Simultaneous Determination of 5'-Monophosphate Nucleotides in Infant Formulas by HPLC–MS

Yiping Ren[1], Jingshun Zhang[2], Xiaodan Song[3], Xiaochun Chen[1], and **Duo Li[2,*]**

[1]Zhejiang Provincial Center for Disease Prevention and Control, Hangzhou, Zhejiang 310051, P.R. China; [2]Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou, Zhejiang 310029, P.R. China; and [3]Qingdao Shengyuan Dairy Industry Co., Ltd., Qingdao, Shandong 266400, P.R. China

Abstract

A method was developed for simultaneous determination of 5'-monophosphate nucleotides, adenosine 5'-monophosphate, cytidine 5'-monophosphate, guanosine 5'-monophosphate, inosine 5'-monophosphate, and uridine 5'-monophosphate in infant formulas by high-performance liquid chromatography-mass spectrometry equipped with electrospray ionization source. The complete chromatographic separation of five nucleotides was achieved through a Symmetry C_{18} column, after a binary gradient elution with water containing 0.1% formic acid and acetonitrile as mobile phase. The multi-reaction monitoring mode was applied for tandem mass spectrometry analysis. The established method was further validated by determining the linearity ($R^2 > 0.999$), recovery (92.0–105.0%), and precision (relative standard deviation ≤ 6.97%). To verify the applicability of the method, thirty commercially available infant formulas were randomly purchased from the supermarkets in Hangzhou, China, and then analyzed. The results showed that the developed method is validated, sensitive, and reliable for quantitation of nucleotides in infant formulas.

Introduction

Nucleotides are composed of one to three phosphate groups and a cyclic nitrogenous base, either a purine or a pyrimidine, linked to a pentose sugar. They play important roles as coenzymes and chemical energy sources in most biological processes in cell metabolism. They are also the primary structure units and momomeric precursors in nucleic acids synthesis (1–4). They can be synthesized endogenously de novo or salvage pathways, and thus they are not considered as essential dietary nutrients (3). However, when the endogenous supply is insufficient for normal function under the conditions of certain disease states, limited nutrient intake, or rapid growth, the exogenous nucleotides may become conditionally essential or semi-essential nutrients (2,5). Nucleotides are involved in the metabolism of long chain polyunsaturated fatty acids and modify the composition of the intestinal microflora and iron absorption in the gut (2,3). Evidence also indicates that nucleotides have other functions such as improvement of gastrointestinal tract repair after damage and participation in the immune response mediated by T cells (2,3,5–7). Concerning their important biological and trophochemical role of maintaining normal growth and development in infants, recently there has been an increasing interest in dietary nucleotides in infant nutrition (4,8).

Human milk is assumed to be the best source of nucleotides for young infants and serves as a gold standard in manufacturing infant formulas (2,8,9). Although most infant formulas are currently made from bovine milk, the compositions of the nucleotides are different from those found in human milk (5,6,8,10). So, the question of specific nucleotides supplementation has been raised, and infant formulas with nucleotides supplemented in amount similar to those in human milk have been adapted and recommended in several countries (1–3,8). For controlling and monitoring the quality of infant formulas, it is required to determine the amounts of supplemented nucleotides.

Because nucleotide supplementation was permitted and accepted as a safe practice in the USA and some other countries (1,4), nucleotide analyses have been performed for years. To date, a series of methods have been developed to simultaneously determine nucleotides and related compounds in different substrates. The focus of the previously reported chromatographic analyses of nucleotides and related compounds was generally for use in clinical, pharmaceutical and genomic studies (11–16). The methods including thin-layer chromatography (TLC), paper chromatography, ion-exchange chromatography, enzymatic assay, and high-performance liquid chromatography (HPLC) of determining nucleotides and nucleosides in various mammalian milk, human milk and infant formulas were reviewed by Gill and Indyk (2007) (5). Unfortunately, these methods have some disadvantages such as being time consuming, having low sensitivity, or requiring complicated operation (4,8).

