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ARTICLE

Development Rapid Analytical Methods for Inositol as a Trace Component by HPLC and LC-MS/MS in Infant Formula

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Abstract

A rapid and simple analytical method, using liquid chromatography tandem mass spectrometry (LC-MS/MS), was developed to detect *myo*-inositol (MI) in infant formulas. For protein removal: acid hydrolysis and lipid removal through organic solvent extraction. The operating conditions for instrumental analysis were determined based on previously reported analogous methods that used LC-MS/MS. Quantitative analysis was used for the detection limit test, infant formula recovery test, and standard reference material (SRM) 1849a to verify the validity of our LC-MS/MS analytical method, which was developed to quantify MI. For validation, the results of our method were compared with the results of quantitative analyses of certified values. The test results showed that the limit of detection was 0.05 mg/L, the limit of quantitation was 0.17 mg/L, and the method detection limit was 17 mg/kg. The recovery test exhibited a recovery between 98.07-98.43% and a relative standard deviation between 1.93-2.74%. Therefore, the result values were good. Additionally, SRM 1849a was measured to have an MI content of 401.84 mg/kg and recovery of 98.25%, which is comparable to the median certified value of 409 mg/kg. From the aforementioned results, we judged that the instrumental analysis conditions and preparation method used in this study were valid. The rapid analytical method developed herein could be implemented in many laboratories that seek to save time and labor.

Keywords: *myo*-inositol, LC-MS/MS, analytical method, infant formula

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Introduction

The isomer *myo*-inositol (MI) is a meso compound with an optically inactive plane of symmetry; *meso*-inositol is an obsolete name that refers to MI. In addition to MI, the other naturally occurring stereoisomers (in minimal quantities) are *scyllo*-, *muco*-, D-*chiro*-, and *neo*-inositol; other possible isomers are L-*chiro*-, *allo*-, *epi*-, and *cis*-inositol. D- and L-*chiro*-inositol constitute the only pair of inositol enantiomers; they are enantiomers of each other, not of MI.

Inositol and some of its mono- and polyphosphate derivatives function as the basis for various signaling and secondary messenger molecules. They are involved in the following biological processes: insulin signal transduction (Larner, 2002), cytoskeleton assembly, nerve guidance (epsin), intracellular calcium (Ca^{2+}) concentration control (Gerasimenko *et al.*, 2006), cell membrane potential maintenance (Kukuljan *et al.,* 1997), breakdown of fats (Rapiejko *et al.,*1986), and gene expression (Shen *et al.,* 2003; Steger *et al.,* 2003). MI is a compound necessary for infant development and when this substance cannot be administered through breast milk, it must be supplied in the diet by either inositol-enriched milk or formula (Koletzko *et al*., 2013). Maximum limits permitted for MI in infant formulas range from 4.0 to 40.0 mg/100 kcal (1.0-9.5 mg/100 kJ), while the requirements for premature infants range from 27.0 to 67.5 mg/100 kcal (Codex Alimentarius, FAO WHO, 2007). Effective quantification of MI is necessary to ensure that its amounts in powdered milk or infant formula do not exceed the established limits. At present, since there are numerous MI-enriched food products, rapid and simple methodologies to determine MI content are desirable. Various analytical methods have already been published to determine quantities of inositol: microbiological assays (Difco Manual, 1984), gas chromatography (Troyano *et al*., 1996), high performance liquid chromatography (Hicks *et. al*., 1994; Kargacin *et al*.,

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1987; Lauro *et al*., 1989), and ion chromatography (Cataldi *et al*., 1998). However, the preparation of samples containing emulsified foods, such as infant formulas, is challenging. Generally, the preparation method includes either alkali decomposition or fat-free processes; these were not considered in a previous report of inositol analysis. This study aims to introduce a sample preparation method that takes in consideration the matrix characteristics of formulated milk powder. This study also discusses the development of a simple test method that requires a smaller amount of test materials and time than the conventional method. The method presented herein is considered an improvement upon the AOAC journal method due to its precise instrumental analysis, simple sample preparation method, and utility of LC-MS/MS.

