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ARTICLE

Development and Application of an HILIC-MS/MS Method for the Quantitation of Nucleotides in Infant Formula

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A method for the quantitation of nucleotides (adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP), cytidine 5′-monophosphate (CMP), and inosine 5′-monophosphate (IMP)) in infant formula was developed by hydrophilic interaction chromatography with tandem mass spectrometry (HILIC-MS/MS). The internal standards used (AMP-$^{13}C_{10}$, $^{15}N_5$; GMP-$^{13}C_{10}$, $^{15}N_5$; UMP-$^{13}C_9$, $^{15}N_2$; CMP-$^{13}C_9$, $^{15}N_3$) were prepared with centrifugal ultrafiltration (CUF). Data acquisition was achieved by using multiple reaction monitoring (MRM) of product ions of protonated molecules of the five nucleotides generated by the positive-ion ESI. HILIC conditions were performed with 30 mmol/L ammonium formate in water (pH 2.5, adjusted with formic acid) and methanol. The LOD and LOQ were 5−10 μg/mL and 10−30 μg/mL for standard solution, respectively. Recovery for intra- and interday assays ranged from 98.1 to 108.9% (RSD: 0.7−5.4%) spiked with three concentration levels (5, 25, and 250 μg/g powder infant formula). This method could be applied for the determination of nucleotides in infant formula samples. The detected concentrations of five nucleotides ranged from not detected (n.d.) to 278 μg/g powder infant formula. The total nucleotide level ranged from n.d. to 600.2 μg/g powder infant formula.

KEYWORDS: Nucleotides; infant formula; hydrophilic interaction liquid chromatography (HILIC); tandem mass spectrometry (MS/MS)

INTRODUCTION

Nucleotides are the components of nucleic acids and have been identified as conditionally essential nutrients. Previous studies of nucleotides have shown a statistically significant decrease in the risk of diarrhea, internal microflora, immune function, sepsis, and modulators of lipid metabolism (*1*−*5*). The addition of nucleotides in infant formula has been suggested to have beneficial effects on the growth and maturation of the gastrointestinal tract of healthy infants (*6*). However, the advantage during episodes of acute diarrhea when using nucleotide-supplemented infant formula was indicated, and the noneffects were compared to those of conventional in infant formula (*7*). Until now, many researchers have discussed the effects of nucleotide-supplemented infant formula.

Commonly, five nucleotides such as AMP, GMP, UMP, CMP, and IMP (**Figure 1**) are used as supplements for infant formulas based on the recommendations. European regulations permit a maximum concentration of 5 mg/100 kcal of nucleotides in infant feeds, while recommendations from The USA Life Sciences Research Office allow up to 16 mg/100 kcal (*8*, *9*). Many experts have argued that these nucleotides are present in human milk and have positive effects. Others wish to see more research to critically assess the benefits and appropriate level of nucleotide supplementation (*10*). In 2007, the Codex Alimentarius Commission adopted a revised standard for infant formula that provides a regulatory framework including provisions for its essential composition (*11*). Those analytical data of essential composition would reflect the nutrient levels that formula-fed infants were actually consuming. Recent investigation was reported from the survey of the levels of nutrients in infant formula with the essential composition in the revised Codex standard for infant formula (*12*). Thus, the analytical method of nutrients in infant formula would be a valuable experiment for the assessment of various infant formulas.

Gill and Indyk introduced various analytical methods for the determination of nucleotides in infant formulas and milk samples (*13*). We developed the ion-exchange liquid chromatography (IELC) and centrifugal ultrafiltration (CUF) based protocol for the routine determination of five nucleotides in infant formulas (*14*). Recently, the LC/DAD procedure was successfully applied to the analysis of nucleotides such as AMP, GMP, UMP, and CMP in infant formulas, fermented milk, cereals, and purees (*15*). To the best of our knowledge, there are few reports of useful techniques for the determination of nucleotides in infant formulas and milk samples.

There is a growing interest to apply LC-MS/MS for the evaluation of supplemented compounds to ensure food safety. When the LC-MS/MS method is considered to be developed in the assay of nucleotides in infant formulas, accurate results can be generated without laborious extraction, as well as sample cleanup

