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Determination of nucleotides in infant milk formulas using novel dendrimer ion-exchangers

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ABSTRACT

The main aim of the present study was to develop a method for the separation of 5′-monophosphate nucleotides with the use of ion chromatography. Novel dendrimeric stationary phases were used for this purpose. The effects exerted by the type of anion-exchanger support (silica or polymeric) and the number of stationary phase layers on nucleotide retention were studied. A silica-based dendrimeric anion-exchanger was most suitable for analyzing the studied compounds. An increase in the number of layers enhanced nucleotide retention inside the column. The separation efficiency of the studied compounds was tested at various concentrations of the mobile phase buffer. At higher phosphate buffer concentrations, nucleotide resolutions were achieved in 6 min. Three commercially available infant milk formulas were analyzed to verify the applicability of the studied method. Solid phase extraction was used for sample cleanup and concentration. The limit of quantification of nucleotides was 0.40 μg/ml, and the method was linear in the concentration range of 0.40–20.6 μg ml^{-1}.

1. Introduction

Nucleotides are monomers of nucleic acids. They consist of three principal elements: a nitrogen-containing base (purine or pyrimidine), sugar (pentose) and one or more phosphate groups. These compounds participate in the transfer of genetic information. Nucleotides also play a key role in phospholipid and protein synthesis and in carbohydrate conversion. They participate in signal transduction pathways. Nucleotide separation and identification is particularly important in analyses of biological fluids, including in studies of energy changes inside a cell. The discussed compounds are also present in cerebrospinal fluid and human milk.

Nucleotides are commonly found in food and herbal products. They are used in the production of bakery goods and powdered foods. Nucleotides impart intense aroma and flavor, they improve the organoleptic properties of meat and mushrooms and mitigate undesirable flavors [1]. Dietary nucleotides contribute to normal growth and development in infants, and they play an increasingly important role in infant nutrition. Rapidly growing infants need nucleotides, and these compounds are used as dietary nutrients in milk and infant formulas [2,3]. Nucleotides are also essential for the proper functioning of the immune system [2,3]. They exert beneficial effects by modifying the composition of intestinal microflora and enhancing lipoprotein metabolism [4,5].

The first attempts to separate nucleotides and their derivatives involved thin layer chromatography methods [4,5]. At present, those techniques are used only for preliminary analysis. Due to the presence of electric charges between nucleotide molecules, their structure can be analyzed by electromigration methods [5]. Capillary electrophoresis is commonly used to determine the nucleotide content of fish, and guinea pig tissues, blood plasma and various types of cells [5]. CE is a less popular technique than reversed-phase high-performance liquid chromatography (RP HPLC) [6,7]. RP-HPLC coupled with tandem mass spectrometry supports the separation of five 5′-monophosphates that are commonly added to milk and infant formulas [8]. The main weakness of RP HPLC in nucleotide analysis is insufficient resolution due to the presence of negatively charged phosphate groups. Ion-pair RP HPLC may be used to compensate for the above deficiency. In comparison with RP HPLC, the results delivered by IP RP HPLC are characterized by greater selectivity, resolution and efficiency due to the formation of ion-pairs in the chromatographic process [9]. IP RP HPLC is often used to determine the nucleotide content of dairy products, diet foods, alcohols, blood, cerebellar granule cells and leukemic cells [10–14].

Ion chromatography (IC) is the method of choice for nucleotide analysis on account on nucleotides' anionic character. The best

Abbreviations: IC, ion chromatography; UmPp, uridine 5′-monophosphate; AmP, adenosine 5′-monophosphate; CmP, cytidine 5′-monophosphate; GmP, guanosine 5′-monophosphate; S1L, silica-based stationary phase with one layer of anion exchanger; S2L, silica-based stationary phase with two layers of anion exchanger; S3L, silica-based stationary phase with three layers of anion exchanger; S4L, silica-based stationary phase with four layers of anion exchanger; P11L, polymer-based stationary phase with eleven layers of anion exchanger.

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results in terms of peak shape and resolution were reported for quaternary amine stationary phases [15]. IC has been used in analyses of nucleotide-sugar complexes, deoxribonucleotides and ribonucleotides [15,16]. The discussed method is sensitive, selective and it supports separation of nucleotides in a very short time [15,16].

The objective of this study was to propose a simple and selective method for nucleotide separation. Silica- and polymer-based dendrimeric packing materials differing in the number of reaction cycles (number of anion-exchange layers) were applied. Three mobile phase buffers (pH range of 2.5–4.0) were used and various buffer concentrations were tested. The optimized analytical method was validated and applied to determine the content of 5′-monophosphate nucleotides in an infant milk formula.

2. Experimental

2.1. Materials and reagents

Standards of uridine 5′-monophosphate disodium salt (UmP), adenosine 5′-monophosphate disodium salt (AmP), cytidine 5′-monophosphate (CmP) and guanosine 5′-monophosphate disodium salt hydrate (GmP) were purchased from Sigma–Aldrich (Gillingham, Dorset, UK). Nucleotide mixtures were prepared by dissolving stock solutions in water.