Due to the incorporation of minimal sample preparation and rapid chromatographic separations, HPLC coupled to the diode array detector (DAD) or ultraviolet (UV) detector has superseded other chromatographic methods and has become the dominant technique for analyzing the nucleotides and related compounds in milk and milk products over the past years (5). There are three

*Author to whom correspondence should be addressed: email duoli@zju.edu.cn.

main modes of liquid chromatography applied for nucleotides analysis: ion-exchange chromatography (IEC), reversed-phase liquid chromatography (RP-LC), and ion-pair (IP) RP-LC. Recently, some developed techniques with high speed or sensitivity such as capillary electrophoresis (17), capillary electrophoresis-mass spectrometry (18,19), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (20), liquid chromatography-mass spectrometry (LC–MS) (21–24) have been also applied for the analysis of nucleotides and nucleosides in the field of clinical and pharmaceutical chemistry. Nevertheless, there are some drawbacks of these methods because most of them employ the phosphate buffer with or without the ion-pair reagent as the mobile phase, which increases the risk of crystallization and obstruction of the system (10). The phosphate salt (e.g., ammonium phosphate) and the ion-pair reagent (e.g., tetra-alkylamine) are not compatible with the mass spectrometry because these nonvolatile compounds generally are bringing about ion suppression in the electrospray ionization (25). In addition, they could contaminate the ion source of the mass analyzer and lead to high background noise (26). By further optimizing the operation conditions, these shortcomings are possible to overcome. And then such techniques can be applied to analyze milk-based nucleotides and related compounds.

The technique of HPLC–MS–MS is widely used in bioanalysis due to its inherent sensitivity, specificity and speed. As far as could be determined, there is no report of applying HPLC–MS–MS for nucleotides determination in milk and infant formulas (5). Therefore, the objective of the present study was to establish and validate a rapid and sensitive HPLC–MS–MS method for determining nucleotides in infant formulas. The method herein describes the optimization of the HPLC–MS–MS operating conditions and its analytical merit as well as its application to the determination of 5'-monophosphate nucleotides in commercial available infant formulas in China.

Experimental

Chemicals

Cytidine 5'-monophosphate (CMP) disodium salt (purity ≥ 98%) and uridine 5'-monophosphate (UMP) disodium salt (purity ≥ 99%) were purchased from Fluka Chemical Corp. in Tokyo, Japan. Adenosine 5'-monophosphate (AMP) sodium salt (purity ≥ 99%) and guanosine 5'-monophosphate (GMP) disodium salt (purity 100%) were purchased from Fluka Chemical Corp. and Sigma-Aldrich Chemical Co. in Munich, Germany, respectively. Inosine 5'-monophosphate (IMP) disodium salt (purity ≥ 99%) was obtained from Sigma-Aldrich Chemical Co. in St. Louis, MO. The chemical structures of the five 5'-monophosphate nucleotides are shown in Figure 1. Formic acid and acetonitrile (Merck, Darmstadt, Germany) were HPLC grade purity. Ammonium acetate (purity ≥ 98.0%) was purchased from Fluka Chemical Corp. in Buchs, Switzerland. All reagents used were of analytical grade or better. Ultra-pure water was purified with the resistivity over 18 MΩ using a Milli-Q Gradient A 10 system (Millipore, Billerica, MA).

Apparatus

HPLC analyses were performed using a Waters Acquity Ultra Performance LC system (Waters, Milford, MA). HPLC separation was achieved on Symmetry C_{18} column (150 mm × 2.1 mm, 3.5 µm particle size, Waters, Milford, MA), with water containing 0.1% formic acid (A) and acetonitrile (B) as the mobile phase at a flow rate of 0.3 mL/min. A gradient elution program was applied as follows: 0–5.0 min linearly increased from 0% to 1% B, 5.0–6.5 min linearly increased from 1% to 5% B, 6.5–7.0 min linearly increased from 5% to 100% B hold on for another 0.5 min and then decreased to 0% B. The composition was held at 0% B for a further 2.5 min for reequilibration, giving a total run time 10 min. The injection volume was 10 µL.

Determination was performed using a Waters Micromass Quattro Ultima Pt tandem quadrupole mass spectrometer (Micromass, Manchester, UK) with the following settings: capillary voltage of 3.0 kV, cone voltage of 40 V, desolvation temperature of 350°C and source block temperature of 120°C. The cone and desolvation gas flow were 50 and 500 L/h. Quantitation was performed in multiple reactions monitoring (MRM) mode and the optimized condition for each nucleotide was performed during infusion. The MRM transitions, parameters on the *m/z* and collision energy of precursor ions, primary product ions and secondary product ions selected for the analysis of the five nucleotides in ESI- positive mode are shown in Table I. Data acquisition and processing were performed using MassLynx V 4.1.