Materials and Methods

Standard, Samples, and reagents

MI (Cat. No. 1340960) was purchased from the US Pharmacopeial Convention (USP, USA) for use as the reference standard material. The purity of MI was 99.9% (0.999 mg/mg). A stock solution of 1,000 μg/mL inositol was dissolved in water and diluted to concentrations of 1, 5, 10, 20, and 50 ng/mL to make the standard working solutions. The infant and toddler formulas used in this study were purchased from a local market and stored at 4°C. A certified reference material (CRM), infant formula SRM 1849a (NIST, USA), was used in the recovery tests to develop the method. The amount of inositol in SRM 1849a was 409 mg/kg. Both of 0.1 M hydrochloric acid (HCl) and ammonium acetate were purchased from Junsei Chemical (Japan). Water, methanol, and chloroform (HPLC grade) were purchased from Merck (Germany). Ultrapure water was obtained using a Banstead Diamond TII system (USA). The distilled water had a resistance of 18.0 MΩ.

Sample preparation

A sample preparation method based on the previous study (Schimpf *et al*., 2012) was modified to include the following techniques for protein removal: acid hydrolysis and lipid removal through organic solvent extraction. A sample containing 1 g of infant formula was placed in a 100 mL volumetric flask and dissolved in 15 mL of distilled water and 1 mL of 0.1 M HCl. The mixture was agitated for 5 min. Afterwards, 10 mL of chloroform was added to remove the lipid fraction (Sullivan and Carpenter, 1993) and the solution was covered with a screw cap and vigorously mixed for 1 min in a vortex mixer at maximum speed. The tube was then centrifuged for 10 min at 4,000 rpm at 4°C. The aqueous layer was filtered through a 0.2 μm nylon syringe filter and analyzed by LC-ESI-MS/MS.

Operating conditions

The operating conditions for instrumental analysis were determined based on previously reported analogous methods that used LC-MS/MS (Kim *et al*., 2012). An Agilent 1200 HPLC system (Agilent, USA) equipped with a Prevail Carbohydrate ES column (4.6 mm × 250 mm, 5 μm, Alltech) and a 6410 triple quadrupole LC/MS tandem MS system were used to analyze inositol. The mobile phase was composed of 5 mM ammonium acetate (25%) and acetonitrile (75%) for isocratic flow. The following parameters were employed: 1.0 mL/min flow rate, 30°C column temperature, and 10 μL injection volume. HPLC grade solvents were filtered through a 0.45 μm membrane and ultrasonically degassed prior to use. The specific conditions used in LC-MS/MS analysis are shown in Table 1.

Validation of the method

The developed method was validated by the AOAC guidelines for single laboratory validation of chemical methods (AOAC International, 2002). The method validation evaluated the following parameters: linearity, limit of detection (LOD), limit of quantification (LOQ), method detection limit (MDL), and recovery test results. Linearity was characterized by the average coefficient of determination (r^2) and was calculated using five consecutive standard curves. The LOD and LOQ were determined by diluting standard working solutions of inositol to obtain signal to noise ratios of ~3:1 for LOD and ~10:1 for LOQ. MDL was determined by multiplying the solvent volume (mL) of the LOD and dividing by the sample amount (g). The recovery tests of inositol were conducted with a spiking test involving an infant formula sample and confirmation of the certificated value for standard reference material (SRM) 1849a. For the spiking test, the spiked level of infant formula was 50 mg/kg for 0.3 g of sample and the results from quantitative analysis were compared to those obtained for samples without the standard solution.

Monitoring test

Infant formulas consisting of milk-based powder and cereal-based powder were analyzed for MI content. A total of 16 samples were used for the monitoring tests. All samples were purchased from a local market and stored at room temperature.

Table 1. Liquid chromatography-tandem mass spectrometry conditions for *myo*-inositol

(a) LC

Parameter	Condition
Column	Prevail Carbohydrate ES 4.6 mm × 250 mm, 5 μm, Alltech
Detector	MS/MS
Mobile phase	A : 5mM Ammonium acetate, B : Acetonitrile, Isocratic (A 25% : B 75%)
Flow rate	1.0 mL/min
Column temperature	30
Running time	30 min
Injection volume	10 uL

(b) MS/MS

Parameter	Condition
Ion source	ESI (Electro spray ionization)
Polarity	Negative
Nebulizer gas	N_2
Nebulizer pressure	36 psi
Gas flow	11 L/min
Ion spray voltage	4500 V
Source temp.	300°C
Resolution	Q1 (unit) Q3 (unit)
Scan mode	MRM (Multiple reaction monitoring)

MRM condition

Retention time (min)	Compound	Precursor ion (m/z)	Product ion (m/z)		Dwell (ms)	Fragmentor (V)	Collision energy (V)
16.12	*myo*-inositol	179	87	Quantitative	200	134	12

Results and Discussion

Establishing the optimum conditions of the ESI ion source

According to a study published in the journal, the polarity of the existing ESI mass detector was set when the inositol assay was analyzed in the positive (Flores *et al.*, 2011) or negative mode (Kim *et al*., 2012). In this study, the test was carried out in both positive and negative modes to determine a suitable polarity for the ESI mass detector used in the inositol LC-MS/MS assays.

