Combined affinity and ion pair column chromatographies for the analysis of food folate

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Folate in nature exists in a variety of forms that differ by the state of oxidation, one carbon substitution of the pteridine ring, and by the number of glutamate residues. A method was developed in our laboratory (Selhub, 1989) that uses affinity chromatography followed by ion-pair high performance liquid chromatography (HPLC) for the analysis of folate distribution in tissues with simultaneous information on the structural nature at both ends of the folate molecule. The purpose of this study was to determine if this (affinity/HPLC) method is also suitable for the analysis of food folates. A total of 10 food products were analyzed. The food items were suspended in 10 volumes of 2% ascorbate-10 mmol/L 2-mercaptoethanol at pH 7.8 and heat extracted in an autoclave for 30 min. After centrifugation, folate in the supernatant fraction was purified by affinity chromatography and analyzed by ion-pair reverse HPLC using a diode array UV detector. Results showed variability of folate distribution in the various products ranging from a single derivative of 5-methylH_4PteGlu found in egg yolk to more complex mixtures of pentaglutamyl folates in lima beans, a series of methylated tetrahydrofolates in banana, and a multiplicity of forms in yeast extracts. The method appears to be reliable, as the measured variability amounted to an average of 10%, while total folates obtained by integrating the concentration of individual folates were comparable to total folates estimated using the more traditional microbial assay method.

Keywords: folate; folylpolyglutamates; food folates; affinity chromatography; ion-pair liquid chromatography

Introduction

Folate in foods exists in a variety of forms that differ by the pteridine ring structure and the number of glutamate residues. This multiplicity of forms renders the analysis of folate distribution a difficult and cumbersome task. The traditional method of DEAE-cellulose chromatography with differential microbiological assay,[1-3] is too involved: the chromatographic step is lengthy, and the need to process each fraction individually for treatment with pteroyl-

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polyglutamate hydrolase for separate microbial analyses using two to three assay organisms makes this method impractical for routine use. Alternate methods that are based on the selective cleavage of the C_9-N_{10} bonds[4] or analysis of ternary complexes of 5,10-methyleneterahydrofolate, [^{3}H]deoxyuridinemonophosphate and thymidylate synthase[5-6] represent improvement, but they are also impractical because of the many steps involved. Other methods that employ high performance liquid chromatography (HPLC) with fluorimetric or electrochemical detection require prior digestion by folate conjugases,[7-8] hence the information obtained is incomplete because it concerns only the pteridine ring structures, not the glutamic acid chain lengths.

To date, less than 10 food products have been analyzed for their folate distribution.[9] Undoubtedly, this paucity of analysis is attributable to the lack of a suitable method for routine determination of folate distribution. This laboratory has recently developed a new method (affinity/HPLC method), which uses affinity chromatography followed by ion pair liquid chromatography with a diode array UV detection system for tissue folate analysis.[10,11] The affinity chromatography results in spectrally pure folate compounds while the ion pair liquid chromatography, in combination with a diode array UV detection system, provides direct quantitative information on the various forms of folates in the purified mixture. The present study was undertaken to determine the feasibility of applying this method for the analysis of folate in foods.

Methods and materials

Materials

[3′,5′7,9-^{3}H] folic acid (1.026–1.28 TBq/mmol) was purchased from Moravek Biochemicals Inc (Brea, CA USA). Folic acid casei medium (ATCC 7469) was from Difco Laboratories (Detroit, MI USA); sodium ascorbate, dithioerythritol bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-Tris), 2-mercaptoethanol, and tetrabutylammonium phosphate were from Sigma Chemical Co. (St. Louis, MO USA) and acetonitrile from Fisher Co. (Fairlawn, NJ USA).