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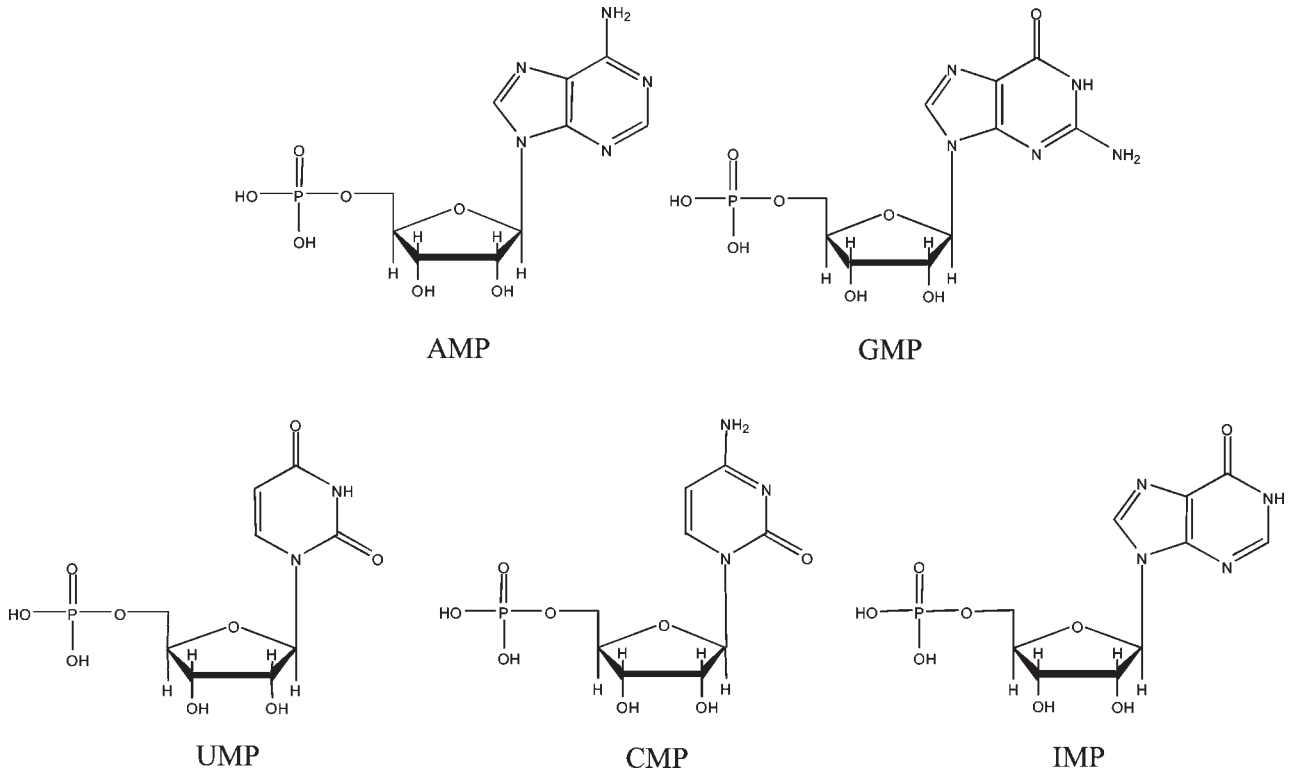

Figure 1. Chemical structures of supplemented nucleotides: adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP), cytidine 5′-monophosphate (CMP), and inosine 5′-monophosphate (IMP).

Table 1. MS/MS Conditions of Nucleotides and Corresponding Internal Standards[a]

analytes	identity	precursor ion (*m*/*z*)	product ion (*m*/*z*)	cone voltage (V)	collision energy (e V)	dwell time (ms)
AMP	$[M + H]^+$	348	136[b]/97	22	20[b]/33	100
AMP-$^{13}C_{10}$, $^{15}n_5$	$[M + H]^+$	363	146	22	20	100
GMP	$[M + H]^+$	364	152[b]/135	20	20[b]/45	100
GMP-$^{13}C_{10}$,$^{15}N_5$	$[M + H]^+$	379	162	20	20	100
UMP	$[M + H]^+$	325	97[b]/213	20	18[b]/10	100
UMP-$^{13}C_9$,$^{15}N_2$	$[M + H]^+$	336	102	20	18	100
CMP	$[M + H]^+$	324	112[b]/97	23	15[b]/35	100
CMP-$^{13}C_9$,$^{15}N_3$	$[M + H]^+$	336	119	23	15	100
IMP	$[M + H]^+$	349	137[b]/97	20	15[b]/30	100

[a] LC-MS/MS system: Waters Alliance 2965/Micromass Quattro Premier (Waters, Milford, MA). HILIC: TSK-gel NH_2-100 column (3 μm, 2.0 × 150 mm, Tosoh, Tokyo, Japan). The mobile phase: solvent A, 30 mmol/L ammonium formate in water (pH 2.5, adjusted by formic acid), and solvent B, methanol. The ionization: ESI (positive ionization mode) condition. [b] The most abundant ion (also used for analyte quantification).

and further isolation. Recently, the HILIC mode was used widely for the separation of highly polar substances including biologically active compounds such as pharmaceutical drugs, neurotransmitters, nucleosides, nucleotides, and amino acids (*16*). HILIC on silica columns with a low aqueous/high organic mobile phase was emerging as a valuable supplement to the reversed-phase LC-MS/MS. Thus, the aim of this study was to develop a quantitative HILIC-MS/MS that would be useful for the regulatory analysis of five nucleotides in infant formula samples.

