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A Mass Spectrometric Validated High-Performance Liquid Chromatography Procedure for the Determination of Folates in Foods

ROBERT J. PAWLOSKY,* VINCENT P. FLANAGAN, AND ROBERT F. DOHERTY

Food Composition Laboratory, Beltsville Human Nutrition Research Center, ARS, USDA, Beltsville, Maryland 20705

A series of five food reference materials (RM) that had certified values of folate concentrations and five frozen food samples were analyzed for 5-methyltetrahydrofolic acid (5-MTHFA) and folic acid (FA) using a high-performance liquid chromatography (HPLC) method with fluorescence detection that was validated using an HPLC mass spectrometry (MS) method with electrospray ionization. Identical sample specimens were extracted and analyzed in triplicate using both instrumental methods, and a comparison was made of the mean values of 5-MTHFA and FA resulting from these determinations. The analytes were isolated on either a high capacity strong anion exchange solid phase extraction column (HPLC method) or a phenyl Bond Elut column (MS method) prior to analyses. For quantification of the analytes by MS, ^{13}C-labeled 5-MTHFA and FA were added to samples as internal standards prior to enzymatic digestion and conversion of the polyglutamate forms of 5-MTHFA to the monoglutamic acid. Quantification of FA and 5-MTHFA using the HPLC analysis was carried out using external standards. With the exception of one RM (pig liver), the values established for 5-MTHFA using these methods were highly comparable. In determining the variance associated with these two procedures, it was observed that the mean relative standard error for 5-MTHFA was 12 (range, 2−27%) and 11% (range, 5−25%) for the HPLC and MS methods, respectively. FA was detected in only three of the samples, and the values obtained for it by either method were similar. This is the first paper that describes a mass spectrometric method used in the validation of an HPLC determination of food folates across a wide range of sample matrixes. The comparable values for 5-MTHFA and FA suggest that HPLC analysis with fluorescent detection may be used to accurately quantify folates present in a variety of food matrixes.

KEYWORDS: Food analysis; 5-methyltetrahydrofolic acid; folic acid; mass spectrometry; HPLC; isotope dilution

INTRODUCTION

Folates belong to a group of water soluble vitamin cofactors (**Figure 1**) that are essential for the synthesis of purines and pyrimidines and in the production of methionine from homocysteine (*1*). They are required in the human diet, and an insufficiency of dietary folates has been implicated with several health-related illnesses (*1, 2*). Folic acid (FA) is a synthetic fortificant, which is added to many grain-based foods in the United States and is therefore a major source of folate in the American food supply (*3*). However, FA must undergo a two step reduction to tetrahydrofolate before it can be utilized in the biosynthesis of nucleotides and methionine. The current fortification level for grain-based foods in the U.S. is 140 μg of FA per 100 g of product (*4*).

Several analytical methods have been developed for the determination of folates in biological matrixes. High-performance liquid chromatography (HPLC) methods have been put forward with electrochemical detection, UV, and/or fluorescence detection to identify different forms of folates in foods (*5*−*16*). Recently, an HPLC technique with fluorescence detection was developed for the determination of FA and 5-methyltetrahydrofolic acid (5-MTHFA) in foods (*17*). The availability of $^{13}C_5$-labeled folates has facilitated the development of highly specific quantitative methods for the determination of folates using mass spectrometry (MS) detection (*18*−*20*).

We report here procedures for the determination of 5-MTHFA and FA in several foods and reference materials using an HPLC technique with fluorescence detection that was validated using a mass spectrometric assay employing stable isotope dilution HPLC/electrospray ionization (ESI)-MS analyses. Both 5-MTHFA and FA were quantified in the food products using separate analytical procedures, and the mean values from those determinations were compared.

* To whom correspondence should be addressed. Present address: Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcoholism and Alcohol Abuse, NIH, Room 114, 12420 Parklawn Dr., Rockville, MD 20851. Tel: 301-443-9029. Fax: 301-594-0035. E-mail: bpawl@mail.nih.gov.