It is worth noting that the precursor ion [M + H] (m/z = 181.2) of *myo*-inositol was not detected in positive mode. Meanwhile, the precursor ion [M-H] (m/z = 179.2) was detected in negative mode. Therefore, negative mode was used in the mass spectrometry process (Fig. 1).

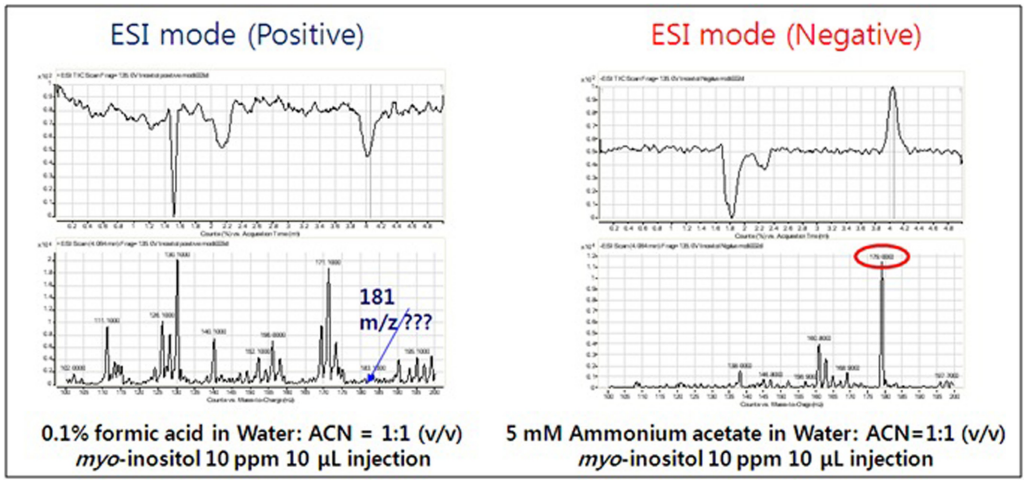


Fig. 1. Test results for optimal ESI mass detector's polarity mode as a *myo*-inositol trace component by LC-MS/MS.

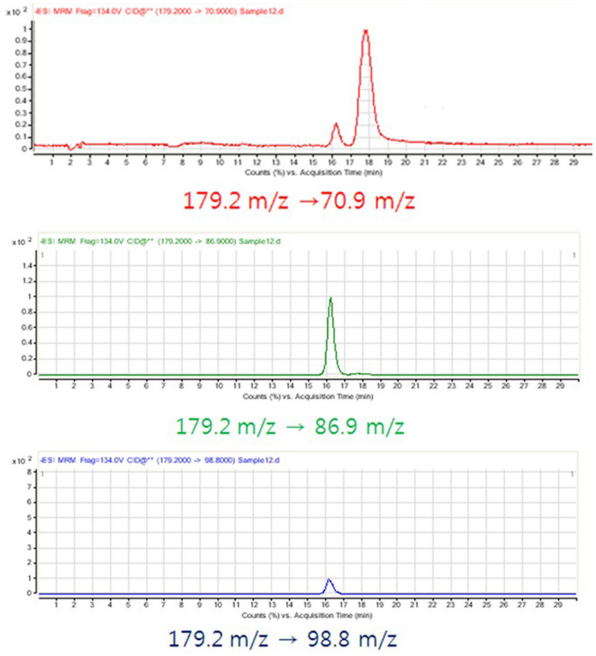


Fig. 2. Test results for quantitative and qualitative ion conditions of the product ion for ion precursor ion.

