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RESEARCH PAPER

Analysis of nucleosides and nucleotides in infant formula by liquid chromatography–tandem mass spectrometry

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Abstract A method for the simultaneous analysis of nucleosides and nucleotides in infant formula using reversed-phase liquid chromatography–tandem mass spectrometry is described. This approach is advantageous for compliance testing of infant formula over other LC-MS methods in which only nucleotides or nucleosides are measured. Following sample dissolution, protein was removed by centrifugal ultrafiltration. Chromatographic analyses were performed using a C_{18} stationary phase and gradient elution of an ammonium acetate/bicarbonate buffer, mass spectrometric detection and quantitation by a stable isotope-labelled internal standard technique. A single laboratory validation was performed, with spike recoveries of 80.1–112.9 % and repeatability relative standard deviations of 1.9–7.2 %. Accuracy as bias was demonstrated against reference values for NIST1849a certified reference material. The method has been validated for the analysis of bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulae.

Keywords Nucleotides · Nucleosides · Infant formula · LC-MS

Introduction

The structure of nucleosides and nucleotides and their importance to infant nutrition have been described previously [1–3]. In view of their physiological benefits, nucleotides are routinely supplemented into infant formulae as sodium salts of adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP) and uridine 5′-monophosphate (UMP) [4]. Although nucleosides are not supplemented into infant formulae, dephosphorylation of nucleotides to the corresponding nucleosides—adenosine (Ado), cytidine (Cyd), guanosine (Guo), inosine (Ino) and uridine (Urd)—can occur under certain processing conditions [5].

Analytical methods for nucleosides and nucleotides in infant formulae and milk have previously been reviewed [6]. Sample preparation of infant formulae is frequently achieved by acidic precipitation of casein proteins from the reconstituted sample [5, 7]. Alternatively, centrifugal ultrafiltration has also been reported [8] and offers a simple mechanism to remove high-molecular-weight material. Further cleanup of sample extracts using ion exchange solid phase extraction and a phenylboronate affinity gel has been reported [9–11].

Liquid chromatography, i.e. reversed-phase liquid chromatography (RPLC), ion pair RPLC, ion exchange liquid chromatography and hydrophilic interaction liquid chromatography, with UV detection is commonly used for the quantitation of nucleotides in milk products [5, 7, 8, 12–15]. RPLC is easily adapted for the analysis of nucleosides, although the retention of nucleotides is more challenging. However, at the appropriate mobile phase pH, polar nucleotides are retained on a C_{18} column and an organic solvent gradient is able to remove late-eluting nucleosides.

The use of mass spectrometry (MS) offers potential advantages with respect to accuracy and simplicity by incorporating the addition of stable isotope-labelled (SIL) internal standards, whilst the selectivity of tandem MS reduces the need to remove other components that often compromise UV analyses [16–18]. The aim of this study was, therefore, to develop an LC-MS/MS method for the

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simultaneous analysis of nucleosides and nucleotides in infant formulae. The method described involves a simple centrifugal ultrafiltration procedure followed by high-performance liquid chromatography (HPLC) with detection and quantitation by tandem MS. Confidence in analytical accuracy is assured through the use of a SIL standard for each analyte. This technique has been validated for a range of bovine milk-based, caprine milk-based, soy-based and hypoallergenic infant formulae.

Experimental

Reagents

Ammonium acetate (NH_4CH_3COO), ammonium bicarbonate (NH_4HCO_3), AMP sodium salt, CMP disodium salt, GMP disodium salt, IMP disodium salt, UMP disodium salt, and corresponding nucleosides were obtained from Sigma-Aldrich (St. Louis, MO, USA). SIL nucleoside standards—$^{13}C_5$ Ado, $^{13}C_9{}^{15}N_3$ Cyd, $^{15}N_5$ Guo, $^{15}N_4$ Ino and $^{13}C_9{}^{15}N_2$ Urd—were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). SIL nucleotide standards—$^{13}C_{10}{}^{15}N_5$ AMP, $^{13}C_9{}^{15}N_3$ CMP, $^{13}C_{10}{}^{15}N_5$ GMP and $^{13}C_9{}^{15}N_2$ UMP—were purchased from Sigma-Aldrich. $^{13}C_{10}{}^{15}N_4$ IMP was purchased from Medical Isotopes (Pelham, NH, USA).