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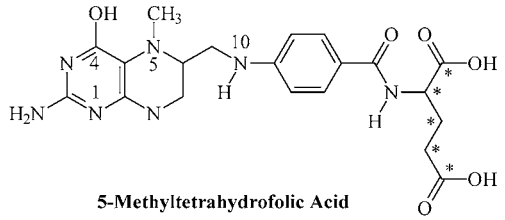
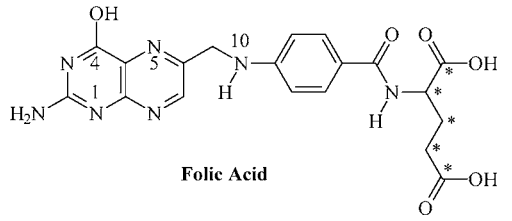


5-Methyltetrahydrofolic Acid



Folic Acid

Figure 1. Chemical structures of 5-MTHFA and FA. The vitamers consist of a pterine ring coupled to *p*-aminobenzoic acid that is linked to glutamic acid. The asterisks denote labeled positions on $^{13}C_5$ analogues that were used as internal standards.

MATERIALS AND METHODS

Both HPLC and liquid chromatography (LC)-MS procedures used here have been reported recently, and only brief descriptions of these methods are given here (*17*, *20*).

Chemicals and Reagents. FA, 5-MTHFA, lyophilized rat plasma, *Aspergillus oryzae* α-amylase, and *Streptomyces griseus* protease were obtained from Sigma (St. Louis, MO). The $^{13}C_5$-glutamyl-5-MTHFA and $^{13}C_5$-glutamyl-FA were obtained from Merck Eprova AG (Schaffhausen, CH). The ^{13}C atoms occupied the five carbons of the glutamic acid portion of each molecule. Standard stock solutions were prepared according to previously described procedures (*17*, *20*) and stored at −60 °C. For LC-MS analyses, internal standards were added in commensurate amounts to samples prior to enzymatic digestion and extraction. All solvents were HPLC grade and were obtained from commercial suppliers and used without further purification. Solid phase extraction (SPE) columns, 500 mg of strong anion exchanger (SAX) with a polystyrene-*p*-divinylbenzene matrix, and the HPLC column were obtained from Alltech Associates (Deerfield, IL). The Bond Elut Ph columns (100 mg) were obtained from Varian (Walnut Creek, CA).

Buffers and Solutions. The extraction buffer was 0.1 M potassium phosphate, pH 6.0, with 10 mM mercaptoethanol, 10 mM ascorbic acid, and 10 mg/L sodium azide. The SAX SPE elution buffer contained 0.1 M phosphate, pH 6.0, and 1 M sodium chloride containing 250 mL/L of acetonitrile. The amylase solution was prepared by dissolving 1 g of α-amylase (*A. oryzae*) in 25 mL of extraction buffer and filtering. The protease solution was prepared by dissolving 25 mg of protease (*S. griseus*) in 25 mL of extraction buffer and filtering. The conjugase solution was prepared by reconstituting lyophilized rat plasma with extraction buffer.

Food Samples and Reference Materials. BCR 121 whole meal flour, BCR 421 dried milk, BCR 485 freeze-dried mixed vegetables, and BCR 487 pig liver were obtained from the European Commission, Institute for Reference Materials and Measurement (Brussels, Belgium). SRM 1846 infant formula was obtained from the National Institute of Standards and Technology (Gaithersburg, MD). Samples of frozen spinach, broccoli, oranges, potatoes, and strawberries were prepared at the Food Science Laboratory of the Virginia Polytechnical Institute and State University (Blacksburg, VA).

Sample Extraction. The procedure for sample extraction has been described elsewhere (*17*). Briefly, a slurry of the sample (2 g) was prepared with 25 mL of extraction buffer. In the case of SRM 1846 infant formula, 2 g of material was suspended in 50 mL of hexane and stirred for 30 min to extract the fat from the sample. The suspension was filtered, and the solid phase was washed with additional hexane. It was then suspended in 25 mL of extraction buffer and treated as the other samples. Two hundred microliters of amylase solution was added, and the solution was degassed under a stream of nitrogen and incubated at 37 °C for 1 h. Two hundred microliters of protease solution was then added to the mixture under a blanket of nitrogen and incubated for 3 h at 37 °C. The mixture was then placed in a boiling water bath and heated to 95 °C for 15 min and cooled, and 50 μL of reconstituted rat plasma was added. The mixture was blanketed with nitrogen, capped, and incubated overnight at 37 °C. The mixture was again heated to 95 °C in a water bath for 15 min, cooled, and then centrifuged in a Beckmann J2 centrifuge (Mountain View, CA) at 40 000*g* for 20 min. The supernatant was applied to the solid phase column. For SRM 1846, the floating pellet was carefully removed and washed with extraction buffer, and the wash was combined with the supernatant.

