Simultaneous Quantification of Nutritional Nucleotides and Nucleosides in Infant Formula Using HPLC-UV-MS/MS

Leo Jinyuan Wang, Bill Schnute

Thermo Fisher Scientific, Sunnyvale, CA, USA

Overview

Purpose:

To develop and evaluate a quantitative high performance liquid chromatography UV and tandem mass spectrometric method (HPLC-UV-MS/MS) for simultaneous determination of nutritional nucleotides and nucleosides in infant formula.

Methods:

Infant formula samples were centrifugated after acid protein precipitation and filtered before analysis. Separation was achieved on an Acclaim C30 reversed phase column using an Ultimate RSLC UHPLC system. UV and selected reaction monitoring (SRM) were used in series to provide the flexibility for routine and trace quantitation purposes.

Results:

Total chromatographic resolution for ten nucleotides and nucleosides was achieved within 12 minutes. Linearity was achieved from 20 to 4000 ppb with coefficient of determination (r^2) greater than 0.999 for each analyte. Precision was evaluated at 50 ppb and 2000 ppb and was achieved within 5% RSD and accuracy within the range from 90.3% (guanosine 5'-phosphate, GMP, 2000ppb) to 112% (GMP, 50 ppb). Powdered and liquid infant formula samples were successfully analyzed using this method.

Introduction

Nucleosides (adenosine, cytosine, guanosine, inosine, uridine) and their monophosphate nucleotides (AMP, CMP, GMP, IMP and UMP respectively) are the building blocks of nucleic acids, and are fully involved in essential cell functions such as energy production, transfer and storage, cellular signaling, as well as enzymatic activities. In recent years, a great number of studies show the growing interests in their dietary nutritional roles, especially in foods for infants. Human milk is the only source of nucleotides for neonates and it presents a significantly different nucleotide profile than other sources of milk such as bovine or soy. Researchers have reported the beneficial effects on immune and gastrointestinal systems of dietary nucleotides supplementation based on human and animal models. Thus nucleotides are considered as conditional essential nutrients.

Nucleotides and nucleosides were traditionally analyzed by microbiological and enzymatic assays, and chromatographic methods were used preferentially for the simultaneous analysis for both nucleotides and/or nucleosides. Anion exchange chromatography is suitable for the highly polar nucleotides, and reversed phase liquid chromatography was used extensively for nucleosides with ion pairing reagents for better on column retention.

In this study, we demonstrate a HPLC-UV-MS/MS method for simultaneous determination of all target nutritional nucleotides and nucleosides using a C30 reversed phase column without using an ion pairing reagent. The Acclaim C30 column demonstrates sufficient retention and provides complete chromatographic resolution for all target analytes within 12 minutes. The combination of UV and MS detection provides the flexibility for daily routine analysis and the sensitivity and selectivity for compound quantitation at trace level. The method is validated with respect to linearity, detection limits, carryover, precision and accuracy, and recovery as well as method ruggedness. This method has been successfully applied to the analysis of nucleotides and nucleosides in liquid and powdered infant formula samples incorporating simple and fast sample preparation.

Methods

Sample Preparation

Each liquid form infant formula sample was homogenized and 5 mL aliquot was transferred to a 15 mL polypropylene centrifuge tube. (Powdered infant formula sample was dissolved in DI water at 5 gram per 40 mL before homogenization.) 2 mL 3% acetic acid was added to each sample and vortex mixed for 15 seconds. 3 mL DI water was added to each sample after let sit for 20 minutes, then centrifuged at 6000 rpm for 20 minutes at 20 ºC. Clear solution was transferred to a 10 mL syringe, and filtered through a syringe filter (25 mm, 0.2 µm). 0.2 mL of filtrate was reconstituted in 0.8 mL DI water and 10 µL was injected for analysis. For MS/MS quantitation, each sample was spiked with 50 µL of 10 ppm mixed isotope-labeled internal standard solution before injection.

Liquid Chromatography (or more generically Separations)

Separation was achieved on an Acclaim C30 reversed phase column (2.1 × 150 mm, 3 µm) at 15 ºC using a Thermo Scientific UltiMate 3000 RSLC UHPLC system. Mobile phase was delivered at a flow rate at 0.4 mL/min with gradient elution. Mobile phase consisted of three components: A). DI water; B). Ammonium acetate buffer, 100 mM, pH 5.0; C). Methanol. Component B was kept constant throughout the gradient to keep the mobile phase buffer concentration at 20 mM. Methanol was hold at 0% from 0 to 3 minutes, increased to 10% in 2.9 minutes, then ramped to 40% in 2.6 minute, hold for 3.4 minutes and then return to 0% in 0.1 minutes followed by 5 minutes column equilibration at initial condition. Detailed gradient events are listed in Figure 1. UV chromatogram was collected at 260 nm.