MATERIALS AND METHODS

Reagents and Standard solutions. Adenosine 5′-monophosphate (AMP) was purchased from Oriental Yeast Co. (Tokyo, Japan). Guanosine 5′-monophosphate (GMP) was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Uridine 5′-monophosphate (UMP), cytidine 5′-monophosphate (CMP), inosine 5′-monophosphate (IMP), adenosine-$^{13}C_{10}$,$^{15}N_5$ 5′-monophosphate (AMP-$^{13}C_{10}$,$^{15}N_5$), guanosine-$^{13}C_{10}$,$^{15}N_5$ 5′-monophosphate (GMP-$^{13}C_{10}$,$^{15}N_5$), uridine-$^{13}C_9$,$^{15}N_2$ 5′-monophosphate (UMP-$^{13}C_9$,$^{15}N_2$), and cytidine-$^{13}C_9$,$^{15}N_3$ 5′-monophosphate (CMP-$^{13}C_9$,$^{15}N_3$) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Ammonium formate, formic acid (LC/MS grade), and methanol (HPLC grade) were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Purified water was obtained from a Milli-Q purifying system (Millipore, Bedford, MA, USA). Infant formula samples were purchased from local stores in Japan and USA.

Pure standard solutions (1.0 mg/mL) of each nucleotide was prepared in water. Mixed standard solutions were prepared by diluting an aliquot of the standard in water/methanol (50/50, V/V). Internal standard solutions (0.5 mg/mL) of each ^{15}N,^{13}C-labeled nucleotide were prepared in water. Mixed solutions were diluted with water to required concentrations. These solutions were used as internal standards for the calibration curves of nucleotides for the quantification.

HILIC-MS/MS Analysis. The LC-MS/MS system was a Waters Alliance 2965/Micromass Quattro Premier (Waters Alliance 2695, Milford, MA). HILIC was performed using a TSK-gel NH_2-100 column (3 μm, 2.0 × 150 mm, Tosoh, Tokyo, Japan) at 40 °C. The injection volume was 10 μL. The mobile phase consisting of solvent A, 30 mmol/L ammonium formate in water (pH 2.5, adjusted by formic acid), and solvent B, methanol, was delivered at a flow rate 0.2 mL/min. The stepwise elution was as follows: 0.0 min [A/B: 85/15], 10.0 min [A/B: 85/15], 10.1 min [A/B: 98/2], 30.0 min [A/B: 98/2], 30.1 min [A/B: 85/15], and 40.0 min [A/B: 85/15]. The ESI (positive ionization mode) conditions were as follows;; capillary voltage

Table 2. HILIC-MS/MS Analytical Factors of Nucleotides[a]

			calibration curve	
analytes	LOD (μg/mL)	LOQ (μg/mL)	linearity (r^2)	calibration range (μg/mL)
AMP	0.005	0.010	0.999	0.01−5
GMP	0.005	0.025	0.999	0.03−5
UMP	0.010	0.020	0.999	0.02−5
CMP	0.005	0.010	0.999	0.01−5
IMP	0.005	0.030	0.999	0.03−5

[a] The calculation of LOD and LOQ: signal to noise ratio (S/N) = 3 and 10. The calibration curves: peak area ratios of nucleotides to each corresponding stable isotope nucleotide.

was 2.5 kV, extractor voltage was 4.0 V, RF lens voltage was 0.0 V, source temperature was 110 °C, and desolvation temperature was 390 °C. The cone and desolvation gas flows were 50 and 900 L/h, respectively, and were obtained using a nitrogen source. We used argon as the collision gas and regulated it at 21 mL/h, setting the multipliers to 650 V. Cone voltages and the collision energies of analytes are summarized in **Table 1**.

CUF Extraction of Infant Formula Sample. Previous preparations of nucleotides in infant formulas reported that the liquid samples were ultrafiltered using a CUF cartridge regenerated cellulose of 3,000 M.W. from Millipore (*14*). In this study, we used a silently modified preparation of the previous method. The infant formula sample (1.0 g) was added in 50 mL of water. For the infant formula sample, the sample was used for the direct preparation. The necessary internal standard was added at this stage. A certain amount of the internal standard solution (10 μg/mL, 50 μL) was used. This infant formula sample was vortex-mixed for 5 min. Nucleotides in infant formula samples were pretreated with Amicon Ultra-4, Ultracel-3k, regenerated cellulose 3,000 M.W. for volumes < 4 mL (Millipore Co., Ltd., Billerica, MA, USA). The 0.5 mL sample solution was eluted through CUF by centrifuging at 3500 rpm (rcf 2328 g) for 15 min (Kubota 5420, Kubota Co., Tokyo, Japan). The filtrate diluted by the same amount of methanol was used as a sample solution.

Analytical Validations. Method validation was carried out by recovery tests (intra- and interday). The calibration curves were prepared using peak area ratios of nucleotides to each corresponding stable isotope nucleotides. The calibration range is shown in **Table 2**. The evaluation of linearity was performed by using the correlation coefficient (r^2). The recovery test was performed using nucleotide-free infant formula samples spiked with five nucleotides. Spiked levels were 5, 25, and 250 μg/g of powdered infant formula, respectively. The intraday test was assessed by the recovery test of six times/one day. The interday test was assessed by the recovery test of two times/one day for three days. The calculations of LOD and LOQ were based on signal-to-noise ratios (S/N) = 3 and 10, respectively.