Standard solutions preparation

Before preparing standard solutions, the nucleotide standards were dried to invariant weight under a vacuum condition at room temperature. Aqueous stock solutions of approximately

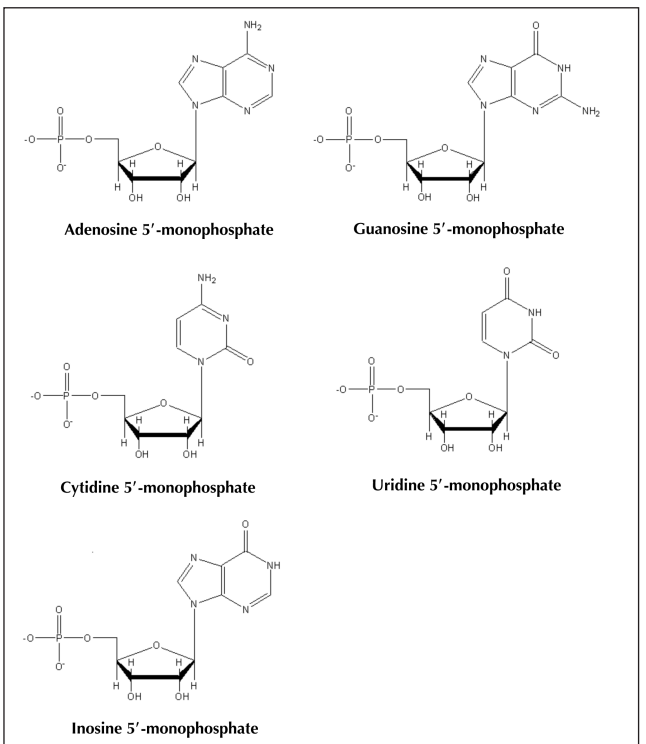

Figure 1. Chemical structures of the five 5'-monophosphate nucleotides.

1mg/mL were prepared for CMP, AMP, UMP, GMP, and IMP, and stored in the dark at –20°C. A mixed working solution containing the five nucleotides standards was prepared by step-wise dilution of the standard stock solutions.

Sample collection

Thirty brands of commercially available infant formulas (27 bovine milk-based formulas and three soy-based formulas) were randomly purchased from the supermarkets in Hangzhou, China, and stored at room temperature until analysis. Eighteen of the bovine milk-based infant formulas (numbered 13–30) had the straightforward label claim of "nucleotides supplemented."

Sample preparation

Just prior to analysis, 1.0 g of previously homogenized infant formula samples were dissolved in approximately 45 mL distilled water at 50°C, cooled to room temperature then adjusted the pH of the solutions to 4.6 with 1% formic acid and diluted to 50 mL with distilled water in a volumetric flask. The samples were ultrasonicated for 10 min and the extracts were centrifuged at 15000 rpm for 15 min. Finally, the solution was passed through a 0.22-µm nylon filter and ready for injection.

For samples with higher concentrations out of standard calibration range, the sample amount should be reduced by an appropriate factor.

Table I. Mass Parameters for the 5'-monophosphate Nucleotides Under ESI- Ionization Mode

Nucleotides	Molecular Weight (*m/z*)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
CMP	323.20	322.5	139.6*	15.0
			279.0	15.0
AMP	347.23	346.5	134.2*	20.0
			211.4	15.0
UMP	324.18	323.5	280.5*	15.0
			151.0	20.0
GMP	363.22	362.5	211.3*	15.0
			150.2	25.0
IMP	348.21	347.5	135.3*	25.0
			151.4	20.0

* Quantificational ion.

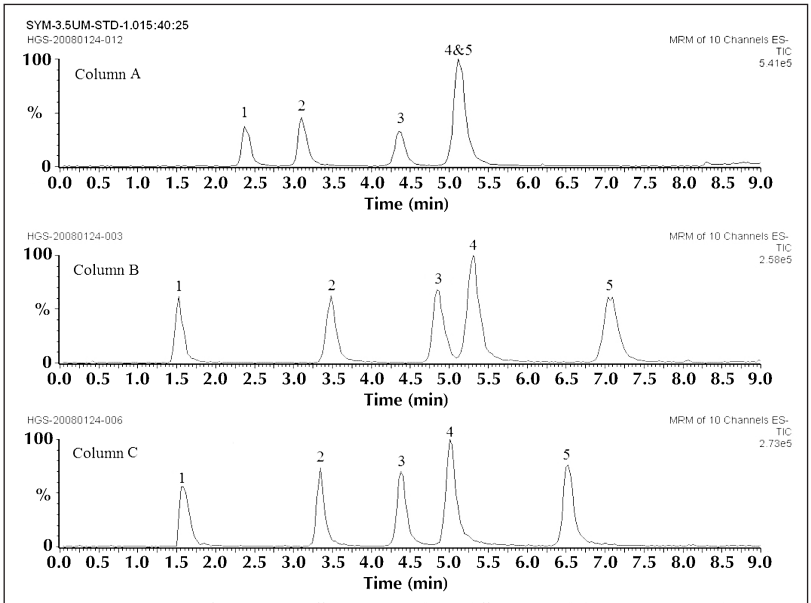

Figure 2. Comparison of separation effects among three different candidate columns. Peaks: CMP (1), AMP (2), UMP (3), GMP (4), and IMP (5).