Optimum condition settings to detect precursor and product ions

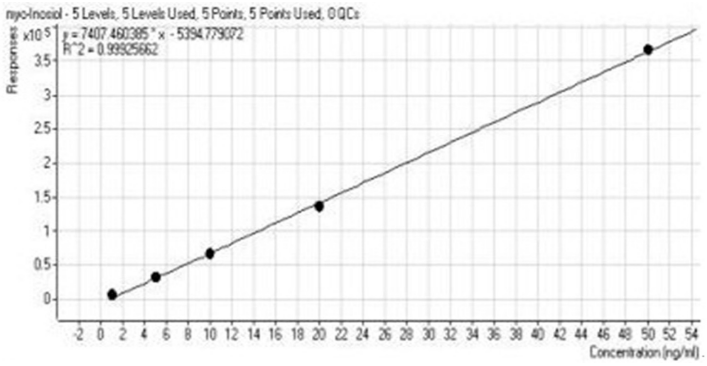
In negative mode, the precursor ion (179.2 m/z) was search for the quantitative and qualitative condition among many product ions. The results are shown in Fig. 2. Showed 86.9 m/z is the highest sensitivity in Product ion, the second is 70.9 m/z, the third was a result of the high sensitivity to 98.8 m/z. In this study, we selected m/z = 86.9 as a quantitative ion, of the ion dose and the highest sensitivity to select the 98.8 m/z as qualitative ion. The 70.9 m/z ion is higher than 98.8 m/z from the surface sensitivity, sample analysis TIC (total ion chromatogram) and EIC (extract ion chromatogram) were detected in the peak that interference, which results in a high concentration of the sample during analysis was determined to be affected.

Linearity and range

The MI content in infant formula was quantified to be approximately 409 mg/kg, and the certified value range of SRM 1849a was 401.84 ± 0.74 mg/kg. In case of completing sample preparation with final volume of 10 mL in comparison with sample of 0.3 g proposed by this study, the calibration curve was predicted to be within the range of 3-5 mg/L (ppm). The test results are shown in Fig. 3 and include an r^2 value of 0.9993; linearity was observed in solutions at concentrations of 1, 5, 10, 20, and 50 μg/L. Therefore, we employed solutions at concentrations within the range of 1-50 μg/L for the calibration curves in this test, and accordingly diluted the final solutions 200-fold for instrumental analysis.

Method validations

Quantitative analysis was used for the detection limit test, infant formula recovery test, and certified reference material SRM 1849a to verify the validity of our LC-MS/MS analytical method, which was developed to quantify MI. For validation, the results of our method were compared with the results of quantitative analyses of certified values. The test results showed that the limit of detection (LOD) was 0.05 mg/L, the limit of quantitation (LOQ) was 0.17 mg/L, and the method detection limit (MDL) was 17 mg/kg. The recovery test exhibited a recovery between 98.07-98.43% and a relative standard deviation between 1.93-2.74%. Therefore, the result values were good. Additionally, SRM 1849a was measured to have an L-carnitine content of 401.84 mg/kg and recovery of 98.25%, which is comparable to the median certified value of 409 mg/kg. From the aforementioned results, we judged that the instrumental analysis conditions and prep-

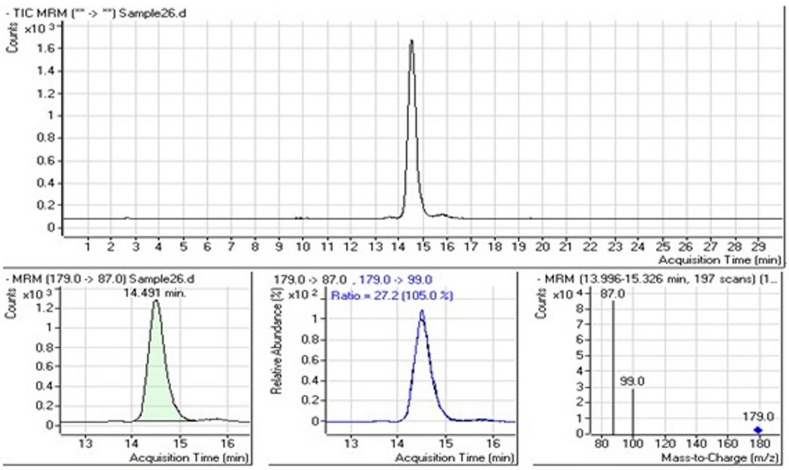


Linear Regression	y=ax+b
Slope (a)	7407.46
y-intercept(b)	-5394.78
r^2 value	0.9993
Range	1 ~ 50 mg/L