Potassium dihydrogen phosphate (KH_2PO_4), acetic acid, orthophosphoric acid, potassium hydroxide and methanol were supplied by Merck. Water was purified with resistivity ≥18 M Ω using an E-pure water system (Barnstead, Dubuque, IA, USA).

A standardising buffer (KH_2PO_4, 0.25 M, pH 3.5) was made by dissolving 34.02 g of KH_2PO_4 in 900 mL of water, adjusting the pH to 3.5 with orthophosphoric acid and then making the solution to 1 L. Mobile phase A (NH_4CH_3COO, 10 mM; NH_4HCO_3, 5 mM, pH 5.6) was made daily by dissolving 0.771 g of NH_4CH_3COO and 0.395 g of NH_4HCO_3 in 950 mL of water, adjusting the pH to 5.6 with acetic acid solution (10 %, *w*/*v*) and then making to 1 L with water. Mobile phase B consisted of 100 % methanol.

Standard solutions

SIL nucleoside and nucleotide stock standards were prepared by accurately weighing 50 mg each of $^{13}C_5$ Ado, $^{13}C_9{}^{15}N_3$ Cyd, $^{15}N_5$ Guo, $^{15}N_4$ Ino, $^{13}C_9{}^{15}N_2$ Urd, $^{13}C_{10}{}^{15}N_5$ AMP, $^{13}C_9{}^{15}N_3$ CMP, $^{13}C_{10}{}^{15}N_5$ GMP, $^{13}C_{10}{}^{15}N_4$ IMP and $^{13}C_9{}^{15}N_2$ UMP into separate 50-mL volumetric flasks. To each flask, 40 mL of water was added and then shaken (with gentle warming if necessary) until the standard was completely dissolved before water was added to volume. Aliquots (~1.5 mL) of SIL stock standards were immediately dispensed into individual cryogenic vials and frozen at −15 °C for later use. Prior to analysis, cryogenic vials containing each SIL nucleoside and nucleotide stock standard were allowed to thaw to room temperature.

Non-isotopically labelled (NL) nucleoside and nucleotide stock standards were prepared in a similar manner by accurately weighing approximately 50 mg of each into separate 50-mL volumetric flasks and making to volume with water. These were refrigerated at 4 °C for up to 1 month.

Estimation of the moisture content of nucleosides was performed using the oven moisture method (102±2 °C, 4 h) and the concentration was calculated on a dry weight basis. Extinction coefficients at UV absorbance maxima were then determined for each nucleoside. These were compared with the values previously determined for nucleotides [5], with correction for molecular weight. The values obtained for each nucleoside were in close agreement with those for the corresponding nucleotide. Mean extinction coefficient values (nucleoside and corresponding nucleotide) were calculated by adjusting for molecular weight and are reported in Table 1. The concentration of each nucleoside and nucleotide stock standard was determined by adding 500 μL of each stock standard into separate 25-mL volumetric flasks, diluting with standardising buffer and measuring the absorbance at the appropriate λ_{max}.

A mixed SIL intermediate standard was prepared by diluting 2.0 mL of each SIL stock standard into a 25-mL volumetric flask and making to volume with water. A mixed NL intermediate standard was made by adding 1.0 mL of each NL stock standard into a 25-mL volumetric flask and making to volume with water.

Four calibration standards were prepared by pipetting 1.0, 1.0, 0.5 and 0.2 mL of SIL intermediate standard and 2.0, 4.0, 5.0 and 8.0 mL of NL intermediate standard into

Table 1 Mean extinction coefficients at UV absorbance maxima of nucleosides and corresponding nucleotides

Nucleoside/nucleotide	Extinction coefficient ($E^{1\ \%}$)	λ_{max} (nm)
AMP	428.6	257
Ado	557.0	
CMP	390.9	280
Cyd	519.5	
GMP	392.0	254
Guo	502.8	
IMP	356.5	249
Ino	462.7	
UMP	312.7	262
Urd	415.1	

Ado adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *AMP* adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate

50-, 50-, 25- and 10-mL volumetric flasks, respectively. The calibration standards were then made to volume with water and mixed thoroughly.

Samples

A range of different infant formula samples were evaluated during the validation of the method. These included a partially hydrolysed bovine milk-based powder, a partially hydrolysed soy-based powder, an infant elemental powder, a bovine milk-based powder, a soy-based powder and a caprine milk-based powder.

