# **ORIGINAL ARTICLE**

# A new analytical technique in capillary electrophoresis: studying the levels of nucleotides in human breastmilk

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## Summary

The effect of nucleotides in the newborn is a determinant in this first stage of life, and their correct level in breastmilk is vital. We have designed a new method for the assay of nucleotides in milk by capillary electrophoresis (CE) after acid hydrolysis. Breastmilk samples were collected from healthy mothers (ages, 25–35 years) of one month lactation, and stored at -20 °C. The duplicated samples were dissociated by acidic hydrolysis (HClO<sub>4</sub>) and the CE assay was performed in an uncoated fused-silica capillary using an alkaline (borate) electrophoretic separation system.

The method gave good recoveries of 5'-mononucleotides. Under the conditions used, the actual CE analysis time was less than 20 minutes. The physiologically and nutritionally important nucleotides were detected at concentrations of 387  $\mu$ g/100ml for UMP-5P, 385.3  $\mu$ g/100ml for AMP-5P, 67  $\mu$ g/100ml for CMP-5P, 172  $\mu$ g/100ml for TMP-5P and 315  $\mu$ g/100ml for GMP-5P. Nucleotides are a significant nutrient in infant growth, and capillary electrophoresis is a sensitive and efficient tool for the assay of nucleotides with a purine or pyrimidine base in breastmilk.

Keywords: nucleotides - capillary electrophoresis - breastmilk

# **INTRODUCTION**

Mother's milk is the best food for infants, and so knowledge of its composition would contribute to the understanding of infant nutrition. Approximately 15–25% of the total nitrogen

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content of human milk is in non-protein compounds, including the nucleotides (Atkinson et al. 1980).

Nucleotides are the primary units of the nucleic acids (RNA, DNA) which control the reproduction, growth, and metabolism of living systems. They consist of a cyclic nitrogencontaining base (purine or pyrimidine), a sugar (pentose), and one or more phosphate groups. There has been increasing interest in the nutritional aspect of dietary nucleotides. They may play an important role in human physiology, especially in infants. Dietary nucleotides seem to influence several aspects of neonatal development, such as modulating lipoprotein metabolism, modifying the composition of the gut's microflora, and participating in the immune response mediated by T-cells and sleep (Gil and Sanchez-Medina 1981, Barnes 1994, Carver 1994, Uauy and Gil 1994, Gil and Uauy 1995, Leach et al. 1995, Schlimne et al. 1997, Boza et al. 1998). Moreover, rapidly growing tissues such as the intestinal epithelium and lymphoid cells lack any significant capacity for *de novo* synthesis of nucleotides and require exogenous sources of the compounds (Oliveira et al. 1999). Neither should one forget the influence that uridine and adenosine have on the regulation of formula-fed infants' sleep (Cubero et al. 2006), as well as the hormonal regulation that they exert through their ingestion in milk in the first stages of life (Vasques-Garibay et al. 2006), in particular the regulation of insulin-like growth factors such as IGF-I and IGFBP-3.

Nucleotides	Authors	Samples	Analysis	Concentration
5'-UMP	Janas and Picciano 1982	Breastmilk	HPLC	321µg/100ml
	Sugawara et al. 1995	Breastmilk	HPLC	0.23 µmol/100ml
	Perrin et al. 2001	Starter formula	HPLC	63 mg/kg
5'-AMP	Janas and Picciano 1982	Breastmilk	HPLC	143 µg/100ml
	Sugawara et al. 1995	Breastmilk	HPLC	0.23 µmol/100ml
	Perrin et al. 2001	Starter formula	HPLC	21 mg/kg
5'-GMP	Janas and Picciano 1982	Breastmilk	HPLC	163 µg/100ml
	Sugawara et al. 1995	Breastmilk	HPLC	n.d
	Perrin et al. 2001	Starter formula	HPLC	11 mg/kg
5'-CMP	Janas and Picciano 1982	Breastmilk	HPLC	321 µg/100ml
	Sugawara et al. 1995	Breastmilk	HPLC	4.29 µmol/100ml
	Perrin et al. 2001	Starter formula	HPLC	108 mg/kg
5'-IMP	Janas and Picciano 1982	Breastmilk	HPLC	290 µg/100ml
	Sugawara et al. 1995	Breastmilk	HPLC	n.d.

