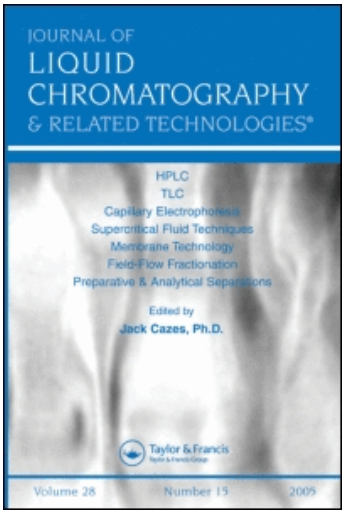

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Validation of an HPLC Method for the Determination of Nucleotides in Infant Formulae

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Abstract: This paper describes the development and validation of a simple, fast, and sensitive high performance liquid chromatographic method for the determination of the 5′-mononucleotides: adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate, and uridine 5′-monophosphate in infant formulae in defined labour conditions. Following deproteinisation and filtration, the sample extract was analysed by reversed-phase liquid chromatography. The method was developed by using a C_{18} reverse-phase column. Isocratic elution was used with a mobile phase consisting of 0.1 M potassium phosphate buffer and 4 mM tetrabutylammonium hydrogen phosphate. The pH of the solution was adjusted to 6.0. The analysis of nucleotides was performed with a PDA detector at 260 nm and 278 nm. Analytical validation parameters, such as specificity and selectivity, linearity, accuracy, precision, robustness, and system suitability were evaluated. During evaluation of the analytical parameters, it was shown that the method is linear ($r = 0{,}999$). The recoveries ranged 100 ± 3 and the relative standard deviation was ≤ 3. The precision of the method was achieved with a coefficient of variation (CV %), which is less than 3%. Standard solutions are stable during 30 hours and in the range of method robustness. Validation of the HPLC method for determination of nucleotides has shown that the developed analytical method is acceptable for its intended purpose in defined labour conditions.

Keywords: HPLC, Infant formulae, Method, Nucleotides, Validation

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INTRODUCTION

Breastfeeding is the best way of feeding infants that normally provides an adequate supply of nutrients to support their healthy growth and development. Certain unique biochemical and immunological factors provide protection to the newborn against infective agents in his/her new environment.[1] Owing to the special biochemical needs of the developing neonate a group of specific compounds considered semi-essential or conditionally essential, namely, taurine and other free amino acids, L-carnitine and free nucleotides are identified as such compounds.[2]

These compounds became conditionally essential when the endogenous supply is inadequate during periods of infants rapid growth or after their injury.[3]

Infants who cannot be breast fed or who should not receive breast milk, or for whom breast milk is not available, require breast milk substitutes of high quality for the first 4 to 6 months of life.[4] Progressive attempts have been made by the infant nutrition industry to bring the composition of infant formulae closer to that of human milk, not only with regard to major components, but also with regard to micro compounds that may be involved in the development of the newborn.[2] Human milk is known to contain a significant amount of free nucleotides when compared with the composition of mature cow's milk.[5]

All infant food formulas have to be prepared according to Food and Drug Administration (FDA) regulations which specify minimum and, in some cases, maximum nutrient level requirements, based on recommendations by the American Academy of Paediatrics Committee on Nutrition.[6] These regulations and the Infant Formula Act help to ensure that all infant formulas are nutritionally complete and safe for babies.[7]

Until now, nucleotides are introduced as supplements to infant formulas in several countries, and their biological effects are published in several fields.[8] Investigations have shown that nucleotides in infant nutrition have a beneficial effect on various components of the immune system.[9–11] They can enhance intestinal absorption of Fe, improve lipoprotein and long chain polyunsaturated fatty acid metabolism, and partly modulate intestinal micro flora.[12,13] In some experimental studies, they have shown to have a beneficial influence on intestinal mucous and liver.[14] Some randomized controlled trials have reported beneficial effects of nucleotide supplementation on reducing the incidence of diarrhea in infants and enhanced growth of infants born small for gestation age.[15,16] Rapidly proliferating tissues, such as the immune system or the intestine are not able to fulfill the needs of cell nucleotides exclusively by *de novo* synthesis and they preferentially utilize the *salvage* pathway, recovering nucleosides and nucleobases from blood and diet. An exogenous

supplement of these compounds through the diet may be essential for sustaining growth and to maintain the cellular function in these tissues.[17–19]