RESULTS AND DISCUSSION

HILIC-MS/MS. Recently, we developed a HPLC/UV method for the determination of five nucleotides (*14*), which is useful for the routine analysis of regular infant formulas. However, this routine HPLC/UV method could not be applied to hypoallergenic infant formula samples. For hypoallergenic infant formulas, the allergenic potential of milk proteins is reduced by using the hydrolyzed process. The degree of hydrolysis and composition of hydrophilic peptides may vary a great deal depending on the hydrolyzed preparation (*17*). Thus, it is difficult that hydrophilic nucleotides in hypoallergenic infant formulas can be separated by LC/UV detection at an absorbance of 254 nm. In this study, stable isotope dilution MS/MS was applied to the detection of nucleotides for selective, sensitive, accurate, and universal methods. For stable isotope dilution, we used AMP-$^{13}C_{10}$,$^{15}N_5$, GMP-$^{13}C_{10}$,$^{15}N_5$, UMP-$^{13}C_9$,$^{15}N_2$, and CMP-$^{13}C_9$,$^{15}N_3$. The ^{13}C,^{15}N-labeled IMP was not obtained, and we used GMP-$^{13}C_{10}$,$^{15}N_5$ in place of ^{13}C,^{15}N-labeled IMP. They have similar structures (a purine backbone with a ketone group) and retention times. These nucleotides were observed in precursor and product ions in ESI positive mode

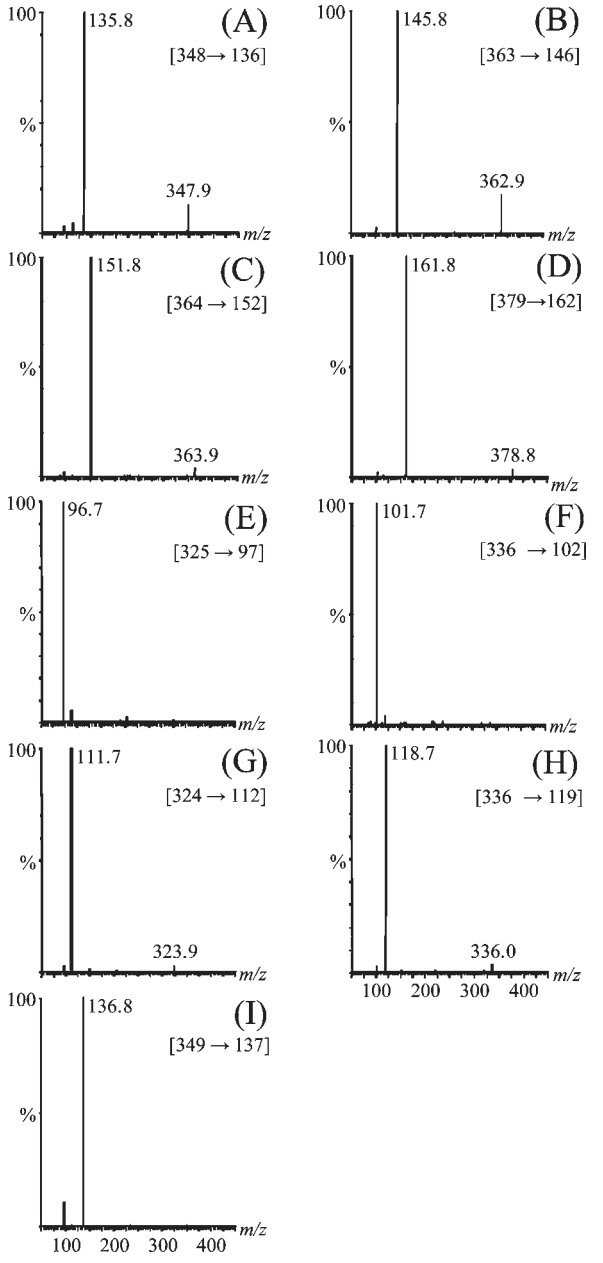

Figure 2. Product ion mass spectra of the protonated molecules of the five nucleotides and four internal standards used. MS/MS conditions: ESI and positive ionization mode as follows; capillary voltage was 2.5 kV, extractor voltage was 4.0 V, RF lens voltage was 0.0 V, source temperature was 110 °C, desolvation temperature was 390 °C, cone flow was 50 L/h, and desolvation gas flow was 900 L/h. Cone voltages and collision energies of analytes are summarized in **Table 1**. (**A**) AMP, (**B**) AMP-$^{13}C_{10}$,$^{15}N_5$, (**C**) GMP, (**D**) GMP-$^{13}C_{10}$,$^{15}N_5$, (**E**) UMP, (**F**) UMP-$^{13}C_9$,$^{15}N_2$, (**G**) CMP, (**H**) CMP-$^{13}C_9$,$^{15}N_3$, and (**I**) IMP.

using infusion analysis. MS/MS spectra, monitoring ions, cone voltages, and collision energies are shown in **Figure 2** and **Table 1**. The major fragment ions at m/z 348 → 136 for AMP, m/z 364 → 152 for GMP, m/z 325 → 97 for UMP, m/z 324 → 112 for CMP, and m/z 349 → 137 for IMP in multiple reaction monitoring (MRM) can be used for the LC-MS/MS analysis of five nucleotides.