Sample preparation

Approximately 5.0 g of infant formula powder was weighed accurately into a 50-mL polypropylene centrifuge tube (Biolab, Auckland, New Zealand) and dissolved in 25 mL of water. To this was added 1.0 mL of the SIL intermediate standard and the tube was capped and vortex-mixed. The sample was allowed to stand for 10 min to ensure complete hydration before dilution to a final volume of 50 mL with water.

A 4.0-mL aliquot of sample solution was added to an Amicon Ultra-4 3000 MWCO centrifugal ultrafiltration unit (Millipore, Billerica, MA, USA) and centrifuged at 3,500×*g* for 60 min. The filter was then removed and discarded and a 1-mL aliquot of filtrate was transferred to an HPLC vial ready for analysis.

Instrumentation

The HPLC system used consisted of a CBM20A system controller, two LC20ADXR pumps for high-pressure gradients, a CTO20AC column oven and a SIL20ACXR autosampler equipped with a 50-μL injection loop (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved using a Gemini column, 5 μm, 4.6×250 mm (Phenomenex, Torrance, CA, USA), with a high-pressure gradient elution programme as described in Table 2.

The MS/MS system consisted of a 3200 QTRAP quadrupole mass spectrometer with a Turbo V ion source equipped with an electrospray ionisation (ESI) probe. Analyst 1.5.1 software was used for instrument control and data processing (ABSciex, Foster City, CA, USA). The mass spectrometer was operated in ESI^+ mode with nitrogen utilised as the drying and collision gas. The instrumental parameters were set as follows: curtain gas at 30 psi, nebuliser gases GS1 and GS2 at 50 and 70 psi, respectively, desolvation temperature at 700 °C, collision-induced dissociation gas at medium and ion spray voltage at 5,500 V. Instrument settings and multiple reaction monitoring (MRM) transitions for the generation of product ions for nucleosides and nucleotides are given in Table 3.

Table 2 Gradient procedure for chromatographic separation

Time (min)	Flow rate (mL min^{-1})	Phase composition	
		%A[a]	%B[b]
0.0	0.75	100	0
3.5	0.75	100	0
10.0	0.75	80	20
20.0	0.75	80	20
21.0	0.75	100	0
35.0	0.75	100	0

[a] Mobile phase A: NH_4CH_3COO, 10 mM; NH_4HCO_3, 5 mM, pH 5.6

[b] Mobile phase B: 100 % methanol

Method validation

Six mixed nucleoside and nucleotide solutions covering the expected working range were prepared and linearity was evaluated by least-squares regression analysis of the SIL-corrected response (ratio of NL/SIL analyte peak area versus ratio of NL/SIL analyte concentration). A minimum value of

Table 3 MS/MS parameters

Analyte	Precursor ion $[M-H]^+$ (*m/z*)	Product ions (*m/z*)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
CMP	324.1	112.0	26	3.5	32	27	4
SIL CMP	336.1	119.0					
UMP	325.0	97.0	21	9.5	18	23	4
SIL UMP	336.1	102.1					
GMP	364.1	152.0	36	4.5	22	23	4
SIL GMP	379.1	160.0					
IMP	349.1	137.0	31	6.0	28	23	4
SIL IMP	362.1	145.0					
Cyd	244.1	112.0	11	6.5	22	19	4
SIL Cyd	256.1	119.0					
Urd	245.1	113.0	16	5.0	16	19	4
SIL Urd	256.1	119.0					
AMP	348.1	136.0	36	1.0	24	25	4
SIL AMP	363.1	146.0					
Ino	269.1	137.0	16	4.5	18	23	4
SIL Ino	273.1	141.0					
Guo	284.1	152.0	11	4.0	24	23	4
SIL Guo	289.1	157.0					
Ado	268.1	136.0	21	8.5	16	39	4
SIL Ado	273.1	136.0					

SIL stable isotope-labelled, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *AMP* adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *DP* declustering potential, *EP* entrance potential, *CEP* collision cell entrance potential, *CE* collision energy, *CXP* collision cell exit potential

0.997 for the correlation coefficient (r^2) was deemed to be suitable. Plots of standard residuals were visually assessed as a further test of linearity.

Repeatability was estimated by analysing replicate pairs (n=9 pairs) of a bovine milk-based infant formula and NIST 1849a. Intermediate precision was determined from replicate analyses (n=6) of a bovine milk-based infant formula and NIST 1849a tested on three different days. Method detection limits (MDLs) were estimated in accordance with US Environmental Protection Agency procedures [19].

