INFANT FORMULA AND ADULT NUTRITIONALS

Analysis of 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography: First Action 2011.20

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A method for the routine determination of 5′-mononucleotides (uridine 5′-monophosphate, inosine 5′-monophosphate, adenosine 5′-monophosphate, guanosine 5′-monophosphate, and cytidine 5′-monophosphate) in infant formula and adult nutritionals is described. After sample dissolution and addition of internal standard, potential interferences were removed by anion-exchange SPE followed by HPLC-UV analysis. Single-laboratory validation performance parameters include recovery (92–101%) and repeatability (1.0–2.3% RSD). The method was approved for Official First Action status by an AOAC expert review panel.

Nucleotides are compounds of critical importance to cellular function, and although not essential dietary nutrients, it has been demonstrated that supplementation of pediatric formulas with nucleotides is of benefit in neonatal nutrition. The described method was developed to provide an accurate, rapid, and robust technique for the routine compliance testing of uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP) in infant formula and adult/pediatric nutritional formula, and was recently reported (1).

In September 2011, the method was reviewed by an AOAC expert review panel and, based on the published single-laboratory validation (SLV) data as compared with the standard method performance requirements (AOAC SMPR **2011.008**; 2) set by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN), it was approved for Official First Action status as AOAC *Official Method*^{SM} **2011.20**.

Submitted for publication February 8, 2012.
The method was approved by the Expert Review Panel on Infant Formula and Adult Nutritionals as First Action. *See* "Standards News," (2011) *Inside Laboratory Management*, September/October issue.
The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author.
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DOI: 10.5740/jaoacint.CS2011_20

AOAC Official Method 2011.20
5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula
Liquid Chromatography
First Action 2011

(Applicable to the determination of 5′-mononucleotides in infant formula and adult/pediatric nutritional formula.)

Caution: Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment, and follow good laboratory practices.

A. Principle

Sample is dissolved in high-salt solution to inhibit protein and fat interactions. The 5′-mononucleotides—uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-phosphate (CMP)—are separated from the sample matrix by strong-anion exchange solid-phase extraction (SPE), followed by chromatographic analysis using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique using thymidine 5′-monophosphate (TMP).

B. Apparatus

(**a**) *HPLC system*.—Equipped with pump, sample injector unit with a 50 µL injection loop, degasser unit, column oven, and photodiode array detector.

(**b**) *C_{18} column*.—Gemini C_{18}, 5 µm, 4.6 × 250 mm (Phenomenex, Torrance, CA).

(**c**) *Spectrophotometer*.—Capable of digital readout to 3 decimal places.

(**d**) *pH meter*.

(**e**) *Polypropylene centrifuge tubes*.—50 mL.

(**f**) *Disposable syringes*.—3 mL.

Table 2011.20A. UV absorbance maxima and extinction coefficients for 5′-mononucleotides

Nucleotide	λ_{max}, nm	$E_{1cm}^{1\%}$
Adenosine 5′-monophosphate	257	430.4
Cytidine 5′-monophosphate	280	398.0
Guanosine 5′-monophosphate	254	393.3
Inosine 5′-monophosphate	249	357.3
Uridine 5′-monophosphate	262	313.5
Thymidine 5′-monophosphate	267	288.5

(**g**) *Syringe filters*.—0.2 µm with cellulose acetate membranes.

(**h**) *SPE vacuum manifold*.

(**i**) *Chromabond SB polypropylene strong-anion exchange SPE cartridges.*—6 mL × 1000 mg (Macherey-Nagel, Düren, Germany).

(**j**) *Filter membranes.*—0.45 µm nylon.

C. Reagents

(**a**) *Standards.*—Should be ≥99% pure (Sigma or equivalent). Nucleotide sodium salts or sodium salt hydrates may be substituted if free acid forms are not readily available.

(*1*) *TMP.*—CAS No. 365-07-1.
(*2*) *AMP.*—CAS No. 61-19-8.
(*3*) *CMP.*—CAS No. 63-37-6.
(*4*) *GMP.*—CAS No. 85-32-5.
(*5*) *IMP.*—CAS No. 131-99-7.
(*6*) *UMP.*—CAS No. 58-97-9.

(**b**) *Potassium bromide*.
(**c**) *Potassium dihydrogen phosphate*.
(**d**) *Orthophosphoric acid*.
(**e**) *Potassium hydroxide*.
(**f**) *Ethylenediaminetetraacetic acid*.
(**g**) *Sodium chloride*.
(**h**) *Methanol*.
(**i**) *Water.*—Purified with resistivity ≥18 MΩ.

D. Reagent Preparation

(**a**) *Standardizing buffer (KH_2PO_4, 0.25 M, pH 3.5).*—Dissolve 34.02 g KH_2PO_4 in 900 mL water and adjust pH to 3.5 with orthophosphoric acid. Dilute to 1 L.