LC is generally used for the determination of nucleotides in infant formula after sample extraction/cleanup. Three main modes of LC have been appraised for the measurement of these nucleotides: reversed-phase, ion-pair, and ion-exchange modes. In RPLC analysis, nucleotide separation with a C_{18}-based column

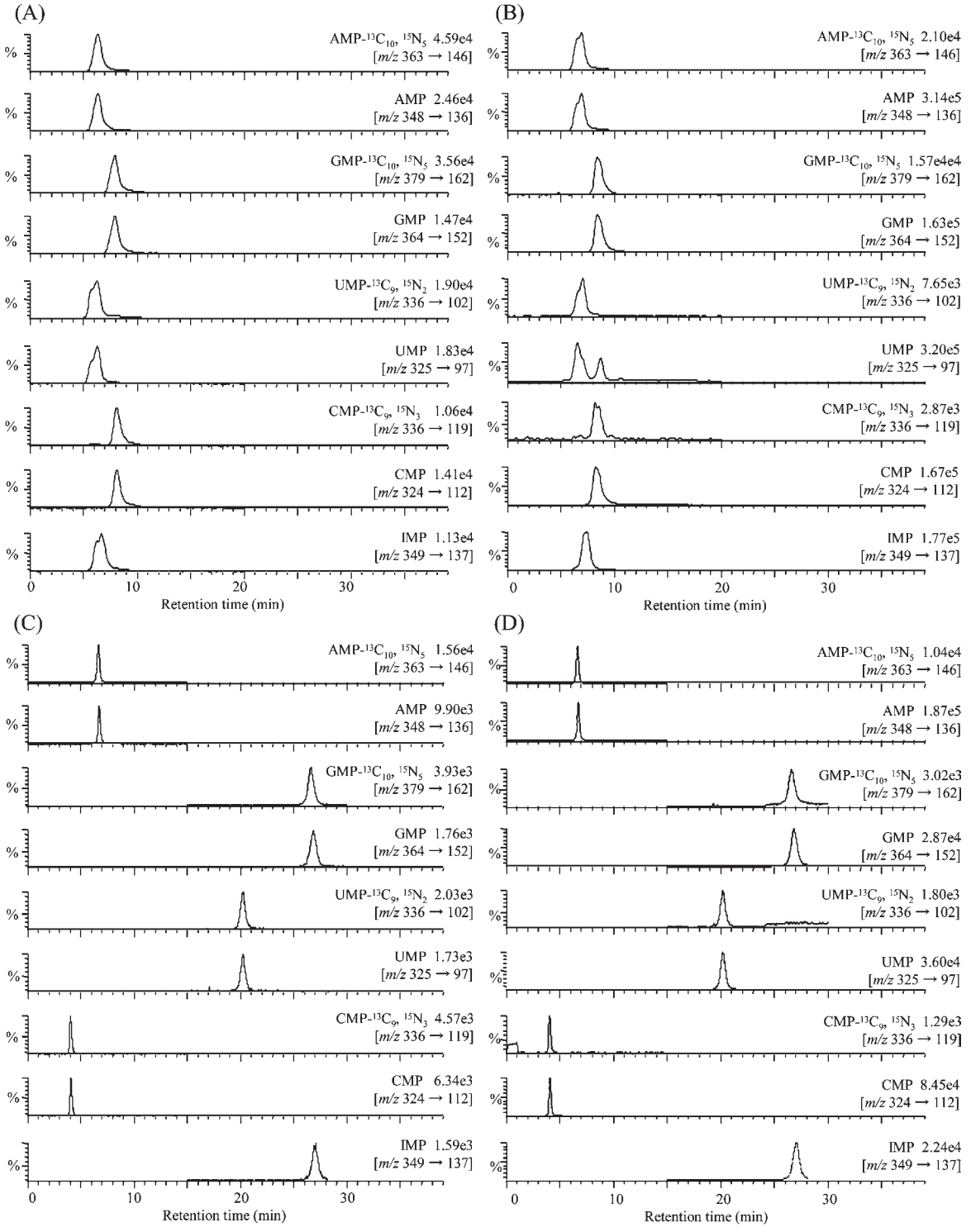

Figure 3. MRM ion chromatograms of five nucleotide standards and recovery test. HILIC conditions: (**A**) column; TSK-gel Amide-80, mobile phase; aquatic ammonium acetate (80 mM, pH 4.5, adjusted with acetic acid) and acetonitrile, 250 ppb standard solution. (**B**) Column; TSK-gel Amide-80, mobile phase; aquatic ammonium acetate (80 mM, pH 4.5 adjusted with acetic acid) and acetonitrile, recovery test (5 μg/g). (**C**) Column; TSK-gel NH_2-100, mobile phase; aquatic ammonium formate (30 mM, pH 2.5 adjusted with formic acid) and methanol, 250 ppb standard solution. (**D**) Column; TSK-gel NH_2-100, mobile phase; aquatic ammonium formate (30 mM, pH 2.5 adjusted with formic acid) and methanol, recovery test (5 μg/g).

