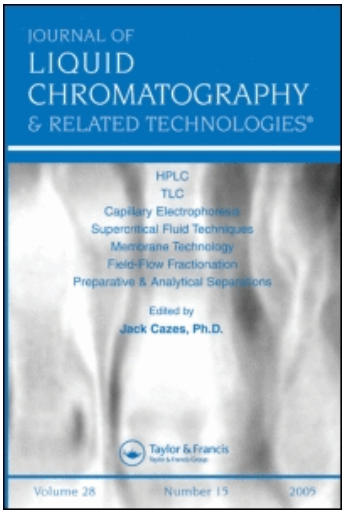

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Carla Oliveira[a]; Isabel M. P. L. V. O. Ferreira[a]; Eulália Mendes[a]; Margarida Ferreira[a]
[a] Faculdade de Farmácia da Universidade do Porto, Porto, Portugal

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DEVELOPMENT AND APPLICATION OF AN HPLC/DIODE ARRAY METHODOLOGY FOR DETERMINATION OF NUCLEOTIDES IN INFANT FORMULAE AND FOLLOW-UP MILKS

Carla Oliveira, Isabel M.P.L.V.O. Ferreira,
Eulália Mendes, Margarida Ferreira

CEQUP/Laboratório de Bromatologia
Faculdade de Farmácia da Universidade do Porto
Rua Aníbal Cunha, 164
4050 Porto, Portugal

ABSTRACT

This paper describes a procedure performed by high performance liquid chromatography/diode array detection for quantification of 4 nucleotides (cytidine 5-monophosphate, uridine 5-monophosphate, guanosine 5-monophosphate and adenosine 5-monophosphate) in infant formulae and follow-up milks. The sample preparation was simple and involved protein removal and filtration. The chromatographic separation was achieved using reverse-phase column C_{18} ($S_{10}ODS_2$). Isocratic elution was used, with 90% buffer A and 10% buffer B. Buffer A consisted of 5 mM tetrabutylammonium hydrogensulphate (TBAHS) and 20 mM potassium dihydrogenphosphate (KH_2PO_4) and buffer B of 5 mM TBAHS, 100 mM di-potassium hydrogen phosphate (K_2HPO_4), and 10% (v/v) acetonitrile. The pH of both solutions was adjusted to 5.2. The effluent was monitored using a diode array detector at 260 nm.

A linear relationship was found between peak area and concentration of nucleotides, over the concentration range of 2-30 mg/L for cytidine and uridine and of 4-30 mg/L for guanosine and adenosine.

Validation of the proposed method was carried out by the standard additions method. The recoveries ranged from 99-103%. The precision of the method was also evaluated and reported a CV% as less than 3%. Twelve samples of commercial brands were successfully monitored applying this methodology.

INTRODUCTION

Dietary nucleotides seem to influence several aspects of neonatal development[1-3] such as, modulate lipoprotein metabolism via their involvement in the synthesis of phospholipids, modify the composition of the intestinal microflora, improve gastrointestinal tract repair after damage, and participate in the immunity response mediated by T cells. Moreover, rapidly growing tissues such as the intestinal epithelium and lymphoid cells lack significant capacity for "de novo" synthesis of nucleotides and require exogenous sources of these compounds.

Nucleic acids, as well as free nucleotides, are found in human milk in significant amounts,[4] whereas in cow's milk these are present at lower amounts. Thus, the different nucleotide patterns occurring in human milk and cow's milk justifies the recommendation of the Committee for Food of the European Community[5] considering nucleotide supplementation of adapted milk formulae.

The need to maintain a close quality control of this supplementation practice, as established by the SCF,[5] warrants the development of a rapid and accurate analytical procedure to estimate cytidine 5-monophosphate (CMP), uridine 5-monophosphate (UMP), guanosine 5-monophosphate (GMP), and adenosine 5-monophosphate (AMP), in infant formulae and follow-up milks.

The analysis of milk nucleotides has usually been carried out using ion-exchange column chromatography[4-6] or enzymatic methods[4-6] in procedures that are not easily applicable to the large number of samples required for routine analysis. The research in this field has been greatly accelerated by the use of reverse-phase high performance liquid chromatography.[7]

With respect to the detection method, different detectors can be used, including fluorescence detection, MS detection, and UV detection.[8] Owing to the large number of conjugated double bonds in their nitrogen bases all nucleotides show absorption maximum in the near-ultraviolet range. For these

compounds the respective spectrum is a useful tool to determine peak purity. Because milk is a complex matrix, UV detection using the Diode Array detector was chosen. Simple sample preparation, rapidity, accuracy, and a lesser chance of detection error are advantages of the proposed method.