Extraction and analysis of folate in foods

Analysis of folate was conducted in 10 food items including baker's yeast, frozen cow liver, egg yolk, lima bean, soybean, wheat germ, cabbage, lettuce, orange juice, and banana. Egg yolk, lima beans, banana, and baker's yeast were used to test the feasibility and reproducibility of the affinity/HPLC method for food folate determination. The entire list of items was used to compare total folate concentrations by the affinity/HPLC method with that obtained by microbiological assay. Before extraction, soft foods were sliced. Dried foods were finely ground. These were suspended in a solution consisting of 2% sodium ascorbate, 10 mmol/L 2-mercaptoethanol and 100 mmol/L Bis-Tris buffer, pH 7.8. These suspensions were placed in an autoclave and heated for 30 min at 120°C and 15 psi. The samples were then cooled in an ice-water bath, homogenized for 1 min in a Waring blender, and centrifuged for 15 min at 30,000*g*. After homogenization and centrifugation, an aliquot of each of the supernatant fractions with an estimated total folate content not exceeding 15 nmol was mixed with a trace amount of [^{3}H]folic acid (18.3×10^3 Bq) and applied to a 1-mL (bed volume) FBP-Sepharose 4B affinity column.[12] The column was washed sequentially with 15 mL of 1 mol/L potassium phosphate buffer (pH 7.0), 15 mL water and eluted with 4 1-mL portions of 20 mmol/L trifluoracetic acid containing 10 mmol/L dithioerythritol. The acid fractions were promptly neutralized with 1 mol/L piperazine, and an aliquot was used for Tritium counting (model 2000 CA, Packard Instrument Company Inc., Meriden, CT USA) to assess folate recovery. A radioactivity recovery of 90% or more was taken as indication that the affinity column was not overloaded with folate from the food sample. The neutralized fractions containing [^{3}H] were combined and an aliquot of 0.9 mL was used for analysis of folate composition by ion-pair liquid chromatography combined with a diode array detection system as was recently described.[10,11] Briefly, the sample was injected into a C-18 column (Econosphere, 5u, 4.6 × 100mm; Alltech, Deerfield, IL USA), which has been equilbrated with 5 mmol/L tetrabutyl ammonium phosphate, 25 mmol/L NaCl, 5mmol/L dithioerythritol, and 10% acetonitrile. After-sample-application folates were eluted from the column by an acetonitrile linear gradient in the same equilibration solu-

Table 1 Molar peak coefficients expressed as integrated peak areas of various folates

Folate tested	Molar peak coefficient (area units/nmol)		
	280 nm	350 nm	258 nm
PteGlu	875	210	472
H2PteGlu	787	152	321
H4PteGlu	682	7	266
10-FormylH4PteGlu	323	0	513
5-methylH4PteGlu	787	0	314
5-FormylH4PteGlu	812	0	323

The data were obtained using [3H]PteGlu before and after conversion to the various reduced forms. Aliquots containing about 1, 3, and 10 nmol were each injected into the column, and the column was subsequently eluted under the same conditions described above. Fractions of 0.5 mL were collected to determine folate contents based on radioactivity counts. The molar peak coefficient (unit area/nmol) was calculated from the amount of folate, which was eluted and the corresponding peak area determined at the wavelength indicated in the table.[10]

tion.[10,11] Folate elution under these conditions was in the order of increasing number of glutamate residues. Folates containing the same number of residues eluted as a separate cluster arranged in three groups. Group I consists of 10-formylH_4Pte-$Glu_n(F_n)$, $H_4PteGlu_n(T_n)$ and $H_2PteGlu_n(D_n)$. Group II consists of 5-formylH_4Pte-$Glu_n(L_n)$; and group III of 5-methyl$H_4PteGlu_n(M_n)$ and $PteGlu_n(P_n)$. The diode array detection system provided monitoring of the column effluent at 280 nm for information on the number of glutamate residues of any eluting folate as well as information on which of the three groups within the cluster the eluting folate corresponded to. A final identification of the pteridine structure of the eluting folate was made on the basis of information on the UV absorption values at 350 and 258 nm also provided by the diode array detector. Absorption values at 350 nm were used for the identification of $PteGlu_n$ and $H_2PteGlu_n$. Activities and 258 nm were used to resolve between $H_4PteGlu_n$ and 10-formyl$H_4PteGlu_n$. Integrated peaks areas were used for estimation of folate concentrations of the individual peaks and the sum of these integrated areas was used to determine total folate in the sample based on the "molar peak coefficients" described in *Table 1*.[10,11]

Reproducibility studies

Boiled egg yolk, thawed frozen cow liver, uncooked lima beans, and baker's yeast were divided into 3–5 equal portions. Each portion was individually extracted and its folates purified then analyzed by the ion-pair HPLC method as described above. Individual activity peaks were used to estimate folate distribution and the coefficients of variation (CV) of the individual folates within the same food product. The sum of the integrated peak areas was used to estimate total folates and coefficients of variation for the same food product.