Table 1. Values found in the literature on the analysis of nucleotides in milk

Uridine 5'monophosphate (5'-UMP); adenosine 5'monophosphate (5'-AMP); guanosine 5'monophosphate (5'-GMP); cytidine 5'monophosphate (5'-CMP); inosine 5'monophosphate (5'-IMP).

The nucleotide levels in milk have been analyzed by different authors principally using HPLC as the analytical technique. Janas and Picciano (1982) initiated the use of this technique for the analysis of nucleotides in human milk, for which Sugarawa et al. (1995) later quantified three nucleosides and six nucleotides. Finally, Perrin et al. (2001) quantified 5 nucleotides and 5 nucleosides in formula milk. These results are summarized in Table 1.

The last ten years have seen the development of the very effective technique of capillary electrophoresis (CE) for nucleotide assay (Uhrova et al. 1996, Adam et al. 1999, Hornik et al. 2007), although not specifically for human milk. The purpose of the present work was therefore to develop a reliable and fast method of nucleotide assay in human milk using acid hydrolysis and quantification by CE.

# MATERIALS AND METHODS

#### Equipment

The CE system used is a P/ACE MDQ Systems 5510 equipped with a diode array detector (Beckman Coulter, Inc., USA). The system can be

rapidly reconfigured from a flexible research platform to a tightly regulated routine use platform. Automated fractionation of a detected peak allows isolation of newly resolved compounds for external identification.

# Capillary cartridges

The capillaries are housed in user-assembled cartridges which are compatible with all current CE capillaries. For the present study of nucleotides, the CE separations were carried out in an uncoated-silica capillary (75  $\mu$ m i.d.  $\times$  375  $\mu$ m o.d.; Polymicro Technologies, LLC, USA) with an effective length of 20 cm.

# Detector modules

To allow for flexible method development and rugged routine use, the P/ACE MDQ's design makes it easy to interchange high-sensitivity diode array (DAD), UV/Vis, and laser-induced fluorescence (LIF) detection modules. An external detector adapter allows the capillary to be extended to additional detection systems.

# Software for the CE analysis

The 32 Karat<sup>TM</sup> software package specific to capillary electrophoresis includes mobility plot generation, advanced reports, and new 2D algorithms to couple mobility and spectral signatures for peak identification. All of this results in a fully integrated CE control and data analysis workstation.

The methods are defined and edited in table format. All functions for the system are handled in a single window, including programming of the buffer array for the automation of strategies for the development of methods, using filters such as scan range, wavelength maximum, and mobility.

#### Control and analysis

Peak identification using either time or mobility, coupled with spectral signature confirmation, creates powerful 2D peak identification schemes. Velocity-Calibrated Peak Area and CAESAR integration ensure reproducible quantification at low limits of detection.

#### Reagents

Adenosine 5'monophosphate, uridine 5'monophosphate, guanosine 5'monophosphate, thymidine 5'monophosphate, cytidine boric 5'monophosphate, acid, sodium and dodecylsulfate were purchased from Sigma-Aldrich, USA. All other chemicals were of analytical purity grade. Perchloric acid 60%, sodium hydroxide pellet and potassium hydroxide 85% pellets were purchased from Panreac, Spain.

All solutions were prepared using de-ionized water (*Milli-Q System*).

## Procedure

#### Preparation of stock solutions

The values reported in the literature indicated that the nucleotide concentrations in human milk would be in the range 0  $\mu$ g/ml to 9  $\mu$ g/ml.

Stock nucleotide solutions were therefore prepared in the following concentrations:  $10 \times 10^{-3}$ ,  $5 \times 10^{-3}$ ,  $1 \times 10^{-3}$ , and  $0.5 \times 10^{-3}$  mg/ml of adenosine 5-P, cytidine 5-P, guanosine 5-P, thymidine 5-P, and uridine 5-P.