(**b**) *Extraction solution (NaCl, 1 M:EDTA, 5 mM).*—Dissolve 58.5 g NaCl and 1.46 g EDTA. Dilute in 1 L water.

Table 2011.20B. Nominal concentration of calibration solutions

Calibration solution	AMP, CMP, GMP, IMP, UMP, µg/mL	TMP, µg/mL
1	0.4	3.2
2	0.8	3.2
3	3.2	3.2
4	8	3.2

(**c**) *Wash solution (KBr, 0.3 M).*—Dissolve 3.57 g KBr in 100 mL water.

(**d**) *Eluent (KH_2PO_4, 0.5 M, pH 3.0).*—Dissolve 6.805 g KH_2PO_4 in 90 mL water and adjust pH to 3.0 with orthophosphoric acid. Dilute to 100 mL.

(**e**) *Mobile phase A (KH_2PO_4, 10 mM, pH 5.6).*—Dissolve 1.36 g KH_2PO_4 in 900 mL water and adjust pH to 5.6 with KOH solution (10%, w/v). Dilute to 1 L with water. Make daily as microbial growth often occurs at room temperature in phosphate buffers that contain little or no organic solvent.

(**f**) *Mobile phase B (100% methanol).*

E. Standard Preparation

See Table **2011.20A** for the UV absorbance maxima and extinction coefficients for 5′-mononucleotides.

(**a**) *Stock standards (~1 mg/mL).*—(*1*) Accurately weigh approximately 50 mg each nucleotide into separate 50 mL volumetric flasks. (*2*) Add 40 mL water, mix until dissolved, and fill to volume with water.

(**b**) *Purity standards*.—Pipet 1.0 mL each stock standard into separate 50 mL volumetric flasks, make to volume with standardizing buffer (KH_2PO_4, 0.25 M, pH 3.5), and measure absorbance at the appropriate λ_{max} to determine the concentration of each nucleotide stock standard.

(**c**) *Internal standard solution (~80 µg/mL).*—Dilute 4 mL TMP stock standard into 50 mL water.

(**d**) *Working standard solution (~40 µg/mL).*—Pipet 2 mL each stock standard (AMP, CMP, GMP, IMP, and UMP) into a single 50 mL volumetric flask and make to volume with water.

(**e**) *Calibration standard solutions*.—*See* Table **2011.20B** for nucleotide concentrations of the calibration standard solutions.

(*1*) *Calibration solution 1.*—Pipet 0.25 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

(*2*) *Calibration solution 2.*—Pipet 0.5 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

(*3*) *Calibration solution 3.*—Pipet 2 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

(*4*) *Calibration solution 4.*—Pipet 5 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

F. Sample Preparation

(**a**) Accurately weigh approximately 1 g powder, or 10 mL liquid milk or ready-to-feed infant/nutritional formula, into a 50 mL centrifuge tube.

(**b**) Dissolve in 30 mL extraction solution (NaCl, 1 M:EDTA 5 mM).

(**c**) Add 1.0 mL TMP intermediate standard (~80 µg/mL).

(**d**) Cap the tube and vortex mix.

(**e**) Allow sample to stand for 10 min to ensure complete hydration.

(**f**) Dilute to a final volume of 50 mL with water.

(**g**) Cap the tube and vortex mix.

G. Extraction

Throughout the extraction procedure, do not let the cartridge run dry but drain to the top of the cartridge bed only. When draining the cartridge the flow rate should be <2 mL/min.

Table 2011.20C. Gradient procedure for chromatographic separation

Time, min	Flow rate, mL/min	Phase A, %	Phase B, %
0	0.6	100	0
25	0.6	80	20
26	0.6	100	0
40	0.6	100	0

(**a**) For each sample, place a single SPE cartridge on a vacuum manifold.

(**b**) Condition the columns by elution with 4 mL methanol followed by elution with 2 × 5 mL water.

(**c**) Load the cartridge with 4 mL sample solution.

(**d**) Wash the cartridge with KBr (0.3 M, 4 mL) to remove interferences.

(**e**) Elute the nucleotides with SPE eluent solution (KH_2PO_4, 0.5 M, pH 3.0, 4 mL) into a test tube.

(**f**) Filter an aliquot (~2 mL) of the eluent through a 0.2 µm syringe filter into an autosampler vial.

H. Chromatography

(**a**) Form gradients by low pressure mixing of the two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in Table **2011.20C**.

(**b**) Acquire spectral data between 210 and 300 nm by the photodiode array detector with chromatograms monitored at the specified wavelengths below for quantitation.

(*1*) IMP wavelength at 250 nm.

(*2*) AMP, GMP, and TMP wavelengths at 260 nm.

(*3*) CMP and UMP wavelengths at 270 nm.

(**c**) Set column oven to 40°C.