Nucleotide supplementation of infant formulae is recommended by European legislation up to the levels found in human milk. Only nucleotides as monophosphate salts can be added to infant formulas and supplementation is allowed to a maximum of 2.50 mg/100 kcal for cytidine 5′-monophosphate, 1.75 mg/100 kcal for uridine 5′-monophosphate, 1.50 mg/100 kcal for adenosine 5′-monophosphate, 0.50 mg/100 kcal for guanosine 5′-monophosphate and 1.00 mg/100 kcal for inosine 5′-monophosphate. But, the total concentration cannot exceed 5 mg/100 kcal.[2,20]

Until recently, analysis of nucleotides was carried out by enzymatic methods or by ion-exchange chromatography.[21] Recent studies for determination of nucleotides in infant formulae and follow-up milks using an ion-pair HPLC and diode array detection were published by some authors. Thus, Oliveira et al. published the analysis of four nucleotides: 5′-AMP, 5′-CMP, 5′-GMP, 5′-UMP in infant formulae and follow-up milks.[22] Perrin et al. developed the method for determination of nucleotides and nucleosides in infant formulae.[23] Leach et al. developed the method for determination of the total potentially available nucleosides (TPAN) of pooled and individual milk samples.[24]

To improve the determination of nucleotides in infant formulas, in this work, a simple, accurate, rapid, and precise HPLC method was developed.

EXPERIMENTAL

Apparatus

The determination of nucleotides was carried out using an HPLC system composed of a Shimadzu Class-VP HPLC system (Shimadzu, Tokyo, Japan), equipped with LC-10AD-vp pump, SPD-M10Avp diode array detector, an SCL-10Avp controller and a DGU-14A degassing unit. The separation was performed with a Supelcolsil LC-18-T column (4.6 mm × 150 mm, 3 μm, Supelco).

Reagents and Standards

Nucleotides (adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate and uridine 5′-monophosphate) were obtained from Sigma Chemical Co (Steinheim, Germany). Tetrabutylamonium phosphate, potassium dihydrogenphosphate were purchased from Fluka (Sigma Aldrich, Steinheim, Germany).

Perchloric acid 65% and *ortho*-phosphoric acid 85% were purchased from Carlo Erba Reagents (Rodano, Italy). Potassium hydroxide was obtained from Kemika (Zagreb, Croatia) and methanol was Merck (Darmstadt, Germany) "gradient grade for chromatography." Water used for chromatography possessed a resistance greater than 15 MΩ.

Chromatography

Standard and sample solutions were analyzed by HPLC under the following conditions: stationary phase: HPLC-column Supelcolsil LC-18-T, 3 μm, 150 × 4.6 mm; flow rate: 1,0 mL/min; oven temperature: 25°C; photodiode array detector: detection wavelength 260 nm for 5′-AMP, 5′-CMP, 5′-IMP, 5′-UMP and 278 nm for 5′-GMP; spectrum acquisition range between 200 and 300 nm; injection volume: 20 μL; run time: 30 min; mobile phase: 13.6100 g potassium dihydrogen phosphate and 1.358 g tetrabutylammonium dihydrogen phosphate were dissolved in 1000 mL distilled water and the pH was adjusted to 6.0 with 5 mol/L potassium hydroxide. Prior to use, the mobile phase was filtered and degassed using a filtration apparatus with a 0.45 μm nylon filter membrane. The 5′-mononucleotides were identified by comparing retention times and photodiode array spectra for standards and samples. Purity of each peak is checked so as to exclude any contribution from interfering peaks. Quantification is then carried out by comparing the areas of the corresponding peaks.[25]

At the end of each working day, the whole chromatographic system was rinsed with water – methanol 80:20 (v/v) for 45 min.

Preparation of the Standard and Sample Solutions

Stock solutions of the standards (adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate and uridine 5′-monophosphate) were prepared in water at a concentration of 1 mg/mL. Further dilutions were made in the range of 5 μL/mL, 4 μL/mL, 3 μL/mL, 2 μL/mL, and 1 μL/mL, respectively, followed by filtration through a 0.45 μm membrane syringe filter. Multilevel calibration was performed using linear least-squares regression, with quantification of nucleotides estimated by the external standard technique. Sample solutions were prepared just prior to analysis. Five grams of powdered product were dissolved in approximately 35 mL water at 50°C, then cooled to room temperature and diluted to 50 mL with water in a volumetric flask. Ready-to-feed formulae (RTF) was homogenised by shaking prior to analysis. Fifteen millilitres of dissolved powder solution or RTF formula was transferred to a centrifuge tube and diluted

with exactly 15 millilitres of 13% (v/v) perchloric acid. The mixture was stirred for 15 min and then centrifuged at 4000 g for 20 min. Twenty five millilitres of the supernatant was introduced into a beaker and the pH was adjusted to about 6.0 with 5 M KOH. Then, the sample was diluted to 50 mL with water and left in an ice bath for at least 1 hour to precipitate all the potassium perchlorate. A few millilitres of the supernatant were filtered through a 0.45 μm membrane filter before analysis.

