Determination of Folic Acid in Fortified Cereals by High Performance Liquid Chromatography with Diode Array Detection

Hurtado AAH¹, Rocha NCC¹, Torres NWD¹ and Torres LRN^{1*}

¹Universidad Autonoma de Nuevo Leon, Facultad de Medicina, Departamento de Química Analítica, Av. Madero y Dr. Eduardo Aguirre Pequeño S/N Mitras Centro. CP 64460, Mexico

Article Information

Received date: Feb 29, 2016

Accepted date: Apr 11, 2016

Published date: Apr 12, 2016

*Corresponding author

Naccha-Torres LR, Universidad Autónoma de Nuevo Leon, Facultad de Medicina, Departamento de Química Analítica, Mexico, Email: nocavazos@yahoo.com

Distributed under Creative Commons CC-BY 4.0

Keywords Folic acid; Analysis; Cereals; HPLC; Fortified cereals; Chromatography

Abstract

Cereal samples fortified with folic acid obtained in the metropolitan area of Monterrey were analyzed by a chromatographic method developed in our laboratory. The results were compared with the product's label nutritional information. All tested cereal samples matched the folate labeled. The chromatographic method applied is quick, simple, low cost and suitable for cereal folic acid determination.

Introduction

Folic acid is a compound belonging to the vitamin B complex. Its structural analogs, the folates, are the metabolically active compounds [1]. Folate deficiency is associated with problems in cell division and alterations of protein synthesis which play a very important role in Neural Tube Defects (NTD) as anencephaly, bifida spine, and encephalocele. These birth defects are public health problems of worldwide scope. In Nuevo León, México, these congenital malformations are the most common problem which contributes to mortality and morbidity in children [2]. Diverse strategies have been developed in several countries to reduce NTD defects, such as the oral administration of folic acid and by encouraging pregnant women to eat a balanced diet including vegetables and fortified cereals. These products are usually fortified with a very simple and stable form of the vitamin in order to achieve optimal nutritional levels [3]. Several reports confirm the beneficial effects of folic acid fortification to prevent the NTD [4,5].

Given the importance of folic acid in children and pregnant women, quality control of fortified products is necessary. The aim of this research was to determinate the levels of folic acid in fortified cereals consumed in the metropolitan area of Monterrey. Folic acid was determinate by a validated chromatographic method implemented in our laboratory. The results were compared with the product label information.

Material and Methods

Materials

Chemicals: Methanol and acetonitrile were HPLC grade from Fisher Scientific (Waltham, MA USA). Tetrabutylammonium phosphate (TBA) was Baker Analyzed HPLC grade (Fischer Scientific, Waltham, MA USA). Trifluoroacetic acid, α -amylase 250,000 U and folic acid standard were purchased from Sigma Aldrich. (St. Louis, MO, USA). Potassium phosphate dibasic (K_2HPO_4) and phosphoric acid (H_3PO_4), were from J.T. Baker (Fischer Scientific, Waltham, MA USA). Nylon filters 0.45 μ m (Waters, Milford MA, USA).

Samples: Fortified cereals samples were purchased from local markets. In addition, a virgin cereal sample provided by the Kellogg's Company was analyzed. This sample was stored in a hermetically sealed metal container to protect from moisture and light.

Methods

Pretreatment of the sample: Two g of finely ground samples were homogenized with 45ml of 0.1 M K_2HPO_4 , pH 8-9, and was stirred for 1 hour, then, the pH was adjusted to 7 and 2ml of α -amylase solution (25mg / ml) were added. The mixture was incubated at 65°C for 1 hour following by heating to 90°C for inactivates the enzyme. The whole digest was cooled to room temperature and transferred to volumetric flask to fill with phosphate buffer to 50ml. Before chromatographic analysis the solutions were filtered through a 0.45 μ m nylon membrane (Millipore).