is limited because of the inherently poor interaction of the highly polar nucleotides. Moreover, the ion-pair technique was confusing; various optimal ion-pair reagents were investigated, and an ion-pair reagent was found to be the contamination of the instrumental source. However, the ion-exchange mode is useful for the analysis of nucleotides in infant formulas because only pH is optimized in the mobile phase (*14*). However, it is difficult to use volatile acids in mobile phase for the separation of nucleotides. Recently, it was reported that the separation of nucleosides was studied by using the HILIC mode (*18*). HILIC stationary phases reported the separation of nucleobases, nucleosides, and nucleotides such as their chemotherapic derivatives 5-fluoro-uracil, 5-fluoro-cytosine, and acyclovir, or the modeling and predicting of retention times of thymine, uracil, uridine, cytidine, adenosine,

adenine, cytosine, and guanine (*19*, *20*). Thus, HILIC stationary phase may be used for the routine and universal analysis of the retention of polar analytes in various biological samples (*21*). In this study, we describe the behavior of five nucleotides on commercial HILIC columns, namely, the TSK-gel Amide-80 and NH_2-100 for infant formulas. For optimal conditions, ammonium formate (0.1−50 mM) and acetate (0.1−80 mM) were investigated for buffered solutions in acetonitrile because of its volatile ability for HILIC-MS/MS. Retention and peak shape were influenced by the presence of ammonium formate and acetate at different concentrations. An increase in salt concentration was evidenced, accompanied by an improvement in peak shape passing from water to 80 mM ammonium acetate (pH 4.5 adjusted with acetic acid) on TSK-gel Amide-80 column (**Figure 3A**). However, these peaks of IMP and GMP are not in the shapes when using TSK-gel NH_2-100 with aquatic ammonium acetate and acetonitrile. For TSK-gel NH_2-100, the retention and peak behavior were improved with aquatic ammonium formate (30 mM, pH 2.5 adjusted with formic acid) and methanol (**Figure 3C**). The results on the effect of the mobile phase are in agreement with the HILIC-MS/MS analysis of nucleotides for standard solutions based on both columns. However, when we tried to measure the five nucleotides from the infant formula for the recovery test, TSK-gel NH_2-100 conditions showed good separation between analytes and sample matrixes (**Figure 3D**). However, it is impossible and difficult to determine the separation of AMP and UMP, and matrix peaks of infant formula were completed in TSK-gel Amide-80 conditions (**Figure 3B**). Therefore, we decided to measure five nucleotides in various infant formulas using TSK-gel NH_2-100 conditions.

A standard solution of five nucleotides was prepared in water/methanol (50/50, V/V) and added to a fixed concentration of AMP-$^{13}C_{10}$,$^{15}N_5$, GMP-$^{13}C_{10}$,$^{15}N_5$, UMP-$^{13}C_9$,$^{15}N_2$, and CMP-$^{13}C_9$,$^{15}N_3$ for a calibration curve covering the concentration range from LOQ to 5 μg/mL. Quantitative analysis was performed using the MRM mode in order to maximize the sensitivity of the quantitative ion and the ratio of the analyte/internal standard. Concentrations were calculated relative to isotopical standards that were added to the samples prior to extraction giving a final extract concentration of 1.0 μg/mL. Eight-point calibrations were performed daily for all analytes with internal standards and showed good correlation values (r^2 = 0.999). The calculated LOD and LOQ were 0.005 or 0.01 μg/mL and 0.01−0.03 μg/mL for the standard solution, respectively, and the S/N was three and ten times, respectively. **Table 2** shows that HILIC-MS/MS was a sensitive and accurate method for determining the five nucleotides.

Sample Preparation and Analytical Validations of Nucleotides in Infant Formulas. Our aim was that more useful, simple, and accurate sample preparation for the quantification of five nucleotides in various infant formulas was developed for HILIC-MS/MS than previous methods. The CUF was applied to the extraction/cleanup procedure of nucleotides in infant formulas (*14*). CUF is easy to use and has a shorter operation time, resulting in high recovery and reproducibility (from 85.0 ± 1.4% to 92.3 ± 2.1%), thereby saving analytical time and solvents for the sample preparation of nucleotides in infant formulas (*14*). Thus, in this study, the CUF preparation was used for MS/MS detection of five nucleotides in infant formulas. Mostly, stable isotopes labeled internal standards were used to compensate for sample-to-sample differences occurring during CUF preparations and HILIC-MS/MS analysis such as absolute recovery, injection volume, and matrix effects. Thus, we used the calibration curves generated using the analyte-to-stable isotope internal standard's peak area ratios by weighted ($1/x^2$) least-squares linear regression on