SPE Procedures. *HPLC.* SAX SPE columns were prepared by swelling with extraction buffer and conditioned with the extraction buffer (15 mL). The supernatant was added and allowed to flow through by gravity. The column was washed with 15 mL of extraction buffer, and the folates were eluted with 6 mL of SPE elution buffer. Nitrogen was bubbled through the eluate for 30 min at 50 °C to remove acetonitrile, and the residue was made up to 5 mL with extraction buffer.

LC-MS. A 0.5 mL aliquot of the SAX SPE column extract of each sample was diluted to 4 mL with 0.03 mM dibasic potassium phosphate with 0.1% each ascorbic acid and mercaptoethanol adjusted to pH 3.5 with trifluoroacetic acid. The sample was loaded onto a 100 mg Bond Elut Ph column that had previously been washed with methanol (1 mL) and 0.03 mM phosphate buffer (1 mL). The column was then washed with 0.03 mM phosphate buffer (1 mL) and 0.1% formic acid (1 mL) to remove traces of salts. The analytes were eluted with 500 μL of ACN:H_2O:MeOH 26:60:14 + 0.1% formic acid. After the mixture was eluted, 200 μL of 0.1% formic acid was added.

Instrumental Conditions and Analyses. *HPLC Analysis.* An Agilent 1050 LC system equipped with a programmable fluorescence detector utilizing Chem Station software was used for HPLC analyses (Agilent Technologies, Wilmington, DE). The in-line photochemical reactor consisted of a 10 W short wavelength mercury lamp (D12) (Atlantic Ultraviolet Corp., Hauppage, NY) and was activated when FA eluted the column using an external programmable controller port of the HPLC. The fluorescence detector was set for excitation at 290 nm and emission at 355 nm for 5-MTHFA and changed to 230 nm excitation and 440 nm emission when the FA photolysis product eluted the column. Samples (100 μL) were injected onto a 15 cm × 4.6 mm column packed with Adsorbosphere C18 HS 3 μm. A flow rate of 1 mL/min was used, and the mobile phase program consisted of 5 min with 99% A (30 mM potassium phosphate, pH 2.2, with 10 mg/L sodium azide) and 1% B (acetonitrile, ACN) followed by a gradient of 20 min to 80% A, 20% B, followed by a step gradient to 60% A for 5 min.

LC-MS Analysis. An HP 1100 HPLC (Agilent Technologies) coupled to an ion trap mass spectrometer (ThermoQuest Corp., San Jose, CA) fitted with an ESI source was used for mass spectrometric analyses. Samples (40 μL) were injected onto a 150 mm × 4.6 mm Luna C-18 HPLC column (5 μm) (Phenomenex, Torrance, CA). The solvent system was a gradient of solvent A (0.1% formic acid in H_2O) and solvent B (ACN:H_2O:MeOH 26:60:14 + 0.1% formic acid). The gradient was applied as follows: isocratic, 0.175 mL min^{-1}, 30% B, 0−9 min; linear, 0.3 mL min^{-1}, 100% B, 9−14 min; isocratic, 0.3 mL min^{-1}, 100% B, 14−25 min; linear, 0.175 mL min^{-1}, 30% B, 25−30 min; isocratic, 0.175 mL min^{-1}, 30% B, 30−45 min. The ESI was operated in positive ion mode for 5-MTHFA using selective ion monitoring (at *m*/*z* 460.2 and 465.2 for ^{12}C-5-MTHFA and $^{13}C_5$-5-MTHFA, respectively). The ESI was switched to negative ion mode for FA using selective ion monitoring (at 440.2 and 445.2 for ^{12}C-FA and $^{13}C_5$-FA, respectively). The spray voltage was set to 4.5 kV, and the capillary temperature was adjusted to 200 °C. The sheath gas was set to 80% of its maximum flow rate.

Quantification of 5-MTHFA and FA. *HPLC.* 5-MTHFA and FA were quantified using external standards, which bracketed the expected concentrations of MTHF and FA in the samples. The calibration curves for both MTHF and FA for both fluorescence detections were linear from concentration values corresponding to 3.5−350 μg/100 g.