Chromatographic analysis: Folic acid was analyzed in a Waters 2690 High Performance Liquid Chromatography with 996 diode array detector. A Sentry Universal Guard Column Holder 50 x75 mm was used. An Atlantis dC₁₈ column (150x4.6 mm with 5µm particle size (Waters) was used. The mobile phase was trifluoroacetic acid 0.1%: acetonitrile (85:15). The elution was isocratic with a flow rate of 1.4 ml/min. The injection volume was 10 µL. Detection was at 280 nm.

Validation: Stock solution of folic acid (50 µg mL⁻¹) was prepared by dissolving in phosphate buffer solution (pH 8-9). By diluting stock solution, standard solutions of folic acid were prepared in Milli-Q water over a range of 0.1 to 4 µg mL⁻¹. The method was validated considering both FDA and ICH guidelines [6,7], taking into account peak area. Linearity was established by calculating the coefficient of variation of the response factors (FR = area/concentration). Limit of Detection (LOD) was calculated as the minimum concentration that produces a response that can differentiate from the noise (ratio signal/noise = 3). The LOQ was calculated as the minimum concentration with suitable precision and accuracy. Precision was evaluated by analysis of five virgin samples fortified at three levels of concentration. Accuracy was calculated as the recovery of spiked samples at three levels of concentration [8].

Results and Discussion

Chromatographic analysis

A C₁₈ reverse phase column as a stationary phase and phosphate buffer and methanol as a mobile phase, with tetrabutylammonium phosphate (ionic-pair reagent), was used as a first chromatographic system. However, the use of ion pair reagent requires longer times for the column conditioning, therefore longer analysis times and a rapid saturation of the column. For these reasons this system was

discarded. A Waters Atlantis dC₁₈ column was used, since its ability to separate non-polar compounds as well as polar compounds, allowing the use of very aqueous mobile phases without ionic pair reagent [9]. Trifluoroacetic acid and acetonitrile were used as a mobile phase. Figure 1a shows a chromatogram of the folic acid standard at 1 µg mL⁻¹. The retention time was 2.99 minutes.

Method validation

The CV of the RF (response factor = concentration/area) was 9.82%. The detection limit and the quantification limit were 0.031 µg mL⁻¹ and 0.098 µg mL⁻¹ respectively. The linear range was 0.1 to 4.0 µg mL⁻¹, which allowed quantify folic acid levels in cereal samples. The results of precision and accuracy are shown in table 1 and table 2 respectively. The stability was tested in the standard as well as in the fortified samples. It was verified that the folic acid standard at 1 µg mL⁻¹, once prepared and stored in the dark at 4°C, was stable up to 74 days after preparation, however, the treated sample must be analyzed the same day as is processed.

Sample analysis

The virgin sample, free of folic acid, was provided by the Kellogg's Company. The analysis of the virgin sample confirmed the natural presence of folic acid. Therefore, to obtain the real amount of folic acid in the enriched samples it was necessary to subtract the amount of folic acid in the virgin sample.

In order to find the best sample preparation, we tried Ossey method [10,11]. However, the folic acid recoveries obtained were very low (29.81%). The Ossey method recommends cleaning the extracts with SAX cartridges [Waters Accell Plus QMA], though we filter the extracts performing a single filtration, the extracts were free of impurities and recovery was increased. In addition to the increase in the percentage of recovery and higher reproducibility, filtration has the advantage of reducing both time and cost. Even though the percentage of recovery was low, the method was precise, so it is acceptable. A correction factor was applied in the quantification of folic acid in the samples studied.

Analysis of commercial samples

Ten fortified cereal samples obtained from the metropolitan area of Monterrey were analyzed (Figure 1b). Results were compared with labeled values of the product (Table 3). Most cereal samples analyzed by our method reported higher concentration of folic acid than the

Table 1: Precision of the method (n=15).

Concentration	t _R CV	Area CV	Height CV
Low (0.2 µg mL ⁻¹)	0.06	1.50	4.77
Medium (1.0 µg mL ⁻¹)	0.03	0.67	2.03
High (3.2 µg mL ⁻¹)	0.03	2.03	4.83

Table 2: Accuracy of the method (n=15).