Mass Spectrometry

A Thermo Scientific Quantum TSQ Access MAX was coupled to the UHPLC system with an Ion Max source and a heated electrospray probe (HESI II). The source parameters were set as following: spray voltage (3000 V), vaporizer temperature (350 ºC), sheath gas pressure (70 arbitrary unit), aux gas pressure (40 arbitrary unit), capillary temperature (200 ºC). Two SRM transitions were used for the quantitation (Q-SRM) and confirmation (C-SRM) of each target analyte with collision energy (CID) optimized for each SRM transition. Detailed SRM scan events are listed in Table 1.

Analyte	Retention Time (min)	Scan Time (min)	Precursor (m/z)	Q-SRM (CID) (m/z)	C-SRM (CID) (m/z)
CMP	2.10	1.5 - 4.0	-322	79 (40)	97 (24)
CMP-IS	2.10	1.5 - 4.0	-334	79 (40)	---
UMP	2.65	1.5 - 4.0	-323	79 (37)	97 (25)
UMP-IS	2.65	1.5 - 4.0	-334	79 (37)	---
Cytidine	5.60	4.0 - 8.2	+244	112 (15)	95 (37)
GMP	6.15	4.0 - 8.2	-362	79 (37)	211 (21)
GMP-IS	6.15	4.0 - 8.2	-377	79 (37)	---
IMP	6.50	4.0 - 8.2	-347	79 (36)	135 (32)
Uridine	7.35	4.0 - 8.2	+245	113 (14)	96 (34)
AMP	8.90	8.2 - 12.0	-346	79 (37)	134 (36)
AMP-IS	8.90	8.2 - 12.0	-361	79 (37)	---
Inosine	9.55	8.2 - 12.0	+269	137 (22)	120 (42)
Guanosine	9.75	8.2 - 12.0	+284	152 (17)	135 (36)
Adenosine	10.55	8.2 - 12.0	+268	136 (22)	119 (42)

Table 1. SRM Scan Events

Data Analysis

Thermo Scientific Xcalibur 2.1 with Foundation 1.0.2 and TSQ 2.3

Thermo Scientific DCMS^{Link} 2.11 for UV data acquisition and processing

Results

Quantitation with UV Detection

The use of a C30 reversed phase column significantly improved the retention of early eluted nucleotides (CMP and UMP) over conventional C8 and C18 columns. As seen in Figure 1, ten target nutritional nucleotides and nucleosides were baseline resolved thus ensuring accurate quantitation using UV detection.

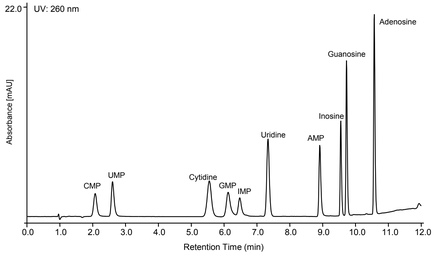
Method performance is evaluated against quality parameters such as linearity, precision and accuracy, and the results are summarized in Table 2.

FIGURE 1. UV Chromatogram of 10 Nutritional Nucleotides and Nucleosides

System: UltiMate 3000 RS UHPLC system
Column: Acclaim C30 reversed phase (2.1 × 150 mm, 3 µm)
Column Temp.: 15 ºC
Mobile Phase: Gradient elution.
A) DI water;
B) NH_4OAC Buffer, 100 mM, pH5;
C) CH_3OH
Flow Rate: 0.4 mL/min
Injection: 10 µL, 1 ppm of each analyte
Detection: UV at 260 nm

Gradient Events

Time	%A	%B	%C
-5.0	80	20	0
3.0	80	20	0
5.9	70	20	10
8.5	40	20	40
11.9	40	20	40
12.0	80	20	0



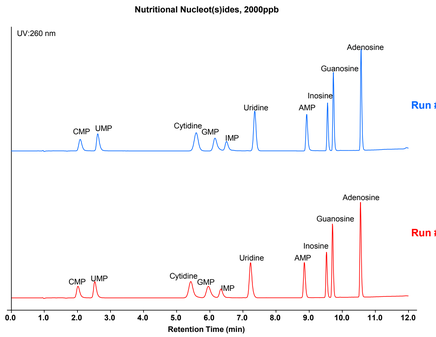
Excellent coefficient of determination (r^2) was achieved with r^2 greater than 0.999 for each analyte. Quantitation precision and accuracy were evaluated at 50 ppb and 2000 ppb (n=7). Excellent precision was achieved within 5% RSD and accuracy within the range from 90.3% (GMP, 2000 ppb) to 112% (GMP, 50 ppb).