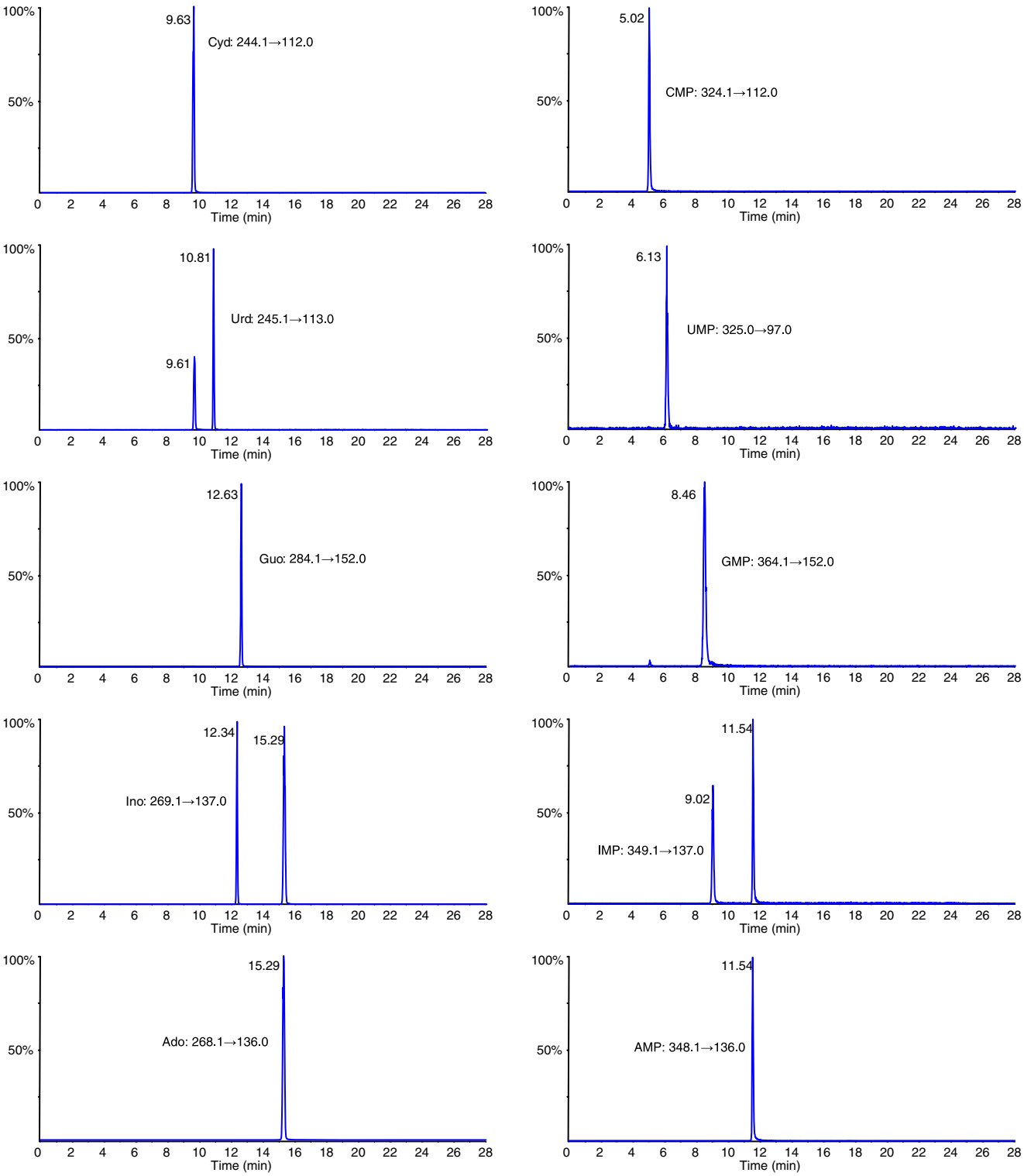

Fig. 1 LC-MS/MS MRM chromatograms of a mixed nucleotide and nucleotide standard solution (~7 μg mL^{-1})

The robustness of the method was assessed by conducting a Plackett–Burman trial [20], as described previously [15]. The seven factors assessed were: initial sample water volume (27 and 23 mL), vortex time (40 and 20 s), wait time (14 and 6 min), centrifuge volume (4.2 and 3.8 mL), centrifuge speed (4,000 and 3,000×*g*), centrifuge time (70 and 50 min) and a dummy factor.