Results and Discussion

Selection of HPLC columns

The selection of HPLC columns with separation efficiency is a prerequisite of the good chromatographic resolution. Conventional C18 columns are not suitable for the separation of nucleotides because the highly polar analytes have inherently poor interaction with the non-polar C18 phase under highly aqueous conditions. In contrast, nucleotides with the charged phosphate groups are relatively well retained on the hybrid and polymer grafted columns and polar embedded C18 phases (5). Therefore, three different HPLC columns, (A) Atlantis dC18 column (150 mm × 2.1 mm, 5 µm particle size), (B) Symmetry C18 (150 mm × 2.1 mm, 5 µm particle size, Waters, Milford, MA), (C) Symmetry C18 column (150 mm × 2.1 mm, 3.5 µm particle size, Waters), were selected to test their separation efficiencies. As shown in Figure 2, GMP and IMP couldn't be separated by column A, although column B and column C could separate the five nucleotides very well. The sensitivity and separation efficiency of column C were obviously better than column B. The reason might be that when the particle size of column reduced from 5 µm to 3.5 µm, the column efficiency was significantly improved as a result of great increasing the superficial area of unit volume. So, the column C was chosen as the analytical column. Under such situation, the elution time of the analytes was also shortened. The run time per sample in this test was only 10 min at a flow rate of 0.3 mL/min.

Selection of mobile phase

After the proper column was ascertained, different mobile phases were also tested in order to obtain better sensitivity and ionization efficiency. Most of previous studies apply phosphate buffer as the mobile phase and employ the ion-pair reagent to improve the chromatographic resolution, although they are not compatible with the mass spectrometry (25). In this work, four mobile phases: (A) acetonitrile–water containing 10 mmol/L ammonium formate, (B) acetonitrile–water containing 10 mmol/L ammonium acetate, (C) acetonitrile–water containing 0.1% aqueous ammonia, (D) acetonitrile–water containing 0.1–0.3% formic acid were prepared and tested. After all factors including good peak shapes, high sensitivities, and totally separation of various 5'-monophosphate nucleotides were considered, the mobile phase was ultimately standardized as water containing 0.1% formic acid and acetonitrile.

Method validation

Linear range and limits of detection

The calibration curves were performed with the concentration sequence of 0.1, 0.2, 0.8, 1.4, 2.0, 2.6, 3.2, and 4.0 µg/mL for CMP and 0.05, 0.1, 0.4, 0.7, 1.0, 1.3, 1.6, and 2.0 µg/mL for AMP, UMP, GMP, and IMP. All measurements were done in duplicate. Table II showed the results of the standard calibration curves of integrated peak area (n = 3) and linearity (R^2). Calibration curves were linear with correlation coefficients > 0.99 for all analytes.

Table II. Linear Regression Data, LOD, and LOQ of Five 5'-monophosphate Nucleotides

	Linear regression data				
Nucleotides	Regressive equation	Test range (µg/mL)	R^2	LOD* (µg/mL)	LOQ† (µg/mL)
CMP	$y = 2205.78x + 649.75$	0.1–4.0	0.9952	0.015	0.033
AMP	$y = 21771x + 568.618$	0.05–2.0	0.9985	0.011	0.016
UMP	$y = 19906.8x + 190.139$	0.05–2.0	0.9990	0.009	0.013
GMP	$y = 9213.27x + 152.659$	0.05–2.0	0.9983	0.011	0.024
IMP	$y = 12688.1x - 63.527$	0.05–2.0	0.9991	0.013	0.021

* LOD = Limit of detection (S/N = 3).
† LOQ = Limit of quantitation (S/N = 10).

