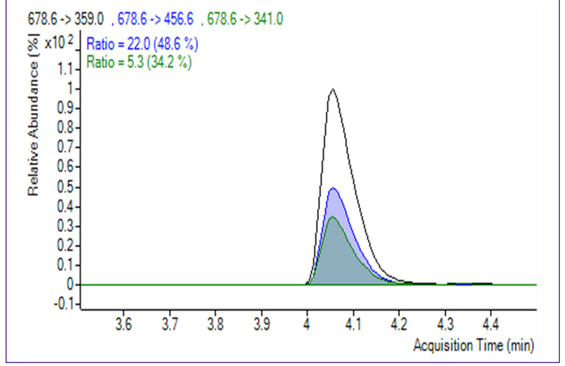

Figure 1. LC-MS/MS method transitions and ratios (Agilent 6490).

Results

A wide variety of samples were analyzed on 12 separate days within a period of more than 3 months. 100% of NIST 1849a infant formula and NIST 3280 tablet results were within the respective certified ranges, although these ranges are quite wide. For at least 60% of over 50 distinct samples analyzed, there was agreement ±10% between results from the two methods. All other results were within ±20%. Sample matrices tested span a wide variety of infant formulas, adult nutritionals, softgels, chews, capsules, tablets, premixes and energy drinks.

Some issues were encountered with ESI+ suppression within certain matrices. Infant formula samples treated with diastase enzyme to aid in bulk extract filtering for SPE concentration resulted in complete signal suppression, and no result was obtained. Select samples were re-extracted without the addition of enzyme, and results here matched original analysis ±10% for all samples.

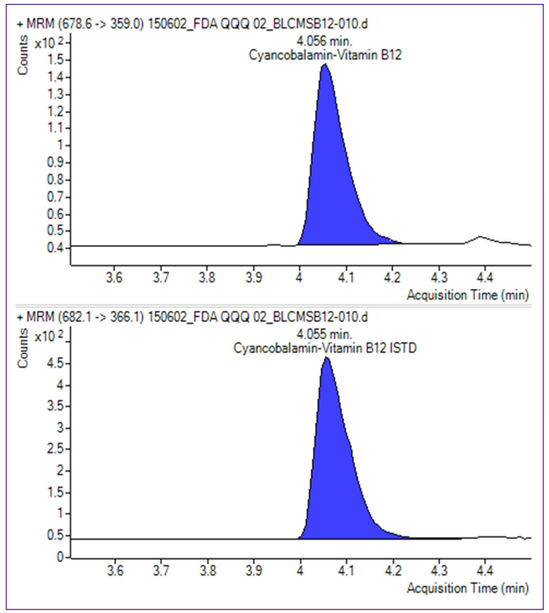

Figure 2. MRM: NIST 1849a sample – Approximately 1.5 ng/mL.

Cyanocobalamin NIST 1849a CRM (n = 22)

Average (mg/kg)	%RSD	Expected (mg/kg)	% Theoretical
0.0473	4.91%	0.0482 +/- 0.0085	99.0

Conclusions

A method was successfully developed for the analysis of cyanocobalamin utilizing LC-MS/MS analysis with $^{13}C_7$ cyanocobalamin as internal standard. Overall, good agreement was seen between results for a wide variety of sample types using the AOAC method and the newly developed method. MS/MS detection is generally considered to offer enhanced specificity when compared to UV detection. The gains in sensitivity realized here allow simple filtration of less than 1mL of the initial extract without SPE concentration/cleanup. Analysis time was reduced from 30 minutes to 6 minutes in this application. This significantly reduces the complexity of the method while also saving significant time and expense

Early efforts made to quantitate several specific forms of B12 were unsuccessful due to instability of these forms in extract solutions. Stability of calibration curves for methylcobalamin and hydroxocobalamin among others were acceptable, but sample extracts would not retain the specific forms and even create others. Converting all B12 to the cyanocobalamin form was eventually seen as a necessary step here.

All ESI+ signal suppression encountered during sample testing was due to enzyme digested matrix components, but there will likely be certain matrices that also exhibit signal suppression using this mode. This is instructive for LC-MS/MS analysis of these matrices, in general. Future efforts are focused on full validation of the method and a simplified SPE cleanup using small sample volumes, as needed. Each class of matrix analyzed here will need to be investigated and validated over a significant period of time.

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