Method accuracy was determined as both recovery and bias. Recovery of both nucleosides and nucleotides was evaluated by spiking a range of sample matrices at 50 and 150 % of the concentration levels typically found in infant formulae. Bias was evaluated by performing a paired *t* test for nucleotides both against reference values of a NIST 1849a powder and against values for a bovine milk-based infant formula tested using AOAC Official Method 2011.20 [21].

Results and discussion

Method optimisation

The simultaneous chromatographic analysis of both nucleosides and nucleotides in infant formulae using LC-UV has previously been described [5]. However, the mobile phase contained a 0.1 M phosphate buffer, which is unsuitable for use in LC-MS. In this study, ammonium acetate (10 mM, pH 5.6) was initially chosen to buffer the mobile phase because of its compatibility with electrospray mass spectrometric detection and a pH buffering range (~3.8–5.8) consistent with nucleoside and nucleotide pK_a values. However, significant peak tailing for nucleotides was observed when this buffer was used.

Conventional LC-UV nucleotide analyses typically contain phosphate in the mobile phase and no significant peak tailing is observed [5, 7]. Unfortunately, the use of non-volatile buffers such as phosphate in LC-MS is generally not recommended because of contamination of the ion source leading to a decrease in sensitivity. Furthermore, the interaction of phosphorylated compounds with metal surfaces in liquid chromatographic applications resulting in peak tailing has been reported [22–26]. Pretreatment of the chromatographic system using phosphoric acid prior to switching to a non-phosphate eluent during analysis [26, 27], the substitution of polyether ether ketone tubing for stainless steel, the use of a high pH mobile phase [28] and the addition of EDTA to the mobile phase [25] have all been employed to overcome this problem.

A number of mass spectrometer manufacturers have evaluated phosphate buffers for use with their instruments and have demonstrated that modern source designs can tolerate the use of non-volatile buffers [29–32]. A phosphate-based ion pair RPLC-MS method was successfully applied to the quantitative analysis of intracellular nucleotides utilising a microbore column to reduce the amount of phosphate introduced to the ion source [33].

In the present study, a low ionic strength phosphate buffer ($NH_4H_2PO_4$ 0.08 mM, pH 5.6) was initially evaluated for compatibility with the mass spectrometer. The chromatographic parameter resolution, retention factor, peak area repeatability, retention time repeatability, plate number and asymmetry were evaluated, with acceptable results being obtained (data not shown). There was some loss of sensitivity as replicate analyses progressed, consistent with a small accumulation of phosphate on the cone. The method was applied to the analysis of nucleotides in infant formula samples in a validation study. Linear response was demonstrated for NL/SIL peak area versus NL/SIL analyte concentration (r^2=0.997–0.999). Accuracy and precision were

Table 4 Chromatographic performance

Parameter	Analyte									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Retention time (min)	9.6 (0.0)[c]	10.8 (0.0)	12.6 (0.0)	12.3 (0.0)	15.3 (0.0)	5 (0.0)	6.1 (0.0)	8.5 (0.0)	9.0 (0.0)	11.6 (0.0)
Capacity factor[a]	2.0 (0.0)	2.4 (0.0)	2.9 (0.0)	2.8 (0.0)	3.8 (0.0)	0.6 (0.0)	0.9 (0.0)	1.6 (0.0)	1.8 (0.0)	2.6 (0.0)
Resolution[a]	1.3 (0.2)	3.5 (0.9)	0.8 (0.2)	2.2 (0.4)	4.6 (0.8)	–	2.8 (0.4)	4.3 (0.6)	0.9 (0.1)	2.8 (0.4)
Tailing factor[a]	1.8 (1.0)	1.2 (0.3)	1.7 (0.5)	1.4 (0.6)	1.8 (0.7)	1.6 (0.3)	1.7 (0.4)	1.6 (0.3)	1.5 (0.3)	1.7 (0.3)
Peak area ratio repeatability[b]	0.42 (0.01)	0.46 (0.02)	0.51 (0.02)	0.55 (0.02)	0.50 (0.01)	0.49 (0.03)	0.36 (0.01)	0.46 (0.01)	5.34 (0.13)	0.51 (0.03)

Ado adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *AMP* adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate

[a] Calculations as defined by US Pharmacopeia

[b] Peak area ratio measured as non-labelled nucleoside or nucleotide/stable isotope-labelled nucleoside or nucleotide