EXPERIMENTAL

Apparatus

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with two type PU-980 pumps, a type MD-910 diode array detector (DAD), and a type AS-950 auto-sampler.

The chromatographic separation was achieved with a Spherisorb $S_{10}ODS_2$ Chromatographic column, 10μm.

Reagents and Standards

Nucleotides were obtained from Sigma Chemical Co. Tetrabutyl-ammonium, potassium dihydrogenphosphate, and di-potassium hydrogen-phosphate were obtained from Merck. Acetonitrile was Merck "gradient grade." Water used for chromatography possessed a resistance greater than 15 MΩ, was filtered through a membrane of 0.45 μm porosity and was, subsequently, degassed.

Sampling

Twelve samples were assayed, which included 6 infant formula samples based on cow´s milk proteins, 3 follow-up milks, 2 infant formulae lactose-free, 1 infant formulae based on whey protein hydrolyzate, commercially available manufactured by the principal companies (Milupa, Mead-Jonhson, Nutricia, Nestlé, Wyeth).

Sample Preparation

After homogenization, 5.0 g infant formula were dissolved in 20 mL of distilled water. Three millilitres of the infant formula solution were added to 2 mL of perchloric acid solution 0.33 M, and after centrifugation (5x1000 rpm) for

10 minutes, 3 mL of supernatant were neutralized with 200µL of potassium carbonate (1.2 M). This solution was then centrifuged (5x1000rpm) for 10 minutes. If necessary the samples were filtered though W42 paper and thereafter, through 0.2 µm filter paper and 20µL were analyzed by HPLC.

Chromatography

The HPLC elution required a mixture of two solvents. Solvent A consisted of 5 mM tetrabutylammonium hydrogensulphate (TBAHS) and 20 mM potassium dihydrogenphosphate (KH_2PO_4) and solvent B of 5 mM TBAHS, 100 mM di-potassium hydrogenphosphate (K_2HPO_4) and 10% (v/v) acetonitrile. The pH of both solutions was adjusted to 5.2. The two solvents were filtered and degassed before use.

Isocratic elution was carried out but in order to reduce separation time a flow gradient was applied as follows: from 0 to 20 minutes the flow rate was maintained at 0.7 mL/min, between 21 and 25 minutes the flow rate was increased and maintained at 1.5 mL/min, at 26 minutes the flow rate dropped back to 0.7 mL/min and was subsequently increased gradually to 1.5 mL/min up to 37 minutes, thereafter it was maintained at 1.5 mL/min up to 44 minutes. At 45 minutes the flow rate returned to the initial experimental conditions.

Nucleotides concentration was determined at 260 nm, which is the maximum wavelength on the spectrum. Figure 1 shows a typical chromatogram for separation of the four nucleotides. Nucleotide peaks were identified by comparison of the retention time and by their spectrum.

RESULTS

Analytical Curve and Detection Limit

Under the assay conditions described, a linear relationship between the concentration of different types of nucleotides and the UV absorbance at 260 nm was obtained.

This linearity was maintained over the concentration range of 0.2-30 mg/L for CMP and UMP and of 1-30 mg/L for GMP and AMP. The correlation coefficient for each standard curve invariably exceeded 0.99 for all the nucleotides.

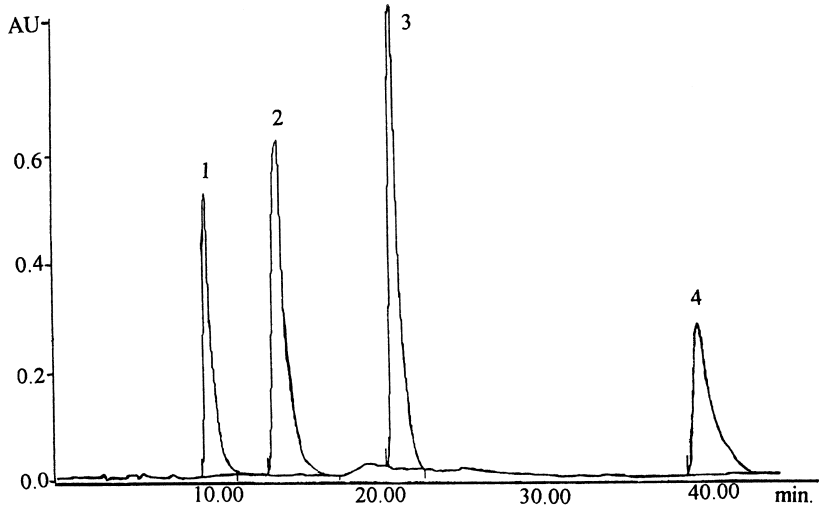

Figure 1. Typical Chromatogram for separation of four nucleotides (chromatographic conditions described in the text): 1 – CMP (RT 9.35), 2 – UMP (RT 14.11), 3 – GMP (RT 21.03), 4 – AMP (RT 39.54). The concentration of each nucleotide injected onto the column was 15 mg/L.