Determination of folate by microbiological assay

Aliquots (0.1 mL) of supernatant fractions from extracted food products were incubated in vacutainers overnight at 37° C with a partially purified chicken pancreas conjugase.[13] Total folate was then determined with *Lactobacillus casei* (ATCC 7469) as described by Wilson and Horne.[14]

Results

In preliminary studies we found the extraction procedure to be adequate as a second extraction[15] did not result in any significant release of additional

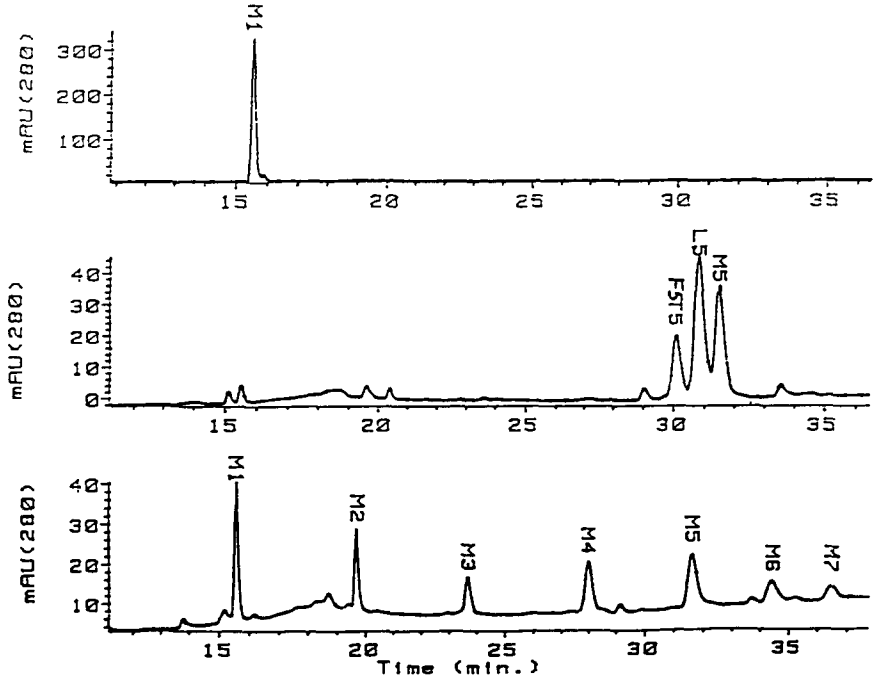

Figure 1 Representative ion-pair high pressure column chromatography of purified food folates. Folates extracted from the various food products and purified by affinity chromatography were subjected to ion-pair high performance liquid column chromatography as described in Methods. Top panel, folate from egg yolk; middle panel, folate from lima beans; bottom panel folate from banana. Symbols on the various peaks denote the following folate derivatives: F, 10-formyltetrahydrofolate; T, unsubstituted tetrahydrofolates; L, 5-formyltetrahydrofolates, and M, 5-methyltetrahydrofolates. Numbers following these letters denote total number of glutamate residues.

folate. *Figure 1* shows representative ion-pair HPLC of purified folates from three food items. Chromatography of purified folate from egg yolk (top panel) yielded a single peak that corresponded to the elution position and the spectral characteristics of 5-methylH_4PteGlu (M1). The middle panel shows purified folates from lima beans. These folates consist of pentaglutamyl derivatives of 10-formyltetrahydrofolate (F5), unsubstituted tetrahydrofolate (T5), 5-formyltetrahydrofolate (L5), and 5-methlytetrahydrofolate (M5). The bottom panel of *Figure 1* shows the results of the chromatography of purified folates from banana. They are composed exclusively of a series of 5-methyltetrahydrofolates with up to a total of seven glutamate residues. Chromatography of affinity purified folates from the other food products (data not shown) also yielded UV absorption peaks with retention time and spectral characteristics of known folate derivatives.

