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A new sample preparation and separation combination for precise, accurate, rapid, and simultaneous determination of vitamins B_1, B_2, B_3, B_5, B_6, B_7, and B_9 in infant formula and related nutritionals by LC-MS/MS

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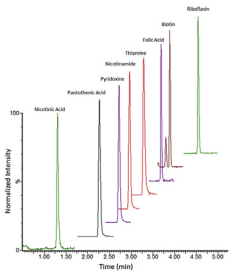
HIGHLIGHTS

- Fast, accurate, and precise water soluble vitamin determination in a wide range of infant formula and nutritional products.
- Improvements in sample preparation and separation conditions are key contributors to method performance.
- The method was validated in eleven different infant formula and related matrixes with excellent demonstrated accuracy and precision.

GRAPHICAL ABSTRACT



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ABSTRACT

An improved method was developed for simultaneous determination of the fortified forms of thiamine (B_1), riboflavin (B_2), nicotinamide and nicotinic acid (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), and folic acid (B_9) in infant formulas and related nutritionals. The method employed a simple, effective, and rapid sample preparation followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). It improved upon previous methodologies by offering facile and rugged sample preparation with improved chromatographic conditions, which culminated in a highly accurate and precise method for water-soluble vitamin determination in a wide range of formulas. The method was validated over six days in ten unique matrices with two analysts and on instruments in two different labs. Intermediate precision averaged 3.4 ± 2.6% relative standard deviation and over-spike recovery averaged 100.2 ± 2.4% (n = 160). Due to refinements in sample preparation, the method had high sample throughput capacity.

1. Introduction

Water-soluble vitamins (WSVs), including: thiamine (B_1), riboflavin (B_2), nicotinamide and nicotinic acid (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), and folic acid (B_9) are essential micronutrients. The body cannot synthesize them in sufficient quantities, so the majority of these vitamins must come from dietary sources or supplementation [1]. Deficiencies can have a negative impact on health and can lead to devastating diseases such as beriberi, microcytic hypochromic anemia, and pellagra or even death [2]. Therefore the concentration of these micronutrients is highly regulated in sole-source foods such as infant formula.

A variety of good measurement techniques are available for each

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vitamin in infant formulas. Historically these methods have been microbiology-based, but recently more modern, separations-based techniques have become the gold-standard for vitamin analysis [3]. Many methods are based upon liquid chromatography with fluorescence or UV-VIS detection. However, liquid chromatography tandem mass spectrometry (LC-MS/MS) with stable isotope dilution has been leveraged to enable simultaneous detection of multiple vitamins because of its sensitivity and selectively, saving significant time and cost. In some cases, multiplexed methods are available which combine four, five, six, and even up to 21 vitamins in a single analysis [3–14].

The latter is an extreme example where both fat and water-soluble vitamins are determined from a simple, multi-vitamin tablet matrix [7]. In our experience, such highly multiplexed methods are typically not suitable to infant formulas. For example, many of the fat-soluble vitamins require extraction conditions that are incompatible with the water-soluble vitamins [15]. To the authors' knowledge, conditions that balance good recovery from both classes have not yet been demonstrated in infant formula. Further, tight regulatory requirements put pressure on method performance in terms of accuracy and precision. Infant formula method requirements published by the AOAC Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN) generally require interlab reproducibility of ≤10% relative standard deviation (RSD) and over-spike recovery of 90–110% [16–22].

Interlaboratory reproducibility of ≤10% RSD puts large onus on the precision of a methodology. Validation in a single lab must therefore achieve intermediate precision of no more than 5% RSD, with 3% RSD being more ideal. Achieving this degree of precision by LC-MS/MS, even with stable-isotope labeled internal standards, is not trivial because of the many different forms of infant formula, which include those made from intact protein, partially hydrolyzed protein, fully hydrolyzed protein, soy protein, milk protein, and others. Non-infant nutritionals bring additional complexity with different flavors, functional ingredients, and stabilizer/emulsifier systems.

A new method was developed and validated to meet the above listed stringent requirements. The method employed a simple, robust, and rapid sample preparation method that, coupled with LC-MS/MS, enabled simultaneous determination of eight water-soluble vitamins with unprecedented accuracy and precision across a wide range of infant formulas and related nutritionals.