Table 3. Recovery Test of Nucleotides in Infant Formula Samples[a]

analytes	spiked levels (μg/g, ppm)	intraday recovery (%)	intraday RSD (%)	interday recovery (%)	interday RSD (%)
AMP	250	100.6	1.2	100.4	2.1
	25	101.0	1.4	100.0	1.1
	5	99.6	3.6	99.6	3.6
GMP	250	101.9	2.4	101.2	1.1
	25	100.4	1.1	104.7	5.4
	5	100.4	2.9	100.4	2.9
UMP	250	101.4	2.2	99.7	1.1
	25	99.0	1.9	100.1	1.3
	5	103.1	2.6	103.1	2.6
CMP	250	100.7	2.9	102.3	2.0
	25	100.1	1.9	100.2	0.7
	5	108.9	4.5	108.9	4.5
IMP	250	101.3	4.2	102.2	2.5
	25	104.5	4.4	98.1	4.0
	5	101.3	4.0	101.3	4.1

[a] The recovery test was performed using nucleotide-free infant formula samples spiked with five nucleotides (spiked levels: 5, 25, and 250 μg/g powder infant formula). Intra-day test: the recovery test of six times/one day. Inter-day test: the recovery test of two times/one day for three days.

consecutive days. Spiked levels for quality control (QC) in nucleotide-free infant formula samples were selected of six replicates of QC at three concentration levels (5, 25, and 250 μg/g powder infant formula). For the determining of intraday accuracy, replicate (n = 6) analytes of QC samples were performed on the same day. The interday accuracy was expressed as the recovery and relative standard deviation (RSD, %), and determined two times per day for three days (n = 6). The results for intraday and interday precision for five nucleotides in QC samples are summarized in **Table 3**. The intraday recovery and precision were from 99.0 to 108.9% and 1.1−4.5%, respectively. The interday recovery and precision were from 98.1 to 108.9% and 0.7−5.4%, respectively. On the basis of our experimental and comparative results, a simple, routine, useful, and universal extraction/cleanup/detection procedure was developed using direct CUF without protein precipitation: stable isotope dilution HILIC-MS/MS for various infant formula samples.

Measurement Five Nucleotides in Common Infant Formulas from Japan and USA. Mononucleotide supplementations of infant formula have been discussed along with their effects and biological benefits. Thus, the total amount and ratio of nucleotides must be determined to evaluate the correct supplementation and biological effects of nucleotides in infant formulas. It was reported that the concentration levels of nucleotides such as AMP, GMP, UMP, and CMP in infant formulas ranged from 1 to 169 μg/g and that the total amount from 38 to 324 μg/g (*22*). Unfortunately, IMP was not measured, and target samples were limited infant formulas (*22*). To the best of our knowledge, analytical data of essential composition and actually consumption of five nucleotides for formula-fed infants have not been reported. Thus, five nucleotides in various infant formulas (n = 25) from Japan and USA were investigated by our developed method. Moreover, the five nucleotides present in actual infant formulas were calculated. These results and sample information are shown in **Tables 4** and **5**. The detected concentrations of five nucleotides ranged from n.d. (not detected) to 278 μg/g powder infant formula. Total nucleotide level ranged from n.d. to 600.2 μg/g powder infant formula. Moreover, the calculated value of total nucleotides was 34.2 ± 26.2 μg/mL milk, and the high concentration level was 100.6 μg/mL milk for formula-fed infants. On the basis of the references, the concentrations of total nucleotides (AMP, GMP, UMP, CMP, and IMP) in human milk

ranged from 6.2 to 464.3 μg/mL (*13*). Thus, this result of five nucleotides in infant formulas may be considered reasonable and proper compared with that from human milk. However, we would need to measure the actual levels of nucleotides in various conditions of human milk. These overall results will be evaluated for the assessment of nucleotide-supplemented infant formulas.

LC/ESI-MS/MS has been more specific and selective for the analysis of nucleotides than HPLC with conventional detectors; however, this method required complex extraction steps and troubled separation techniques for biological samples (*23*, *24*). HILIC-MS/MS assays have the possible value of detecting five nucleotides such as AMP, GMP, UMP, CMP, and IMP in various biological samples. Thus, we focused on the relatively simple, highly sensitive, and specific analytical method for the quantitative determination of five nucleotides using HILIC-MS/MS in MRM mode. This screening assay allowed for the discovery of the interesting significant results in infant formulas compared with the referenced human milk levels. Thus, the safety assessment of the supplied nucleotide levels in various infant formulas would need to be discussed on the basis of this data.