Table III. Average Recovery Tests of the Investigated 5'-monophosphate Nucleotides (n = 3)

	High level*		Mid level†		Low level‡	
Nucleotides	Recovery§ (%)	RSD** (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
CMP	96.0 ± 4.0	4.16	104.2 ± 6.4	4.70	99.9 ± 4.7	6.15
AMP	101.5 ± 5.8	5.14	98.4 ± 3.5	3.54	97.0 ± 4.3	4.47
UMP	104.5 ± 1.5	1.45	101.0 ± 2.1	2.10	95.7 ± 4.8	5.01
GMP	102.2 ± 4.5	4.42	98.1 ± 1.4	1.45	100.5 ± 5.3	6.82
IMP	96.2 ± 2.1	2.21	97.8 ± 3.9	4.00	102.9 ± 3.8	3.71

* 4.0 µg/mL for CMP; 2.0 µg/mL for AMP, UMP, GMP and IMP.
† 2.0 µg/mL for CMP; 1.0 µg/mL for CMP, AMP, UMP, GMP and IMP.
‡ 0.4 µg/mL for CMP; 0.2 µg/mL for CMP, AMP, UMP, GMP and IMP.
§ Mean recovery ± standard deviation tested in triplicate.
**Relative standard deviation.

Table IV. Intra- and Inter-day Precision of the HPLC–MS–MS Method

	Intra-day precision (n = 11)		Inter-day precision (n = 5)	
Nucleotides	Detected amount* (µg/mL)	RSD† (%)	Detected amount* (µg/mL)	RSD† (%)
CMP	1.774 ± 0.051	2.87	1.709 ± 0.119	6.97
AMP	0.877 ± 0.021	2.41	0.751 ± 0.049	6.53
UMP	0.565 ± 0.028	4.90	0.531 ± 0.022	4.19
GMP	0.323 ± 0.008	2.49	0.306 ± 0.014	4.72
IMP	0.151 ± 0.004	2.91	0.163 ± 0.005	3.35

* Mean detected amount ± standard deviation.
† Relative standard deviation.

The detection or quantitation limits were determined by serial dilution of sample solution using the described HPLC–MS–MS conditions until the signal-to-noise ratio was reached to 3:1 or 10:1. The results are shown in Table II.

Recovery and precision

The method of standard addition was employed to determine the recovery. The mixed standards of five nucleotides were added to infant formula samples at high, intermediate and low levels (4.0, 2.0, and 0.2 µg/mL for CMP and 2.0, 1.0, and 0.1 µg/mL for AMP, UMP, GMP, and IMP), respectively. Then, these samples were pretreated as described in 2.4, and the results are summarized in Table III. All recoveries obtained were very well, indicating the good recovery of the method.

The results of repeatability, intra-day precision and inter-day precision for each analyte were listed in Table IV. The RSDs of the intra-day were in the range of 2.41–4.90%, and the RSDs of the

Table V. Quantities of the Five 5'-monophosphate Nucleotides in 30 Infant Formulas

	Nucleotides content (mg/100 g)					
Sample	CMP	AMP	UMP	GMP	IMP	Total
1	nd*	nd	nd	nd	nd	–
2	nd	nd	nd	nd	nd	–
3	1.9†	nd	nd	nd	nd	1.9
4	2.2	nd	nd	nd	nd	2.2
5	2.4	nd	nd	nd	nd	2.4
6	2.2	1.8	nd	nd	nd	4.0
7	3.7	nd	nd	1.5	nd	5.2
8	2.3	nd	nd	nd	nd	2.3
9	5.2	1.6	nd	nd	nd	6.8
10	3.4	nd	nd	nd	7.5	10.9
11	6.6	nd	nd	nd	nd	6.6
12	9.1	2.3	1.4	3.0	nd	15.8
13	4.9	3.1	2.5	4.2	nd	14.7 (18.0)‡
14	10.2 (11.0)	3.7 (3.3)	4.1 (4.3)	1.8 (2.2)	1.1 (1.3)	20.9 (21.1)
15	7.6	4.3	3.5	nd	0.8	16.2 (18)
16	7.4	4.7	2.2	3.1	nd	17.4 (19.1)
17	26.8	5.6	8.5	2.1	nd	42.6 (49)
18	15.9 (17.2)	4.5 (5.3)	9.9 (11.9)	2.8 (2.4)	4.7 (5.2)	37.8 (42)
19	6.7	2.8	2.3	0.7	3.7	16.2 (18.9)
20	19.1 (22)	12.3 (11)	7.3 (11)	8.7 (11)	nd	47.4 (55)
21	25.8	10.7	7.4	5.5	nd	49.4 (35–58)
22	14.9 (17.2)	6.1 (5.3)	10.6 (11.9)	2.6 (2.4)	4.3 (5.1)	38.5 (42)
23	8.9	4.5	3.7	0.8	nd	17.9 (19.1)
24	23.2	7.8	2.1	1.5	nd	34.6 (≥35)
25	19.9	4.3	1.0	0.8	nd	26.0 (23)
26	5.8	nd	nd	nd	nd	5.8
27	4.8	1.7	1.1	nd	1.2	8.8
28	10.1	10.4	4.5	0.8	1.3	27.1 (≥25)
29	24.8 (22)	10.2 (11)	7.5 (11)	9.4 (11)	nd	51.9 (55)
30	12.0 (11)	4.4 (3.7)	4.7 (5.1)	1.9 (2.3)	1.2 (1.6)	24.2 (23.7)