2. Experimental

2.1. Materials

Folic acid, biotin, calcium pantothenate, niacinamide, nicotinic acid, pyridoxine hydrochloride, riboflavin, and thiamin chloride hydrochloride were purchased from USP in Rockville, MD. $^{13}C_5$-biotin was purchased from Alsachim in Illkirch Graffenstaden, France; and $^{13}C_5$-folic acid was purchased from Merck & Cie Schauffhausen in Schauffhausen, Switzerland. $^{13}C_3$,^{15}N-pantothenate calcium and $^{13}C_4$-pyridoxine were purchased from Cambridge Isotope Laboratory in Tewksbury, MA. $^{13}C_4$-thiamine chloride and $^{13}C_4$,$^{15}N_2$-riboflavin were purchased from IsoSciences in King of Prussia, PA. $^{2}H_4$-nicotinamide and $^{2}H_4$-nicotinic acid were purchased from CDN Isotopes in Pointe-Claire, QC. NIST SRM 1849a was from the National Institute of Standards and Technology in Gaithersburg, MD. MS grade ammonium formate was from Fluka, LCMS grade methanol was from EMD, and ACS grade glacial acetic acid was from Mallinckrodt. Laboratory water was 18 MΩ from a Millipore Milli-Q Advantage A10 water polisher.

2.2. Procedure

All sample and standard preparation was carried out under UV shielded lighting, and all pipetting was done with positive displacement pipettes. A mixture containing each vitamin and a mixture containing each internal standard were prepared from stock solutions each day (concentrations given in Table 1). Six calibration standards were prepared by transferring 10, 25, 50, 100, 250, and 500 μL aliquots of the mixed standard solution into individual 2 mL microfuge tubes. Laboratory water was added to bring the total volume to 750 μL, and 60 μL of internal standard mixture was added to each. The calibration standards were then prepared following the same procedure as the samples as described below.

Powdered samples were reconstituted by dissolving 10 g of sample into 90 g of water and liquid samples were used as is. Reconstituted powders must be fully solubilized and liquid products must be well mixed prior to sampling to minimize errors from sample inhomogeneity. Sample size of 0.75 g, 0.50 g, and 0.25 g were used for infant formula, pediatric formula, and adult nutritionals, respectively. Sample was placed in a 2 mL microfuge tube. 60 μL of internal standard mixture was added, and water was added (if necessary) to bring the total volume to 750 μL. (Over-spikes were added at this point for method validation). 60 μL of internal standard mixture was also added to the calibration standards.

After addition of internal standard, each of the samples, over-spiked samples, and calibration standards were vortexed and allowed to equilibrate for 15 min. Next, 750 μL of 1% glacial acid in methanol was added, and the sample or standard was immediately vortexed. Samples and standards were centrifuged together at 13,000 rfu and 20 °C for 10 min. After centrifugation, a 100 μL aliquot of supernatant was diluted with 20 mL of 50 mM ammonium formate. The diluted samples and standards were mixed well and then filtered through a 0.45 μm syringe filters into amber autosampler vials for analysis.

2.3. Analysis conditions

Separation was performed on a Waters Acquity BEH C18, 2.1 × 100 mm, 1.7 μm column. Mobile phases consist of (A) 20 mM ammonium formate and (B) methanol. Separation was performed after a 10 μL injection. Mobile phase flow was 0.35 mL/min at 40 °C. The reversed phase gradient started with a 0.5 min hold at 99% A, followed by a 2.0 min ramp to 92% A, then a 2.5 min ramp to 10% A, and a hold at 10% A for 1.0 min before returning to initial conditions and re-equilibrating for 2.0 min. All compounds eluted between 1.0 and 4.5 min and the total gradient program is 8.0 min. Samples were held at ambient temperature.

The method was developed and validated on a Waters Xevo TQ-S instrument using electrospray ionization (ESI) conditions. ESI+ mode was used with a capillary voltage of 2.5 kV, cone voltage of 20 V, and source offset of 50 V. Desolvation was performed at

Table 1
Approximate concentration of each vitamin in the mixed standards which were prepared daily.

Native standard	μg/mL	Internal standard	μg/mL
Biotin	4.60	$^{13}C_5$-biotin	3.17
Folic	4.09	$^{13}C_5$-folic	3.17
Niacin	53.0	$^{2}H_4$-niacinamide	35.6
Nicotinic acid	4.10	$^{2}H_4$-nicotinic acid	31.7
Pantothenic acid	32.0	$^{13}C_3$,^{15}N-pantothenic acid	25.0
Pyridoxine	6.75	$^{13}C_4$ pyridoxine	4.44
Riboflavin	5.20	$^{13}C_4$,$^{15}N_2$-riboflavin	4.63
Thiamine	7.46	$^{13}C_4$ thiamine	6.35