Validation of the Method

Method validation is an important part of analytical chemistry to confirm that an analytical method is acceptable for its intended purpose.[26] Evaluation of the HPLC method was based on analytical validation parameters such as selectivity, linearity, accuracy, precision, robustness, and system suitability.

RESULTS AND DISCUSSION

Validation of the Chromatographic Method

Selectivity

The ability of this method to separate the nucleotide peaks indicates the specificity of the method. Retention times and UV spectra of the reference substance were used to identify the peaks in the chromatograms.

Linearity

Linearity was studied using five solutions of each nucleotide in the concentration range of 1 to 5 mg/L ($n = 3$). The regression equation was found by plotting the peak area (y) versus the nucleotides concentration (x) expressed in mg/L. The correlation coefficients ($r = 0.999$) obtained in each case for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of nucleotides (Table 1).

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined by application of the analytical procedure to an analyte of known purity or by recovery studies, where a known amount of standard is

Table 1. Linearity of the HPLC method for the assay of nucleotides, peak area *vs.* concentration ($n = 5$)

Nucleotides	Concentration range (mg/L)	Equation for regression line	Correlation coefficient
adenosine 5′-monophosphate	1–5	Y = 24701x628.19	0.9990
cytidine 5′-monophosphate	1–5	Y = 19890x−2156.9	0.9991
guanosine 5′-monophosphate	1–5	Y = 31919x+1311.6	0.9991
inosine 5′-monophosphate	1–5	Y = 37134x−3353.1	0.9995
uridine 5′-monophosphate	1–5	Y = 20295x−4970.0	0.9992

Table 2. Recovery studies

	Applied concentration % of target (n = 3)			
Nucleotides	80	100	120	RSD (%)
5′-AMP	100.4	100.6	100.8	0.6
5′-CMP	100.4	100.7	100.9	0.7
5′-GMP	101.1	100.6	101.4	0.6
5′-IMP	100.5	100.9	101.0	0.4
5′-UMP	100.4	100.5	100.7	0.6

Table 3. Precision of the assay method

	Repeatability intra day ($n = 6$)		Intermediate precision ($n = 3$)			
			t_R(min) (%)RSD		RF (%)RSD	
Nucleotides	t_R (min) (%)RSD	Peak area (%)RSD	Day I	Day II	Day I	Day II
5′-AMP	0.77	0.95	0.63	0.61	1.18	1.21
5′-CMP	0.39	0.38	0.51	0.54	0.56	0.51
5′-GMP	1.62	0.39	1.19	1.17	1.14	1.11
5′-IMP	1.04	1.70	0.65	0.68	1.84	1.81
5′-UMP	0.62	1.70	0.72	0.75	2.30	2.28

Table 4. The robustness of the standard solutions during 30 hours

Nucleotides	t_R (min) %RSD	RF (%) RSD
5′-AMP	0.87	0.35
5′-CMP	0.39	0.90
5′-GMP	0.58	0.13
5′-IMP	0.85	0.65
5′-UMP	0.59	1.53

spiked in the sample. In this study, a number of different solutions were prepared with a known added amount of each nucleotide (80%, 100% and 120%) and injected in triplicate (n = 3). Percent recoveries of response factor (area and concentration) were calculated, as shown in Table 2, and it is observed that the method is accurate within the determined range.

Precision

The precision of the chromatographic method, reported as % RSD, was performed by measuring repeatability (intra-day assay precision) and intermediate precision (inter-day variation). Repeatability was evaluated by assaying six replicate samples, at the same concentration and during the same day. The intermediate precision was studied by comparing the assays on different days (2 days) using three solutions. The % RSD values, presented in Table 3, for the t_R(min) and peak area were found to be less than 2.3% in all cases; this illustrated the good precision of the analytical method. The precision was performed by plotting the nucleotide concentration versus peak area (RF).

Robustness

The stability of the standard solutions was determined. The stock solutions of five nucleotides were prepared in triplicate and left at room temperature during 30 hours. Each solution was analyzed every five hours. The effect on retention time and peak parameters was studied. The results presented in Table 4 show that the stock standard solutions are stable during 30 hours at room temperature and can be kept at least 30 h at 20°C.

CONCLUSIONS

A determination method of five 5′-nucleotides from infant formulae samples has been developed using HPLC. This analytical method showed

good selectivity, linearity, accuracy, precision, and robustness using infant formulae samples. This study suggested that the developed method can be successfully used in quality control for the determination of nucleotide content in infant formulae.

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