LC-MS/MS Method for Sensitive Detection and Quantitation of 8 Water-Soluble vitamins in Infant Milk Powder

Yin Ling Chew[1], Eleanor Wai Yi Hor[2]*, Qi Zong Lee[3]*, Noah Luzheng Ong[3], Muhammad Averus[4]; Suwiton[4]; Martin Uli Tua[4], Jie Xing[1], Zhaoqi Zhan[1], Robert Clifford[5]

[1]Shimadzu (Asia Pacific) Pte Ltd, Singapore, [2]School of Physical and Mathematical Sciences, Nanyang Technological University, [3]Department of Chemistry, National University of Singapore, [4]PT Saraswanti Info Genotech, Bogor City, Indonesia, * Internship Student, [5] Shimadzu USA

Overview

- A highly-sensitive LC-MS/MS method with a simple extraction procedure for quantitation of 8 water-soluble B vitamins is established. Isotope labelled ISs were used to ensure the accuracy of analysis and compensate the matrix effect.
- Method performance was evaluated, obtaining LOQs and LODs, repeatability and recovery for all eight B vitamins.
- The established method is further evaluated using the reference material NIST 1849a. The quantitative results of the eight B vitamins are within the ranges of official values given.

1. Introduction

Water-soluble B vitamins are essential nutrients needed by human body to function properly. Since B vitamins are not produced in adequate amounts in human body, it is important to obtain them from food sources. Low levels of B vitamins in breastfeeding mothers may cause their babies slow growth. Therefore, infant formulas must include proper amounts of nutrients including B vitamins. The Dietary Reference Intake (DRI) and the Food Additive have issued the reference value of infants' daily requirement of B vitamins in infant formula. HPLC and LC/MS/MS methods have used in analysis of B vitamins and additives, either limited to few compounds in HPLC method or required time-consuming sample clean-up method due to the complicated matrix of infant formula. Here, we present a LC-MS/MS method for simultaneous determination of 8 water-soluble B vitamins in infant formula with 5 isotope labelled internal standards, with a simple sample extraction procedure.

2. Experimental

Eight water-soluble vitamin standards namely thiamine (B1), riboflavin (B2), nicotinic acid (B3), nicotinamide (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7) and folic acid (B9) were obtained from Sigma Aldrich. Five isotope labelled internal standards, $^{13}C_4$-thiamine (IS B1) , $^{13}C_4$, $^{15}N_2$-riboflavin (IS B2), $^{2}H_4$-nicotinamide (IS B3), $^{13}C_6$, $^{15}N_2$-pantothenic acid (IS B5) and $^{2}H_2$-biotin (IS B7) were purchased from Isosciences. Vitamins B1, B3, B5 and B6 were diluted using water while vitamins B2, B7 and B9 were diluted using 0.01M NaOH to form individual stock solutions. All the vitamin standards and internal standards were diluted into working solutions to constrcte calibration curves. For sample preparation method, 1 g of infant formula was weighed into a 50 mL polypropylene tube. 10 mL of water was added and vortexed. 30 mL of 1% acetic acid was then added and vortexed, and centrifuged. The supernatant was filtered using a 0.22 nylon filter and diluted 5x further using water. Shimadzu LCMS-8060 with heated ESI, and a Shim-pack GIST C18-AQ (2.1 mm x 50 mm; 1.9 µm) column was used in this analysis.