Validity of the Method

To demonstrate the effectiveness of the extraction and the accuracy of the proposed method, a sample of infant formula was analyzed before and after the addition of known amounts of CMP, UMP, GMP, and AMP. The mean recovery values ranged between 99 and 103 % with a mean value of 101 ± 2.8%. These results confirmed no interference effects due to the complex matrix composition. The precision of the method was also evaluated and reported a CV% as less than 3% (n=5). The detection limits were 0.2 mg/L for CMP, UMP, and GMP and 0.5 mg/L for AMP.

Analysis of Infant Formula and Follow-up Milk Samples

The results of duplicate determinations for a variety of commercial infant formulae and follow-up milks are presented in Table 1.

The results obtained showed that just one of the twelve samples analyzed presented the four nucleotides under study. A further six samples showed only traces of cytidine and no nucleotides were detected in the remaining infant formulae.

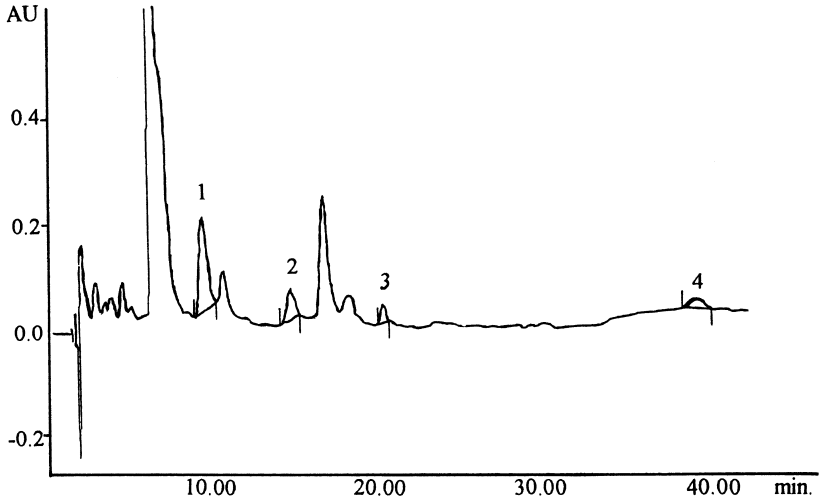

Figure 2. Typical chromatogram for an infant formulae (chromatographic conditions described in the text). The numbers correspond to the numbers in Fig. 1 with respect to peak identification.

Table 1

Contents of CMP, UMP, GMP, and AMP of 12 Infant Formulae and Follow-up Milks

Samples	CMP (mg/100g)	UMP (mg/100g)	GMP (mg/100g)	AMP (mg/100g)
1	Traces	N.D.	N.D.	N.D.
2	Traces	N.D.	N.D.	N.D.
3	Traces	N.D.	N.D.	N.D.
4	Traces	N.D.	N.D.	N.D.
5	N.D.	N.D.	N.D.	N.D.
6	N.D.	N.D.	N.D.	N.D.
7	N.D.	N.D.	N.D.	N.D.
8	8.11±0.20	1.42±0.11	Traces	Traces
9	Traces	N.D.	N.D.	N.D.
10	N.D.	N.D.	N.D.	N.D.
11	N.D.	N.D.	N.D.	N.D.
12	Traces	N.D.	N.D.	N.D.

N.D. - Not detected.

Figure 2 shows the typical chromatogram for an infant formula which presents the nucleotides CMP,UMP,GMP, and AMP. The concentrations of CMP and UMP were 8.1 mg/100g and 1.4 mg/100g respectively; GMP and AMP were presented in trace amounts.

Identification of the nucleotides in sample extracts was achieved through spectral equivalence against standards, calculated by the peak purity analysis program. The unidentified peaks (Fig.2) had different UV spectra when recorded with a diode-array detector; because infant formulae and follow-up milks are complex matrices the presence of other compounds that are extracted together with the nucleotides and that appeared on the chromatograms is understandable but their presence did not interfere with the evaluation of the nucleotides assayed.

CONCLUSIONS

The chromatographic gradient was optimized to achieve chromatograms with no interfering components on the retention times of the nucleotides under study.

Simple sample preparation, precision, accuracy, peak purity analysis, and less chance of detection error were advantages of this method. The use of a diode array detector was crucial to achieve these results.

In conclusion, this study suggests that the technique herein presented is quite useful for the routine analysis of nucleotide supplementation in infant formulae and follow-up milks. For all the infant formulae and follow-up milks analyzed the levels of nucleotides were below the maximum limits allowed for supplementation.

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