Table 4. Sample Details of Infant Formulas[a]

sample number	country	brand	origin	form	information
1	Japan	A	milk	powder	0−9 months
2	Japan	A	milk	powder	follow-up milk
3	Japan	A	milk	powder	lactose-free
4	Japan	A	milk	powder	hypoallergenic formula
5	Japan	B	milk	powder	0−9 months
6	Japan	B	milk	powder	follow-up milk
7	Japan	B	milk	powder	lactose-free
8	Japan	B	milk	powder	hypoallergenic formula
9	Japan	C	milk	powder	0−9 months
10	Japan	C	milk	powder	follow-up milk
11	Japan	C	soy	powder	hypoallergenic formula
12	Japan	D	milk	powder	0−9 months
13	Japan	D	milk	powder	follow-up milk
14	Japan	E	milk	powder	0−9 months
15	Japan	E	milk	powder	follw-up milk
16	Japan	F	milk	powder	0−9 months
17	USA	G	milk	powder	0−12 months
18	USA	G	milk	powder	0−12 months
19	USA	G	soy	powder	hypoallergenic formula
20	USA	G	milk	liquid	0−12 months, lactose-free
21	USA	G	milk	liquid	0−12 months
22	USA	H	milk	powder	0−12 months
23	USA	H	milk	liquid	0−12 months
24	USA	H	soy	liquid	hypoallergenic formula
25	USA	I	milk	powder	0−12 months

[a] Infant formula samples were purchased from local stores in Japan and USA in 2009−2010.

ABBREVIATIONS USED

HILIC, hydrophilic interaction liquid chromatography; MS/MS, tandem mass spectrometry; CUF, centrifugal ultrafiltration; AMP, adenosine 5′-monophosphate; GMP, guanosine 5′-monophosphate; UMP, uridine 5′-monophosphate; CMP, cytidine 5′-monophosphate; IMP, inosine 5′-monophosphatte; UV, ultraviolet; LOD, limit of detection; LOQ, limit of quantitation; S/N, signal-to-noise ratio; QC, quality control; RSD, relative standard deviation; M.W., molecular weight.

Table 5. Nucleotide Levels in Infant Formula Samples[a]

	Concentrations of Nucleotides in Infant Formula											
	AMP		GMP		UMP		CMP		IMP		Total	
sample number	concentration (μg/g)	calculated value (μg/mL)	concentration (μg/g)	calcubted value (μg/mL)	concentration (μg/g)	calculated value (μg/mL)	concentration (μg/g)	calculated value (ug/mL)	concentration (μg/g)	calculated value (μg/mL)	concentration (μg/g)	calculated value (μg/mL)
1	18.4	2.4	9.0	1.2	22.8	3.0	95.9	12.5	8.0	1.0	154.0	20.0
2	12.1	1.7	20.7	2.9	15.9	2.2	50.4	7.1	30.9	4.3	130.0	18.2
3	20.2	2.8	15.1	2.1	31.5	4.4	118.6	16.6	11.4	1.6	196.8	27.6
4	34.0	4.8	20.6	2.9	50.6	7.1	169.3	23.7	19.6	2.7	294.1	41.2
5	25.5	3.6	39.6	5.5	45.5	6.4	114.1	16.0	30.7	4.3	255.4	35.8
6	9.3	1.3	16.6	2.3	13.9	1.9	61.2	8.6	9.7	1.4	110.7	15.5
7	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
8	31.0	4.7	30.5	4.6	46.0	6.9	116.3	17.5	28.5	4.3	252.4	37.9
9	5.0	0.6	6.1	0.8	5.7	0.7	158.0	20.5	5.7	0.7	180.4	23.5
10	2.1	0.3	3.7	0.5	8.1	1.1	89.4	12.5	n.d.		103.3	14.5
11	70.6	10.6	93.8	14.1	14.0	2.1	20.8	3.1	n.d.		199.2	29.9
12	61.6	8.0	29.6	3.8	82.9	10.8	278.0	36.1	31.4	4.1	483.5	62.9
13	60.6	8.5	26.4	3.7	81.4	11.4	258.0	36.1	25.7	3.6	452.2	63.3
14	n.d.		n.d.		n.d.		102.1	13.3	n.d.		102.1	13.3
15	n.d.		4.5	0.6	n.d.		101.9	14.3	n.d.		106.4	14.9
16	2.5	0.3	n.d.		n.d.		94.2	12.2	n.d.		96.7	12.6
17	84.3	14.3	141.9	24.1	110.9	18.9	254.6	43.3	n.d.		591.8	100.6
18	61.4	10.4	25.0	4.2	63.8	10.8	103.2	17.5	3.5	0.6	256.9	43.7
19	2.1	0.3	n.d.		n.d.		2.3	0.3	n.d.		4.4	0.7
20	11.1	5.5	17.2	8.6	14.5	7.3	34.2	17.1	n.d.		77.0	38.5
21	89.0	44.5	14.7	7.3	10.9	5.4	32.2	16.1	n.d.		146.7	73.4
22	82.7	12.4	139.7	21.0	120.7	18.1	257.1	38.6	n.d.		600.2	90.0
23	3.0	1.5	n.d.		n.d.		15.3	7.7	n.d.		18.3	9.2
24	3.8	1.9	n.d.		n.d.		n.d.		n.d.		3.8	1.9
25	29.0	4.3	20.8	3.1	32.1	4.8	126.7	19.0	3.5	0.5	212.1	31.8

[a] Concentration: μg/g of powder or liquid samples. Calculated value: μg/mL of actual infant formula samples.

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