Various reagents were used to prepare infant milk samples. Hydrochloric acid solution (1 M) and sodium hydroxide solution (1 M) were purchased from POCh (Gliwice, Poland). Water was obtained using the Millpore Milli-Q water purification system (Billerica, USA).

Deionized water from the Milli-Q system (Millipore, El Passo, TX, USA) was used to prepare mobile phases for IC. Buffer solutions were prepared with the use of high purity substances: ammonium acetate (CH_3COONH_4), ammonium formate ($HCOONH_4$), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), orthophosphoric acid (H_3PO_4), acetic acid (CH_3COOH), formic acid (HCOOH) and sodium carbonate (Na_2CO_3) (Sigma–Aldrich, Gillingham, Dorset, UK).

2.2. Apparatus

The LC-10Avp (Shimadzu, Kyoto, Japan) high-performance liquid chromatography system equipped with a diode-array detector (Shimadzu, Kyoto, Japan), Rheodyne 7125 manual injection valve (Rheodyne, Berkeley, CA, USA) with 5 μl loop were used in the study. Data was collected and integrated in the CLASS-VP program. The Hettich Rotina 48 R centrifuge (11,000 × *g*) was supplied by Witko Sp. z o. o. (Łódź, Poland). The Visiprep 12-port solid phase extraction vacuum manifold was purchased from Supelco (Buchs, Switzerland).

2.3. Sample preparation procedure

Stock standard solutions containing 0.10 mg ml^{-1} of CmP, UmP, AmP, GmP were prepared in water and stored at 4 °C for three weeks. Standard working solutions were prepared by diluting the stock solution with water to the required concentration in the range of 0.1–33.0 μg/ml. 5 g of powdered infant formula was dissolved in 40 ml water at 60 °C. The solution was cooled to room temperature and diluted to 50 ml with redistilled water in a volumetric flask. 20 ml of the prepared formula was transferred to a centrifuge tube and 400 μl 1 M HCl was added. The solution was shaken and centrifuged at 11,000 × *g* for 20 min. 15 ml of the supernatant was transferred to a flask and diluted with 15 ml of water. pH was adjusted to 7.0 with 1 M NaOH. The solution was transferred to a 50 ml volumetric flask and diluted to the required volume with water.

The samples were cleaned by solid phase extraction (SPE). Several 500 mg Bakerbond Quaternary Amine N (J.T. Baker, Deventer, Holland) SPE columns were placed on a SPE vacuum manifold. In the first step of SPE procedure, the column was conditioned with 10 ml of water. 50 ml of the prepared sample was passed through the SPE column at the flow rate of maximum 1 ml min^{-1}. The columns were rinsed with 10 ml of water and air dried for 5 min. 5 ml of 0.05 M hydrochloric acid was to elute the nucleotides from the quaternary amine support. Eluate pH was adjusted to approximately 2.5 (mobile phase pH). 1 ml of the prepared sample was passed through a 0.2 um nylon membrane filter before IC analysis.

2.4. Chromatographic conditions

Three different mobile phases were used to elute the nucleotides in isocratic conditions: KH_2PO_4/H_3PO_4 (pH 2.5), $HCOONH_4$/HCOOH (pH 3.0) and CH_3COONH_4/CH_3COOH (pH 4.0). Buffer concentrations were in the range of 25–75 mM. Fresh buffer solution was prepared daily. The flow rate was 0.5 ml min^{-1}. Detection wavelength was set at λ = 254 nm.

Five chromatographic columns were used in the study. Four columns were silica-based stationary phases, and one column was prepared with the use of polymer support. All columns were prepared in our laboratory. The column preparation method has been described in detail in previous studies [17,18]. Polymeric packing materials were synthesized through the modification of 1,4-di(2-hydroxy-3-methacryloyloxypropoxy)phenol. The support was coated with polymeric layers formed by condensation polymerization of methylamine and 1,4-butanedioldiglycidyl ether. The same amine and diepoxide were used to prepare four silica-based stationary phases. The anion exchanger had dendrimeric structure. The characteristics of the applied packing materials are presented in Table 1 and Fig. 1. Stationary phases were packed into 150 mm × 2.1 mm I. D. peek columns. The packing procedure was

Table 1
The properties of prepared polymeric and silica adsorbents.

Stationary phase	Shortcut	Particle size (μm)	Specific surface area (m^2 g)	Percentage part of carbon (%)	Nitrogen content (%)
Silica support	S0L	5	219.25	–	–
Silica based, 1 layer	S1L	5	198.22	10.63	1.72
Silica based, 2 layers	S2L	5	179.31	12.67	1.78
Silica based, 3 layers	S3L	5	158.70	14.42	1.82
Silica based, 4 layers	S4L	5	142.97	16.45	1.88
Polymer support	P0L	5–10	190.00	–	–
Polymer based, 11 layers	P11L	5–10	20.30	–	1.295