			50 ppb			2000 ppb		
No.	Analyte	r^2	Mean	%Accuracy	%RSD	Mean	%Accuracy	%RSD
1	CMP	0.9994	50.0	99.9	3.00	1943	97.1	0.99
2	UMP	0.9992	53.4	107	3.04	1950	97.5	0.98
3	Cytidine	0.9993	50.0	99.9	1.84	1963	98.2	0.73
4	GMP	0.9990	55.9	112	3.63	1807	90.3	1.51
5	IMP	0.9996	49.1	98.3	4.39	1875	93.7	1.89
6	Uridine	0.9994	49.0	97.9	1.62	1951	97.6	0.74
7	AMP	0.9990	53.8	108	3.04	1975	98.8	0.83
8	Inosine	0.9994	46.4	92.9	1.80	1918	95.9	0.86
9	Guanosine	0.9994	46.0	92.1	1.13	1922	96.1	0.77
10	Adenosine	0.9994	45.9	91.8	1.20	1932	96.6	0.83

Table 2. Method Performance with UV Detection

The C30 reversed phase column demonstrated excellent long term stability with more than 400 injections of infant formula samples. As seen in Figure 2, retention time, peak shape, resolution were well maintained after a great number of assays by comparing two identical injections: Run #1 and Run #420.

FIGURE 2. Long term stability of the C30 reversed phase column



For certain sample where particular analytes present at low level, accurate quantitation may be difficult using non-specific UV detection. As seen in Figure 3, quantitation of inosine using UV is very challenging. However, inosine and other low level nucleosides can be easily quantified with great confidence using selective and sensitive SRM detection, as seen in Figure 4, SRM chromatograms of infant formula sample B.

FIGURE 3. Difficulty of Inosine quantification using UV

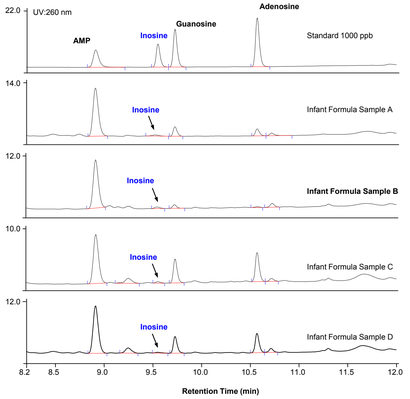
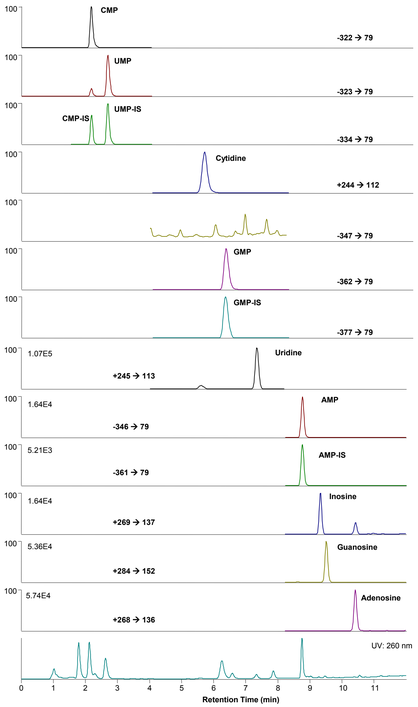


FIGURE 4. SRM Chromatograms of Nutritional Nucleot(s)ides in Infant Formula Sample B



Conclusion

A LC-UV-MS/MS method for simultaneous determination of nutritional nucleotides and nucleosides was developed and demonstrated excellent performance with respect to chromatographic separation, calibration and linearity, quantitation accuracy and precision as well as long term column stability. The use of MS/MS detection in SRM mode significantly improves the detection sensitivity and provides great quantitation confidence for analytes at trace level.