# Extraction of nucleotides for breastmilk

We followed the technique of Perrin et al. (2001) with certain modifications. We started from milk samples of healthy women of at least 4 weeks lactation. Aliquots of 0.75 ml of each sample were hydrolysed with 0.75 ml of 13% perchloric acid, mixing for 45 min on a roller mixer. After centrifuging at 5000 g for 20 min at room temperature, the supernatant was collected, discarding the fatty halo.

The solution was then adjusted to neutral pH with 5M KOH, and left in an ice bath for 1 h for all the potassium perchlorate to precipitate. It was then filtered through a 0.45  $\mu$ m membrane filter (*Millex*, Millipore, USA) before assay.

# CE analysis

All experiments were performed on a P/ACE System 5510 (Beckman Coulter, Inc., USA). The CE separations were carried out in an uncoatedsilica capillary (50  $\mu$ m i.d.  $\times$  375  $\mu$ m o.d.; Polymicro Technologies, LLC, USA) with an effective length of 20 cm. Detection was by ultraviolet over the range 190-300 nm (cartridge detection window 100  $\times$  800  $\mu$ m).

Samples were loaded by low-pressure injection (3.45 KPa) for 6 s (14.3 nl, 2.7% of the total capillary volume injected). Borate buffers were prepared from boric acid, sodium dodecyl sulfate (SDS) was added, and the solution was adjusted with 500 g/l NaOH to the appropriate pH. The capillary was washed at the beginning of each working day with de-ionized water, 0.1M sodium hydroxide, water, and finally with a separation buffer for 5 min.

Between runs, it was rinsed with water for 1 min and with a separation buffer for 2 min. The assays were run at constant voltage using a ramp of 1 min.

The alkaline (borate) separation system as described by Adam et al. (1999) was used as follows. The capillary was operated at 30 °C. The separation buffer was prepared from boric acid (60 mmol/l), sodium dodecylsulfate (80 mmol/l), and adjusted with 2-amino-2-methyl-1-propanol to neutral pH. Assays were run at +10 kV (positive outlet).

The detector's data rate was set at 4 Hz.



Fig. 1. **Typical electropherograms of the standard nucleotides and samples.** Capillary temperature, 30 °C; voltage, 10 kV; detection wavelength, 200 nm.

#### **RESULTS AND DISCUSSION**

Twenty human milk samples from lactating women were analyzed by this method. Each sample presented different dominant peaks.

The system provides electropherograms at 200 nm (the optimal wavelength for these samples) such as the example shown in Fig. 1. All five nucleotides were detectable at both the lowest and the greatest concentrations tested with the alkaline system used. At pH>9, however, the run-to-run reproducibility was lower (data not shown). Inter-day variation was less than 9.5%.

The stability of these nucleotides was tested in water at -20 °C, 4 °C, and room temperature. They were all stable at -20 °C (half-life 12 months).

The results of the breastmilk assays were as follows (see Fig. 2): 387  $\mu$ g/100ml for UMP-5P; 385.3  $\mu$ g/100ml for AMP-5P; 67  $\mu$ g/100ml for CMP-5P; 315  $\mu$ g/100ml for GMP-5P; and 172  $\mu$ g/100ml for TMP-5P. These mean values are significantly greater than the literature values quantified by HPLC by Janas and Picciano (1982) and Sugarawa et al. (1995), presumably reflecting

the greater sensitivity of the capillary electrophoresis technique for the assay of nucleotides in human milk.

#### CONCLUSION

A simple and effective analytical method has been developed for the routine electrophoretic determination of nucleotides in breastmilk in an uncoated fused-silica capillary. It benefits from the important advantages of capillary electrophoresis such as the small demand on sample size, simplicity of operation, low solvent consumption, and short analysis time.

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Fig. 2. Nucleotide levels in breastmilk (Arithmetic mean; X) quantified by capillary electrophoresis (CE), and their comparison with the literature levels determined by HPLC (Janas and Picciano 1982).

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