Concentration	Recovery (%)	CV
Low (0.2 µg mL ⁻¹)	62	1.9
Medium (1.2 µg mL ⁻¹)	60	1.0
High (3.2 µg mL ⁻¹)	62	3.0

Table 3: Folic acid levels in enriched cereal.

Product	Batch	Folic acid $\mu\text{g}/100\text{g}$	Folic acid in Nutritional Information $\mu\text{g}/100\text{g}$
Kellogg's Corn Flakes	B8	642.3	666.6
Kellogg's Corn Flakes	C8	764.1	666.6
Kellogg's Corn Flakes	A10	1174.6	666.6
Kellogg's Corn Flakes	C10	795.8	666.6
Kellogg's Corn Flakes	C12	975.0	666.6
Kellogg's Corn Flakes	B1	1040.0	666.6
Kellogg's Corn Flakes	C3	599.2	333.3
Hill Country Corn Flakes	J1	305.9	400.0
Nestlé Corn Flakes	3B	670.8	400.0
Quaker Corn Flakes	7MA	293.0	320.0

level marked in nutritional information. A possible explanation is that during the fortification larger amount of folic acid were added to ensure that the product met the label specification before product expiration date. Also, it must be considered that folic acid was detected naturally in the product.

Conclusion

Most cereal samples analyzed by our method met the concentration of folic acid marked in nutritional information. The stability test of the fortified samples indicates that the samples must be processed and analyzed the same day. The chromatographic method applied is simple, quick, precise, low cost and suitable for the determination of folic acid in different cereals.

Acknowledgement

We wish to thank CONACYT for its financial support in the form of a fellowship for A.A.H.H.

References

- Mathews C, Van Holde K, Appling D, Anthony-Cahill S, Bioquímica. Pearson Educación. 2013.
- Ramírez-Espitia JA, Benavides FG, Lacasaña-Navarro M, Martínez JM, García AM, Benach J. [Mortality from neural tube defects in Mexico, 1980-1997]. *Salud Publica Mex.* 2003; 45: 356-364.
- FOOD STANDARDS AGENCY. Folic Acid fortification. 2015.
- Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong LY. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA.* 2001; 285: 2981-2986.
- De Wals P, Tairou F, Van Allen MI, Uh SH, Lowry RB, Sibbald B, et al. Reduction in neural-tube defects after folic acid fortification in Canada. *N Engl J Med.* 2007; 357: 135-142.
- Gao L, Li J, Kasserra C, Song Q, Arjomand A, Hesk D, et al. Precision and accuracy in the quantitative analysis of biological samples by accelerator mass spectrometry: application in microdose absolute bioavailability studies. *Anal Chem.* 2011; 83: 5607-5616.
- ICH. Validation of Analytical Procedures: Text and Methodology Q2 (R1). 2015.
- Deconinck E, Crevits S, Baten P, Courselle P, De Beer J. A validated ultra high pressure liquid chromatographic method for the qualification and quantification of folic acid in pharmaceutical preparations. *Journal of Pharmacology and Biomedical Analysis.* 2011; 54: 995-1000.
- Cavazos-Rocha N, Carmona-Alvarado I, Vera-Cabrera L, Waksman-De-Torres N, Salazar-Cavazos M. HPLC Method for the Simultaneous Analysis of Fluoroquinolones and Oxazolidinones in Plasma. *Journal of Chromatographic Science.* 2014.
- Finglas PM, Wigertz K, Vahteristo L, Sue CW, Southon, Froidmont-Gortz ID. Standardization of HPLC techniques for the determination of naturally-occurring folates in food. *Food Chemistry.* 1999; 64: 245-255.
- Osseyi ES, Wehling RL, Albrecht JA. Liquid chromatographic method for determining added folic acid in fortified cereal products. *J Chromatogr A.* 1998; 826: 235-240.