Table 1. Compendium of Determinations of the Concentrations of 5-MTHFA and FA from Five Food Reference Materials and Five Frozen Foods and HPLC Analysis with Fluorescent Detection and by Stable Isotope LC-MS Analyses

	5-MTHFA						FA					
	HPLC			LC-MS			HPLC			LC-MS		
	μg/100 g ($n = 3$)	SD	RSE	μg/100 g ($n = 3$)	SD	RSE	μg/100 g ($n = 3$)	SD	RSE	μg/100 g ($n = 3$)	SD	RSE
whole meal flour BCR 121	6.45	1.77	27%	3.82	0.95	25%	ND			ND		
nonfat dried milk BCR 421	14.87	1.08	7%	15.74	0.74	5%	66.95	2.61	4%	64.85	7.21	11%
mixed vegetables BCR 485	195.82	6.61	3%	197.7	6.65	5%	ND			0.2	0.07	34%
pig liver BCR 487	168.93	79.38	47%	410.54	81.28	20%	80.89	9.9	12%	70.81	12.1	17%
infant formula SRM 1846 (NIST)	3.11	0.24	8%	4.79	0.88	18%	88.02	7.08	8%	109.44	9.98	9%
frozen broccoli	27.6	0.57	2%	34.45	1.81	5%	ND			ND		
frozen oranges	20.46	1.79	9%	30.32	3.28	11%	ND			ND		
frozen potatoes	9.76	0.71	7%	5.25	0.85	16%	ND			ND		
frozen spinach	79.58	8.86	11%	88.69	6.65	8%	ND			0.58	0.03	5%
frozen strawberries	31.98	3.34	10%	27.87	0.87	3%	ND			ND		

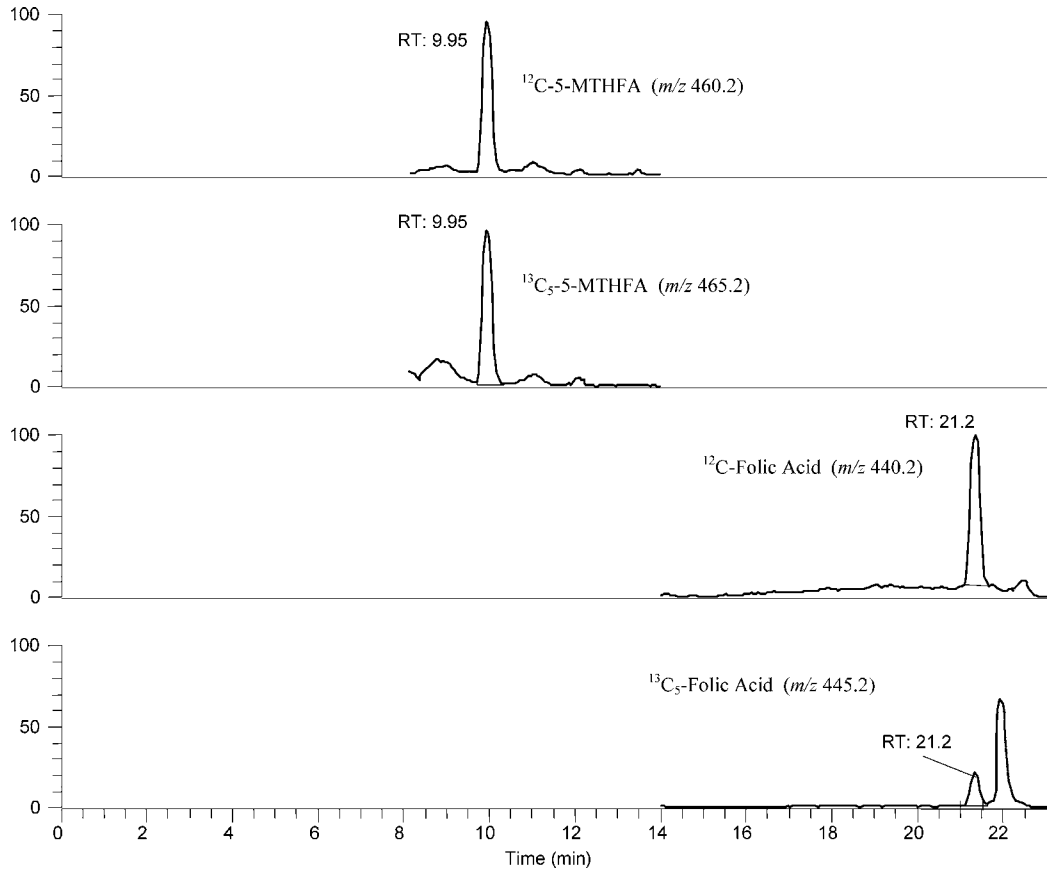


Figure 2. Selected ion chromatograms of molecular cations of labeled and unlabeled 5-MTHFA at *m/z* 460.2 and 465.2, respectively, and the molecular anions of labeled and unlabeled FA at *m/z* 440.2 and 445.2, respectively, using LC-MS with electrospray ionization. The chromatographic profiles are obtained from BCR 421 (nonfat dried milk).