* nd = not detected.
† Mean detected amount in triplicate.
‡ Nucleotide label claim levels
§ Samples 1–3 were soy-based formulas; samples 4–12 were milk-based formulas with no nucleotides supplemented and samples 13–30 were milk-based formulas with label claim.

inter-day were in the range of 3.35–6.97%. All these data revealed that the established HPLC–MS–MS method has an accepted precision.

Advantages of the HPLC–MS–MS method

Among the various modes of liquid chromatography, IP-RPLC is currently the most commonly utilized technique for the determination of nucleotides in milk and milk products (5,14). Nevertheless, there are some disadvantages of the method because phosphate buffer is applied as the elution solution, which increases the risk of crystallization and obstruction of the system. Especially when the ion-pair reagents are employed for analyzing nucleotides, the disturbed column equilibration needs a long re-equilibration time, thus extending the run time of analysis (5,10). While analyzing the intact milk protein infant formulas, the RP-LC analysis is more complicated due to co-elution of the peptides under the conditions suited for the separation of 5'-monophosphate nucleotides (5).

The mass spectrometric detection with electrospray ionization is confirmed more specific than any other HPLC detection method. On this basis, the HPLC–MS–MS method was developed and applied for the analysis of nucleotides in infant formulas. The HPLC–MS–MS method was also compared with the HPLC method of Brendon D. Gill and Harvey E. Indyk (3) by analyzing the infant formulas. The results showed that both methods were basically coincident. However, the HPLC–MS–MS method only requires fewer sample and solvent as well as shorter analysis time. The separation of five nucleotides was achieved in 10 min by the established HPLC–MS–MS method herein, and the HPLC method needed more than 50 min. In addition, the described method avoids the disadvantages as a result of employing ion-pair reagents and phosphate buffer.

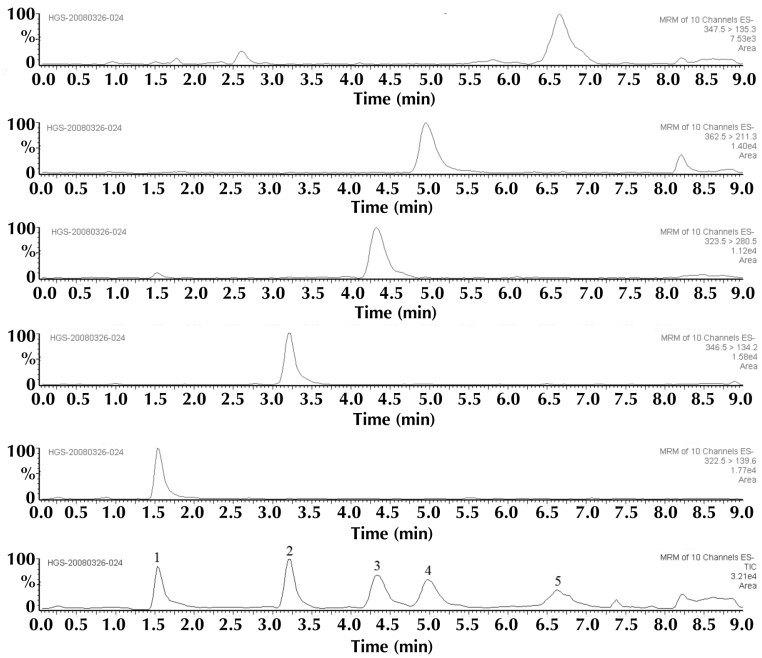

Figure 3. Typical chromatograms of five 5′-monophosphate nucleotides in the nucleotides supplemented infant formula. Peaks: CMP (1), AMP (2), UMP (3), GMP (4), and IMP (5).