[c] Analysis of six replicates of a mixed nucleoside and nucleotide standard reported as the mean (standard deviation)

Table 5 Method performance as linearity, detection limit and precision

Analyte	Range (μg mL^{-1})	Linear regression[a]	r^2	MDL[b] (mg hg^{-1})	RSD_r[c] (%)	$HorRat_r$[d]	RSD_{iR}[e] (%)
Cyd	0.7–58.6	$y=0.737x+0.1053$	0.9996	0.03	4.8	0.4	14.4
Urd	0.8–60.9	$y=0.957x-0.3441$	0.9987	0.12	4.1	0.4	14.1
Guo	0.7–54.9	$y=0.837x+0.2553$	0.9996	0.01	6.2	0.4	7.9
Ino	0.8–62.1	$y=1.059x-0.0417$	0.9982	0.01	7.2	0.4	11.2
Ado	0.7–59.2	$y=0.778x+0.1853$	0.9997	0.01	–[f]	–	–
CMP	0.6–45.3	$y=0.94x+0.0113$	0.9998	0.13	4.0	0.6	4.6
UMP	0.5–42.6	$y=0.872x-0.1152$	0.9997	0.01	5.0	0.6	6.2
GMP	0.6–45.7	$y=0.928x+0.1423$	0.9993	0.01	1.9	0.2	2.9
IMP	0.6–46.8	$y=1.069x+0.5071$	0.9999	0.03	–	–	–
AMP	0.8–60.6	$y=0.787x+0.35$	0.9986	0.01	2.8	0.4	7.8

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine

[a] Linear regression plotted as the ratio of peak area of unlabelled analyte to stable isotope-labelled standard versus the ratio of the concentration of unlabelled analyte to stable isotope-labelled standard

[b] Determined from n replicates at or near the expected detection limit: $MDL=t_{(n-1,\ 1-\alpha)}\times SD$, where $n=7$ and $\alpha=0.01$

[c] Relative standard deviation repeatability (RSD_r)=SD of n duplicate pairs/mean×100 ($n=9$)

[d] Horwitz ratio=$RSD_r/pRSD_R$, where $pRSD_R=2C^{-0.1505}$ at the 10-ppm concentration level (typical range, 0.3–1.3)

[e] Intermediate reproducibility of six replicates measured on three different days ($n=18$). RSD%=SD/mean×100

[f] Concentration at or below the detection limit

evaluated, with both spike recovery (84.2–107.1 %) and repeatability relative standard deviation (1.5–3.1 %) deemed to be acceptable. Despite this performance, a limitation with this phosphate-based approach was that the number of samples within each analytical run was limited due to the requirement for regular maintenance of the source.

An alternative chromatographic system was evaluated based on the observations of Asakawa et al. [22], who found a beneficial chromatographic effect with a number of mobile phase additives. Of those evaluated, only ammonium bicarbonate is volatile and considered suitable for use in LC-MS and was therefore incorporated as an additive in the ammonium acetate eluent.

The optimisation of the MS conditions was performed by infusion of a standard of each nucleoside or nucleotide (~10 μg mL^{-1}) diluted in a mixture of mobile phases A and B (90:10). Initial development focused on ESI^+ for nucleosides and ESI^- for nucleotides. However, it was found that ESI^+ gave superior response for both analytes, with the $[M+H]^+$ ion most abundant and low levels of potassium adduct, thereby simplifying the analysis with the detection of all analytes in positive mode.

The conditions for MRM were optimised by selecting individual fragments and adjusting collision energies to maximise the product ion signal. The most abundant fragment ion observed for nucleosides and nucleotides was

Table 6 Mean recovery of nucleosides and nucleotides in spiked samples at 50 and 150 % of typical concentrations

Sample	Recovery (%)									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
IF powder p/h bovine milk-based	95.4	84.9	104.4	94.1	99.3	101.3	81.8	104.8	112.9	95.1
IF powder p/h soy-based	101.1	98.9	107.2	96.8	100.1	101.8	88.8	101.6	98.4	101.7
Infant elemental powder	98.7	97.2	104.1	98.2	99.0	103.8	91.0	104.8	109.0	98.6
IF powder bovine milk-based	93.4	86.6	102.6	100.1	97.9	95.7	90.7	102.0	101.3	101.8
IF powder soy-based	101.7	80.1	107.9	103.0	95.3	101.8	90.3	103.5	94.8	98.7
IF powder caprine milk-based	96.4	109.1	112.0	100.1	100.5	103.0	97.5	100.9	98.9	110.1