Concentrations of the analytes are reported as mean values from triplicate analyses together with standard deviation and relative standard errors (RSE).

LC-MS. Analytes were quantified by comparing the ratio of the ion current abundances of the internal standards to the analytes and calculating their concentrations from known concentrations of internal standards that had been added to the samples. Values are expressed as mean concentrations from triplicate analyses.

RESULTS AND DISCUSSION

This paper describes an analytical procedure for the determination of 5-MTHFA and FA in foods using an HPLC method with fluorescent detection, which was validated using an independent LC-MS assay. Folates are found in a variety of food matrixes and require complex extraction procedures to render them accessible to instrumental analysis. In addition, the

FA food fortification program in North America has expanded the number of foods that now contain folates. As such, the determinations of these nutrients in the food supply continue to provide demanding analytical challenges and validation procedures utilizing MS and provide several distinct advantages.

The concentrations of analytes in each of the samples were quantified using different instrumental methodologies with separate sets of standards. Therefore, the stock solutions of the standards used in determining the concentrations of the analytes in these assays were calibrated as previously described (*17*). The mean values for the concentration of the analytes given in **Table 1** represent independent determinations of 5-MTHFA and FA from 10 food products (five certified reference materials and five frozen foods). These food products present a fairly broad spectrum of materials that are likely to contain both naturally occurring folates and FA in highly varying concentrations. An example of the chromatographic profile of 5-MTHFA and FA obtained using LC-MS analysis is given in **Figure 2**. In so far as possible, identical sample specimens were analyzed using both sets of analytical procedures. As such, the results illustrate a "best case" scenario for determining folate concentrations of foods across a wide spectrum of background matrixes when side-by-side determinations and comparable analytical procedures are employed. Comparisons of the values of the folate concentrations of the reference materials (as provided by the issuer) to the HPLC determinations have been discussed previously and therefore are not included here (*17*).

The variances observed in the determination of the concentrations of 5-MTHFA in several of the food products using either the HPLC or the LC-MS assay were on the order of 10% or lower. For instance, there was comparatively little variation in the determination of 5-MTHFA in BCR 485 (HPLC, 3% RSE; LC-MS, 5% RSE), BCR 421 (HPLC, 7% RSE; LC-MS, 5% RSE), frozen broccoli (HPLC, 2% RSE; LC-MS, 5% RSE), oranges (HPLC, 9% RSE; LC-MS, 11% RSE), and strawberries (HPLC, 10% RSE; LC-MS, 3% RSE). This suggests that factors affecting the precision of these determinations using either instrumental procedure were fairly uniform and did not interfere in chromatographic peak discrimination. Moreover, there was generally good agreement in the accuracy of these values. There was a 5% difference in the determination of the concentration of 5-MTHFA in BCR 421 between the HPLC and the LC-MS assays. There was only a 1% difference in the values obtained for BCR 485. There was a 17 and 12% difference in the concentration of 5-MTHFA obtained for frozen broccoli and frozen strawberries, respectively.

There was greater variation in the determination of 5-MTHFA in whole meal flour (~25% RSE) and pig liver (20 and 47% RSE) using either analytical procedure. In addition, there was a relatively large variance associated with the LC-MS determination of 5-MTHFA in infant formula (18% RSE). Because the error associated with determination of 5-MTHFA in whole meal flour by either HPLC or LC-MS was similar, this suggested that the low concentration of the analyte and an inherent substrate variability contributed to the lower precision. There was also a high degree of variability in the values obtained for the concentration of 5-MTHFA in pig liver. This appears to be associated, in part, with variances in substrate sampling and in the formation of an emulsion during the extraction phase of the analysis. The use of internal standards did not noticeably improve the precision of these determinations.

Only three samples (nonfat dried milk, infant formula, and pig liver) contained measurable quantities of FA that could be detected by both assays. The values obtained for FA for both the nonfat dried milk and the pig liver were similar (**Table 1**) with a 3 and 12% difference in the concentration of FA in the milk and liver, respectively. Although the variances associated with the determination of FA in infant formula were similar in both assays, the difference in the accuracy of these values was significant (19%).

This is the first paper that describes a mass spectrometric method used in the validation of HPLC determinations of food folates across a wide range of sample matrixes and varying concentrations. The comparable values obtained for 5-MTHFA and FA in many of the samples suggest that HPLC analysis with fluorescent detection may be used to accurately quantify folates present in a variety of food products. However, some food samples such as organ meat (liver) or foods containing a high fat content (infant formula) still require further refinements in this methodology.

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