Method application

To further verify the established method, it was employed to quantify five 5'-monophosphate nucleotides in 30 commercially available infant formulas randomly purchased from the supermarkets in Hangzhou, China. Among them, samples 1–3 were soy-based formulas; samples 4–12 were milk-based formulas with no nucleotides supplemented and samples 13–30 were milk-based formulas with label claim (Table V). Six out of 18 samples supplemented nucleotides declared the values of individual nucleotide and ten samples only declare the total amount of nucleotides. Two samples did not state the values of nucleotides though they declare nucleotides supplement on side of their packet. The chromatograms of a typical sample are shown in Figure 3.

The contents of various 5'-monophosphate nucleotides in the samples are summarized in Table V. Among them, the values in brackets refer to the label claim values on those samples, which have declared nucleotides on side of their packet/can. The results showed that all of the five 5'-monophosphate nucleotides in different samples were reliably identified and determined employing the established method. The measured values are in accordance with the label claim values. The CMP nucleotide was present in all analyzed infant formulas except two pure soy-based formulas without supplemented nucleotides. In all samples containing nucleotides, the content of CMP was the highest, followed by AMP. The possible reason was that CMP and AMP were supplemented into the milk powder, although the two nucleotides were originally contained in the bovine milk according to the previous research work (5,10). The nucleotides such as GMP, UMP, and IMP present in human milk but absent in mature bovine milk were not detected in the samples without added exogenous nucleotides. It was unexpected that AMP was not monitored in some bovine milk-based infant formulas. A previous study showed that nucleotides can be converted to nucleosides by reactivated alkaline phosphoesterase during thermal processing (3). This might explain the absence of AMP in some formulas. There were five samples that contained the whole five 5'-monophosphate nucleotides in the tested samples. It was not surprising because many infant formulas manufactured are not supplemented with IMP and there is little or no endogenous IMP. Different industrial processing techniques are also the potential causes of the obvious differences. As shown in Table V, the measured results by the present HPLC–MS–MS method were in accord with the label claim values on those samples which have declared nucleotides on side of their packets/cans.

Conclusions

A robust HPLC–MS–MS method with excellent sensitivity, selectivity and reliability has been developed that allows for simultaneous quantitative analysis of the five 5'-monophosphate nucleotides

including CMP, AMP, UMP, GMP, and IMP in complicated infant formula matrix. After optimization of the mobile phase composition and the HPLC columns, the absolutely chromatographic resolution of the five 5'-monophosphate nucleotides was achieved in less than 7 min, providing good peak symmetry. Simultaneously, the established method avoided the drawbacks of diminished column lifetime and long equilibration time because it excluded the commonly used ion-pair reagents and buffer salt solution. Compared to other methods, the HPLC–MS–MS method described here was proved to be of excellent validation parameters such as selectivity, precision, recovery, and sensitivity. Finally, the applicability of the method has been demonstrated by analyzing contents of nucleotides in thirty commercially available infant formulas purchased from supermarkets in Hangzhou, China. It is probable that HPLC–MS–MS method can be applied to support the more frequently used HPLC-UV methods currently in use during the analysis of nucleotides in milk and infant formulas.