I. Calculations

(**a**) Percentage purity of each nucleotide (as free acid) in purity standard:

$$\text{Purity, \%} = \frac{Abs_{\lambda max}}{E_{1cm}^{1\%}} \times \frac{50}{wtSS} \times \frac{50}{1}$$

where $Abs_{\lambda max}$ = UV absorbance at maximum wavelength; $E_{1cm}^{1\%}$ = extinction coefficient for nucleotide; *wtSS* = weight of nucleotide in stock standard (g); 50 = total volume of stock standard (mL); 50 = total volume of purity standard (mL); 1 = volume of stock standard added to purity standard (mL).

(**b**) Concentration of nucleotide in stock standards:

$$\text{Stock standard, µg/mL} = \frac{wtSS}{50} \times \frac{P\%}{100} \times 10^6$$

where *wtSS* = weight of nucleotide in stock standard (g); 50 = total volume of stock standard (mL); 10^6 = concentration conversion (g/mL to µg/mL); *P%* = purity (%); 100 = mass conversion from % to decimal.

(**c**) Concentration of TMP in internal standard:

$$\text{Internal standard, µg/mL} = SS \times \frac{4}{50}$$

where *SS* = concentration of nucleotide in stock standard (µg/mL); 4 = volume of stock standard in internal standard (mL); 50 = total volume of internal standard (mL).

(**d**) Concentration of nucleotide in calibration standards:

$$\text{Calibration standard, µg/mL} = SS \times \frac{2}{50} \times \frac{VS}{25}$$

where *SS* = concentration of nucleotide in stock standard (µg/mL); 2 = volume of stock standard in working standard (mL); 50 = total volume of working standard (mL); *VS* = volume of working standard in calibration standard (mL); 25 = total volume of calibration standard (mL).

(**e**) Concentration of TMP in calibration standards:

$$\text{Calibration standard, µg/mL} = IS \times \frac{1}{25}$$

where *IS* = concentration of TMP in internal standard (µg/mL); 1 = volume of working standard in calibration standard (mL); 25 = total volume of calibration standard (mL).

(**f**) Determine the linear regression curve for the ratio of peaks areas (nucleotide/TMP; *y*-axis) vs the ratio of concentrations (nucleotide/TMP; *x*-axis) for calibration standards and calculate the slope with the *y*-intercept forced through 0.

(**g**) Interpolate the nucleotide contents in unknown samples from this calibration curve.

For powders:

$$\text{Nucleotide, mg/hg} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{W_S} \times \frac{100}{1000}$$

For ready-to feed liquids:

$$\text{Nucleotide, mg/dL} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{V_S} \times \frac{100}{1000}$$

where A_{NT} = nucleotide peak area in sample; A_{IS} = TMP peak area in sample; L = linear regression slope of calibration curve; C_{IS} = concentration of internal standard added to sample (µg/mL); V_{IS} = volume of internal standard added to sample (mL); W_S = weight of sample (g); 100 = mass conversion of result (g to hg); 1000 = mass conversion of result (µg to mg); V_S = volume of sample (mL); 100 = volume conversion of result (mL to dL).

J. Data Handling

Report results in mg/hg to 1 decimal place.

Reference: *J. AOAC Int.* **95**, 599(2012)

Results and Discussion

An SLV of the method previously published (1) indicated that this method is suitable for the routine determination of the 5′-mononucleotide content in milk and milk-based pediatric and adult nutritional products. The validation parameters investigated included linearity and working range, method detection limit, accuracy as recovery and bias, precision as repeatability and intermediate precision, and robustness. Linearity was demonstrated for all five nucleotides with correlation coefficients of >0.9999, and a visual inspection of residual plots. The method detection limits for individual nucleotides ranged from 0.06 to 0.19 mg/kg. The working range for individual nucleotides evaluated was from 0.06 to 17.4 mg/kg. Accuracy was determined as recovery, with values measured from 92 to 101%, within the suggested AOAC limits of 80−115% at the 10 ppm level (3), and no bias was found (*P* values all >0.05) when compared with a

previously published method (4). Precision as repeatability was estimated as 1.0–2.3 %RSD with a range for HorRat of 0.3–0.5 and for intermediate precision of 3.8–8.6 RSD%. A Plackett−Burman robustness study (5) was performed and the seven factors evaluated were shown not to affect the final results within typical experimental variations.

The method was applied to the analysis of a number of commercially available pediatric and nutritional powders. The products used for sampling included infant formula, follow-on formulas, and an adult nutritional product. The range of sources for these products included bovine milk, hydrolyzed milk protein, caprine milk, and soy protein. The method was found to be suitable for use with these various product matrixes.

It is recommended that this method be further examined against a set of infant formula and adult nutritional matrixes developed for this purpose by the SPIFAN community, and its performance evaluated against the SMPRs established by SPIFAN.

References

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