Table 1. Analytical conditions of 8 water-soluble vitamins analysis with LCMS-8060

Column	Shim-pack GIST C18-AQ 1.9um, (2.1 mm I.D. x 50 mm L)	**Interface**	**ESI**
Flow	0.3 mL/min	MS Mode	Positive mode
Mobile phase	A : 5mM Ammonium Acetate in MilliQ water 0.1% formic acid B : Methanol	CID gas	Argon, 230 kPa
Oven Temp	40 ºC	Heat Block Temperature	400 ºC
Injection vol	2 µL	DL Temperature	250 ºC
Elution mode	**Gradient Elution**; B% : 0% (0.0 to 2.0 min) ➡ 30% (2.5 min) ➡ 50% (5.0 min) ➡ 99% (5.5 to 7.0 min) ➡ 0% (7.1 to 12.0 min)	Interface Temperature	300 ºC
		Nebulizing Gas Flow	Nitrogen, 3.0 L/min
		Drying Gas Flow	Nitrogen, 10.0 L/min
		Heating Gas Flow	Zero Air, 10.0 L/min

3. Results and Discussion

3.1 MRM method for eight water-soluble B vitamins and internal standards

Automated MRM optimization of 8 water-soluble vitamins standards and 5 internal standards were carried out using the LabSolutions workstation. The precursors were their protonated ions, [M+H]+. Two MRM transitions for each compound were chosen as quantifier and qualifier ions (Table 2).

Linear calibration curves were obtained for all 8 water-soluble vitamin compounds. Good linearity with correlation coefficient (r2) greater than 0.999 across the range of 10 ng/mL – 750 ng/mL was obtained. The calibration curves of vitamin B2, B3 and B5 are shown in Figure 2.

Table 2: MRM transitions for 8 water-soluble vitamins and 5 internal standards

Compound name	Quantifier ion	Qualifier ion	Internal standard	Quantifier ion	Qualifier ion
B1	265.00>81.10	265.00>144.10	IS B1	269.00>122.10	269.00>148.10
B2	377.00>243.15	377.00>172.20	IS B2	383.00>249.15	383.00>175.15
B3 (Nicotinic acid)	124.00>53.10	124.00>78.10	IS B3	127.00>84.10	127.00>81.10
B3 (Nicotinamide)	123.00>53.10	123.00>78.05			
B6	170.00>77.10	170.00>152.10			
B5	220.10>90.10	220.10>202.25	IS B5	224.00>206.10	224.00>188.10
B7	245.00>227.10	245.00>97.10	IS B7	247.00>229.10	247.00>99.10
B9	442.10>295.10	442.10>176.10			

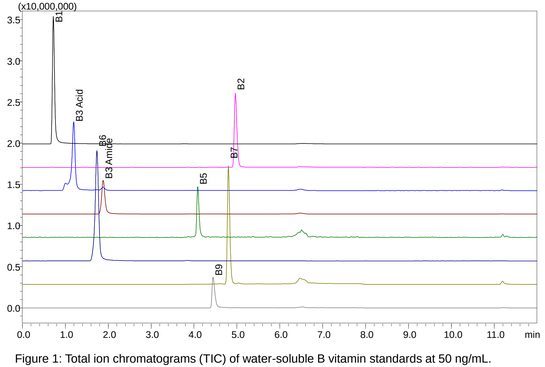

Figure 1: Total ion chromatograms (TIC) of water-soluble B vitamin standards at 50 ng/mL.

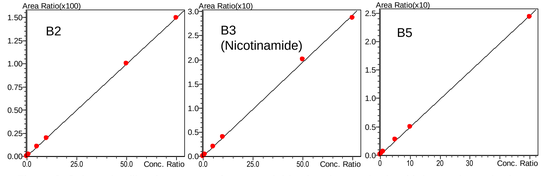

Figure 2: Selected calibration curves of water-soluble vitamin standards with internal standards.

3.2 Performance evaluation of established quantitation method

The limit of detection (LOD) and limit of quantitation were obtained from LabSolutions software with S/N~3 and S/N~10. The LOD and of LOQ of 8 water-soluble B vitamins ranges from 0.08 – 1.36 ng/mL and 0.23 – 4.13 ng/mL, respectively. Repeatability (n=6) tests were performed at concentration of 10 ng/mL for each vitamin standard and the results are tabulated in Table 3.

Table 3: Linearity, LOD, LOQ and repeatability results for 8 water-soluble vitamin compounds.