References

1. E. Schlimme, D. Martin, and H. Meisel. Nucleosides and nucleotides: natural bioactive substances in milk and colostrum. *Brit. J. Nutr.* **84 Suppl 1:** S59–68 (2000).
2. J.D. Carver and W.A. Walker. The role of nucleotides in human nutrition. *J. Nutr. Biochem.* **6:** 58–72 (1995).
3. B.D. Gill and H.E. Indyk. Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas. *Int. Dairy J.* **17:** 596–605 (2007).
4. C. Perrin, L. Meyer, C. Mujahid, and C.J. Blake. The analysis of 5'-mononucleotides in infant formulae by HPLC. *Food Chem.* **74:** 245–253 (2001).
5. B.D. Gill and H.E. Indyk. Determination of nucleotides and nucleosides in milks and pediatric formulas: a review. *J. AOAC. Int.* **90:** 1354–1364 (2007).
6. J.P. Schaller, R.H. Buck, and R. Rueda. Ribonucleotides: Conditionally essential nutrients shown to enhance immune function and reduce diarrheal disease in infants. *Semin. Fetal Neonatal Med.* **12:** 35–44 (2007).
7. P. Aggett, J.L. Leach, R. Rueda, and W.C. MacLean. Innovation in infant formula development: A reassessment of ribonucleotides in 2002. *Nutrition* **19:** 375–384 (2003).
8. I.M.P.L.V.O. Ferreira, E. Mendes, A.M.P. Gomes, M.A. Faria, and M.A. Ferreira. The determination and distribution of nucleotides in dairy products using HPLC and diode array detection. *Food Chem.* **74:** 239–244 (2001).
9. L. Thorell, L.B. Sjoberg, and O. Hernell. Nucleotides in human milk: sources and metabolism by the newborn infant. *Pediatr. Res.* **40:** 845–852 (1996).
10. A. Gil and R. Uauy. Nucleotides and related compounds in human and bovine milks. In *Handbook of Milk Composition*; Jensen, R.G., Ed.; Academic Press: San Diego, 1995; pp 436–464.
11. K.-S. Boos, B. Wilmers, E. Schlimme, and R. Sauerbrey. On-line sample processing and analysis of diol compounds in biological fluids. *J. Chromatogr. A* **456:** 93–104 (1986).
12. F.Q. Yang, J. Guan, and S.P. Li. Fast simultaneous determination of 14 nucleosides and nucleobases in cultured Cordyceps using ultra-performance liquid chromatography. *Talanta* **73:** 269–273 (2007).
13. A. Werner. Reversed-phase and ion-pair separations of nucleotides, nucleosides and nucleobases: analysis of biological samples in health and disease. *J. Chromatogr. A* **618:** 3–14 (1993).
14. P.R. Brown, C.S. Robb, and S.E. Geldart. Perspectives on analyses of nucleic acid constituents: the basis of genomics. *J. Chromatogr. A* **965:** 163–173 (2002).
15. V. Bezy, P. Morin, P. Couerbe, G. Leleu, and L. Agrofoglio. Simultaneous analysis of several antiretroviral nucleosides in rat-plasma by high-performance liquid chromatography with UV using acetic acid/hydroxylamine buffer: Test of this new volatile medium-pH for HPLC-ESI-MS/MS. *J. Chromatogr. B* **821:** 132–143 (2005).
16. S.P. Li, P. Li, C.M. Lai, Y.X. Gong, K.K. Kan, T.T. Dong, K.W. Tsim, and Y.T. Wang. Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured Cordyceps by pressurised liquid extraction and high-performance liquid chromatography. *J. Chromatogr. A* **1036:** 239–243 (2004).
17. R. Qurishi, M. Kaulich, and C.E. Müler. Fast, efficient capillary electrophoresis method for measuring nucleotide degradation and metabolism. *J. Chromatogr. A* **952:** 275–281 (2002).
18. A.V. Willems, D.L. Deforce, C.H. Van Peteghem, and J.F. Van Bocxlaer. Analysis of nucleic acid constituents by on-line capillary electrophoresis-mass spectrometry. *Electrophoresis.* **26:** 1221–1253 (2005).
19. C.F. Yeh and S.J. Jiang. Determination of monophosphate nucleotides by capillary electrophoresis inductively coupled plasma mass spectrometry. *Analyst.* **127:** 1324–1327 (2002).
20. B. Kammerer, A. Frickenschmidt, C.H. Gleiter, S. Laufer, and H. Liebich. MALDI-TOF MS analysis of urinary nucleosides. *J. Am. Soc. Mass. Spectr.* **16:** 940–947 (2005).
21. E.L. Esmans, D. Broes, I. Hoes, F. Lemière, and K. Vanhoutte. Liquid chromatography-mass spectrometry in nucleoside, nucleotide and modified nucleotide characterization. *J. Chromatogr. A* **794:** 109–127 (1998).
22. S.H. Lee, B.H. Jung, S.Y. Kim, and B.C. Chung. A rapid and sensitive method for quantitation of nucleosides in human urine using liquid chromatography/mass spectrometry with direct urine injection. *Rapid Commun. Mass. Sp.* **18:** 973–977 (2004).
23. T. Qian, Z. Cai, and M.S. Yang. Determination of adenosine nucleotides in cultured cells by ion-pairing liquid chromatography-electrospray ionization mass spectrometry. *Anal. Biochem.* **325:** 77–84 (2004).
24. Klawitter, V. Schmitz, J. Klawitter, D. Leibfritz, and U. Christians. Development and validation of an assay for the quantification of 11 nucleotides using LC/LC-electrospray ionization-MS. *Anal. Biochem.* **365:** 230–239 (2007).
25. R.M. Seifar, C. Ras, J.C. van Dam, W.M. van Gulik, J.J. Heijnen, and W.A. van Winden. Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry. *Anal. Biochem.* **388:** 213–219 (2009).
26. J.J. Conboy, J.D. Henion, M.W. Martin, and J.A. Zweigenbaum. Ion chromatography/mass spectrometry for the determination of organic ammonium and sulfate compounds. *Anal. Chem.* **62:** 800–807 (1990).

Manuscript received September 20, 2009;
Revision recieved March 9, 2010.