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *IF* infant formula, *p/h* partially hydrolysed

Table 7 Method bias against NIST 1849a reference values

	Analyte							
	CMP	UMP	GMP	AMP	CMP+Cyd	UMP+Urd	GMP+Guo	AMP+Ado
Measured[a] results	27.0 (0.99)	12.0 (0.66)	14.8 (0.45)	10.3 (0.29)	28.1 (1.00)	14.4 (0.68)	15.0 (0.45)	10.3 (0.29)
Reference values	26.8±2.9	12.9±1.5	14.6±1.1	10.51±0.5	31.7	15.5	14.6	10.8
Bias (*p* value)	0.44	<0.001	0.16	0.03	<0.001	<0.001	0.01	0.05

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Urd* uridine

[a] Mean (standard deviation) of analytical results of NIST 1849a CRM in milligrams per hectogram (n=12 replicates)

formed by cleaving of the glycosidic bond, resulting in the loss of ribose or ribose + phosphate group and the detection of the positively charged nucleobase. The exception to this was UMP, which underwent fragmentation and rearrangement to generate the m/z 97.0 ion. A similar fragmentation scheme has been reported for the generation of a product ion with m/z 81.0 from the fragmentation of deoxycytidine 5′-monophosphate [34].

Using the LC-MS/MS method developed, the simultaneous detection of nucleosides and nucleotides in a standard solution was achieved (Fig. 1).

Method performance

A high degree of selectivity is afforded by an MRM experiment; however, chromatographic separation is required for critical peaks with similar MRM transitions if accurate quantitation is to be achieved. Chromatographic performance was assessed by replicate analyses (n=6) of a mixed nucleoside and nucleotide standard, with satisfactory resolution being obtained between IMP/AMP (6.7), Ino/Ado (6.8) and Cyd/Urd (4.3) critical pairs, compounds which differ in mass by <2 Da (Table 4).

Method validation experiments to determine linearity, detection limits and precision are summarised in Table 5. Linearity was evaluated by least-squares regression analysis, with acceptable values being obtained for the correlation coefficient and with standard residual plots showing no pattern and only a small amount of random variation. The detection limits were appropriate, as defined by the infant formula industry, with the exception of those for CMP and Urd [35]. Although the detection limits of CMP and Urd were higher than those specified, the MDL was two orders of magnitude lower than that found in unfortified milk powder [5]. Precision was evaluated as repeatability (1.9–7.2 %) and intermediate precision (2.9–14.4 %). The suitability of these results was demonstrated by a Horwitz (repeatability) ratio of 0.2–0.6 [36].

The method was found to be robust for the seven method performance parameters studied, with variances in the results obtained not being significantly different from those expected by chance. Given the method's simplicity, two critical steps are required to ensure the accuracy of the results obtained: accurate measurement of the amount of sample weighed and accurate addition of the internal standard.

Accuracy determined as spiked recovery results measured in the six different product types were within the acceptable limits of 80–115 % at the 10-μg g^{-1} level, as suggested by the AOAC [36] (Table 6). Accuracy estimated as bias was evaluated against reference values for NIST 1849a CRM (Table 7) and against an LC-UV method for determining nucleotides in infant formula (AOAC method 2011.20; Table 8). Although there were statistically significant differences for some of the results, the differences were small enough (0–13 %) that they are unlikely to be of practical significance for compliance and labelling requirements.

Conclusions

The optimisation and validation of an LC-MS/MS method for the analysis of nucleosides and nucleotides in infant formulae has been described. The use of SIL internal

Table 8 Method bias against AOAC Official Method 2011.20

	Analyte				
	CMP	UMP	GMP	IMP	AMP
Measured results[a]	12.9 (0.39)	4.1 (0.14)	1.6 (0.04)	0 (0)	3.6 (0.11)
AOAC 2011.20 results[a]	12.3 (0.50)	4.0 (0.21)	1.6 (0.07)	0 (0)	3.2 (0.16)
Bias (*p* value)	<0.001	0.24	0.44	0	<0.001

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *IMP* inosine 5′-monophosphate

[a] Mean (standard deviation) of analytical results for bovine milk-based infant formula in milligrams per hectogram (n=12 replicates)

standards provides confidence in the accuracy of the results obtained. The method has been demonstrated to be precise and accurate and has been validated for the analysis of bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulae.

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