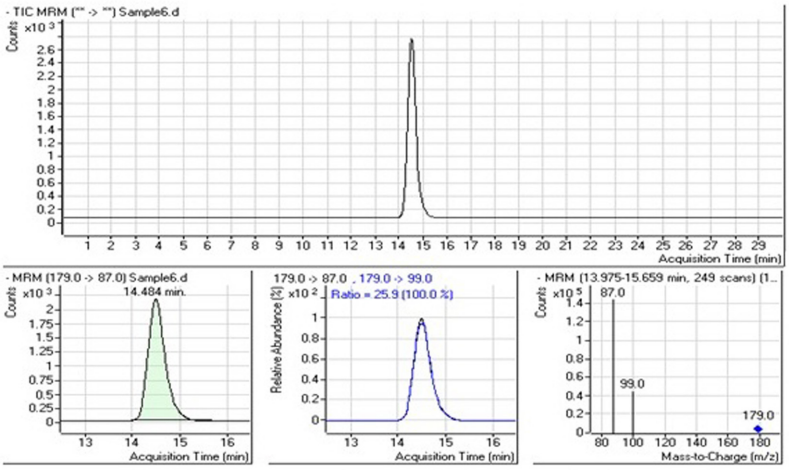
Fig. 3. Monitoring test for *myo*-inositol in certified reference material (SRM 1849a) and infant formula.

Table 2. Validation factors and monitoring test for *myo*-inositol in certified reference material (SRM 1849a) and infant formula using liquid chromatography-tandem mass spectrometry analysis

Recovery test		Tested Value[a] (mg/kg)	RSD (%)	Recovery (%)	
SRM 1849a[b]		401.84 ± 0.74	0.18	98.25 ± 0.18	
Spiked sample[c]	T-1	265.84 ± 3.84	2.74	93.18 ± 1.54	
	T-2	271.91 ± 3.22	2.53	95.64 ± 1.45	
	T-3	265.81 ± 2.65	1.93	95.31 ± 2.03	
Samples		Tested Value (mg/kg)	Samples	Tested Value (mg/kg)	
Infant formula (milk-based, powder)	T-1	941.24	Infant formula (milk-based, powder)	T-9	828.39
	T-2	880.79		T-10	150.23
	T-3	919.68		T-11	1120.10
	T-4	884.88		T-12	556.69
	T-5	940.42		T-13	538.68
	T-6	999.91	Infant formula (cereal-based, powder)	T-1	684.61
	T-7	1118.08		T-2	481.93
	T-8	483.87		T-3	134.85
r^2		0.9993	Linear Regression	y = 7407.46x – 5394.78	
LOD (μg/L)		0.05	Range	1-50 μg/L	
LOQ (μg/L)		0.17			
MDL (mg/kg)		17			



[LC-MS/MS chromatogram and mass spectrum of the sample]



[LC-MS/MS chromatogram and mass spectrum of the standard reference material]

Fig. 4. The LC-MS/MS chromatogram and mass spectrum for MI.

aration method used in this study were valid. The specific validation factors are shown in Table 2.

Monitoring test for infant and toddler formulas

A monitoring test was carried out for 16 samples of infant formula and toddler formula with SRM 1849a, international certified reference material. According to the results of the monitoring test, which are shown in Table 2, the MI content in powder products was quantified at levels between 134.85-1118.08 mg/kg. These levels were accurate from comparison with the contents indicated on the products. Therefore, it was possible to verify that MI indication management of infant formula placed on the Korean market was well accomplished.

Quantity corresponding to appropriate sample volume of each food group was 0.3 g for all sample groups and it was quantity corresponding to sample volume included in calibration curve concentration established by the method. The LC-MS/MS chromatogram and mass spectrum for MI are shown in Fig. 4. The chromatogram and the mass spectrum showed that the standard solution, infant formula and SRM 1849a sample treated by the developed test method and standard solution.

Conclusions

A method employing LC-MS/MS and a simple sample pretreatment procedure was developed to accurately quantify MI in infant formulas. We carried out this study along to suggested material and method. Sample pretreatment time and labor were reduced, and the recovery test exhibited accurate results for infant formula samples. The precursor ion for MI was detected at m/z = 179.2, and product ions were detected at m/z = 86.9 (quantitative) and m/z = 98.8 (qualitative), respectively. The results from the spiked recovery test were in the range of 93.18-95.31% and exhibited relative standard deviations between 1.93% and 2.74%; additionally, the result for certified reference material (SRM 1849a) was within the range of the certificated values. The developed method based on LC-MS/MS in MRM mode, following the described sample preparation, could be an accurate tool to replace conventional analytical methods, as well as reduce time and labor. Moreover, a beginner can be expected to easily perform this analytical procedure due to its simplicity.

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