Compound name	Range (ng/mL)	R2	LOQ (ng/mL)	LOD (ng/mLb)	RSD% (n=6)
B1	1-500	0.9994	0.52	0.17	2.80
B2	1-750	0.9999	0.23	0.08	3.67
B3 (Nicotinic acid)	1-750	0.9993	0.97	0.32	3.20
B3 (Nicotinamide)	1-750	0.9994	0.52	0.17	2.36
B5	5-500	0.9990	4.13	1.36	1.91
B6	1-750	0.9999	0.51	0.17	3.94
B7	1-100	0.9991	0.32	0.11	2.07
B9	1-100	0.9992	1.00	0.33	2.87

3.3 Recovery study of 8 water-soluble vitamins in infant formula powder

A recovery test was performed using infant formula powder as the matrix. The 8 water-soluble vitamins are spiked in the infant formula powder at different concentrations namely, 25 mg/kg for vitamins B3 (both nicotinic acid and nicotinamide) and B5; 5 mg/kg for vitamins B1, B2 and B6; 0.5 mg/kg for vitamins B7 and B9.

The recovery test was performed twice to obtain reliable results. Each duplicate was injected thrice and the average concentration was used for the calculation of recovery. Good recovery of 8 water-soluble vitamins was achieved between 90.1~113.6%.

Table 4: Average recovery of water-soluble vitamins in infant formula (n=2 x 3).

Compound name	Recovery (%)
B1	113.6
B2	105.2
B3 (Nicotinic Acid)	101.6
B3 (Nicotinamide)	109.8
B5	104.9
B6	108.0
B7	90.1
B9	90.1

3.4 Quantitation of 8 water-soluble vitamins in NIST 1849a

The established method was evaluated using the standard reference material, NIST 1849a, which is an infant/adult nutritional formula. Three sets of NIST 1849a milk powder was extracted and each of them injected in triplicates to ensure the accuracy of the method. The results obtained are within the range of content and are tabulated in Table 4.

Table 5: Results of quantitation of 8 B vitamins in NIST 1849 nutritional formula (D.F. = 200).

Vitamin	Content (mg/kg)	Replicate (mg/kg) (n=3)		
		1st	2nd	3rd
B1 – Thiamine	12.57 ± 0.98	13.14	13.23	12.89
B2 – Riboflavin	20.37 ± 0.52	20.59	20.80	20.77
B3- Nicotinamide	108 ± 10	100.92	101.31	100.53
B5 - Pantothenic acid	68.2 ± 1.9	69.02	67.99	67.34
B6 - Pyridoxine	13.46 ± 0.93	13.58	14.03	13.82
B7 - Biotin	1.99 ± 0.13	2.06	2.09	2.09
B9 - Folic acid	2.293 ± 0.062	2.253	2.285	2.277

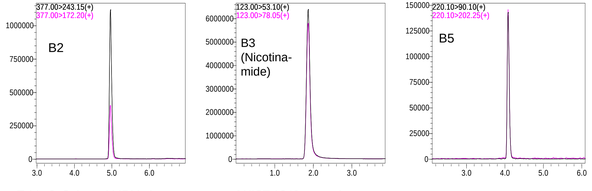

Table 3: Selected MRM chromatograms of NIST 1849a sample.

4. Conclusions

A sensitive and selective LC-MS/MS method with a simple extraction procedure was established for quantitation of 8 water-soluble B vitamins using 5 isotope labelled internal standards. The method performance of analysis and sample preparation were evaluated. The linearity (r2) of all the 8 B vitamins are greater than 0.999. The LOD and LOQ estimated are at 0.08~1.36 ng/mL and 0.23~4.13 ng/mL, respectively. The recovery ranges from 90.1% to 113.6%. The method was used to quantitate the standard reference material, NIST 1849a. The quantitation results fall within the official values given.

References

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2. Goldschmidt, R. J.; Wolf, W. R. *Anal. Bioanal. Chem.* **2010**, *397* (2), 471–481.