Table 2 shows the results of the reproducibility studies for determining individual folates in egg yolk, (thawed) frozen cow liver, lima beans, and baker's yeast. For egg yolk, the coefficient of variation in estimating the amount of 5-methyltetrahydrofolate was 5%. For frozen cow liver, the coefficients of variation in estimating the individual folates ranged from 17–24%. The coefficients of variation in determining the different folate forms in lima beans were in the range of 6–22%, while those in baker's yeast ranged from as low as 6% for the major peak representing 5-methylH_4PteGlu$_6$ (M6) to as high as 30% for the minor peak representing 10-formylH_4PteGlu$_6$ (F6).

Table 3 shows the variability of the method with respect to estimation of total folates. The coefficient of variation was 5% for total folates in egg yolk, 7% in baker's yeast, 12% in lima beans, and 19% in thawed frozen cow liver.

Figure 2 is a comparison of the affinity/HPLC method with that of *L.*

Table 2 Reproducibility of the affinity/HPLC method with respect to the determination of individual folate concentrations*

	Egg yolk (n = 3)	Cow liver (n = 4)	Lima beans (n = 5)	Baker's yeast (n = 5)
M1	1.93 ± 0.09 (5.1%)*			
D1		1.28 ± 0.22 (17.1%)		
M1		1.49 ± 0.26 (17.4%)		
T1		4.92 ± 1.35 (24.2%)		
F5			0.20 ± 0.03 (15.0%)	
T5			0.20 ± 0.03 (15.0%)	
M5			0.80 ± 0.18 (22.5%)	
L5			1.07 ± 0.07 (6.5%)	
M5				6.20 ± 0.54 (8.7%)
F6				2.96 ± 0.88 (29.7%)
T6				2.74 ± 0.70 (25.5%)
M6				16.29 ± 1.18 (7.3%)
F7				1.96 ± 0.52 (26.5%)
T7				1.82 ± 0.15 (8.2%)
M7				37.17 ± 2.18 (5.8%)

*Mean in nmole/g ± SD (CV).

Table 3 Reproducibility of the affinity/HPLC method with respect to the determination of total folate concentrations

Food item	Number of assays	Folate content* (Mean + SD)	Assay variability (CV,%)
Egg yolk	3	1.93 ± 0.09	5.1
Baker's yeast	5	69.13 ± 4.84	7.0
Lima beans	5	2.27 ± 0.26	11.6
Cow liver	4	7.69 ± 1.47	19.1

*nmole/g.

casei for estimating total folates in 10 food products. Correlation of the two methods can be described by Equation 1.

$$Y = -0.0304 + 1.10114X \qquad R^2 = 0.933 \qquad (1)$$

where Y denotes folate concentration determined by the affinity/HPLC method and X denotes the folate concentration determined by the *L. casei* method.

Discussion

This study indicates that the affinity/HPLC method is suitable for the analysis of folate in foodstuffs. An important feature of this affinity/HPLC method is the capacity of the affinity column to concentrate and quantitatively purify the folates in tissue extracts. This enables folate activity to be determined by direct spectrophotometric methods as opposed to other methods, e.g., microbial assay, that are more laborious. The studies described in *Figure 1* show that even though food products are chemically more complex than tissues, folates in extracts of these products are similarly purified by the affinity column. Every UV-absorbing peak in the chromatograms of *Figure 1* corresponds to a specific folate derivative. Moreover, the close similarity between the affinity/HPLC method and that of *L. casei* assay for estimating total folates is a strong indication that the folate purification by the affinity column is quantitative and, to the extent that the microbial assay is the "gold standard" for measuring total folate, it validates the use of the affinity/HPLC method for the analysis of food folate content.

Results of the reproducibility studies have shown consistency with respect

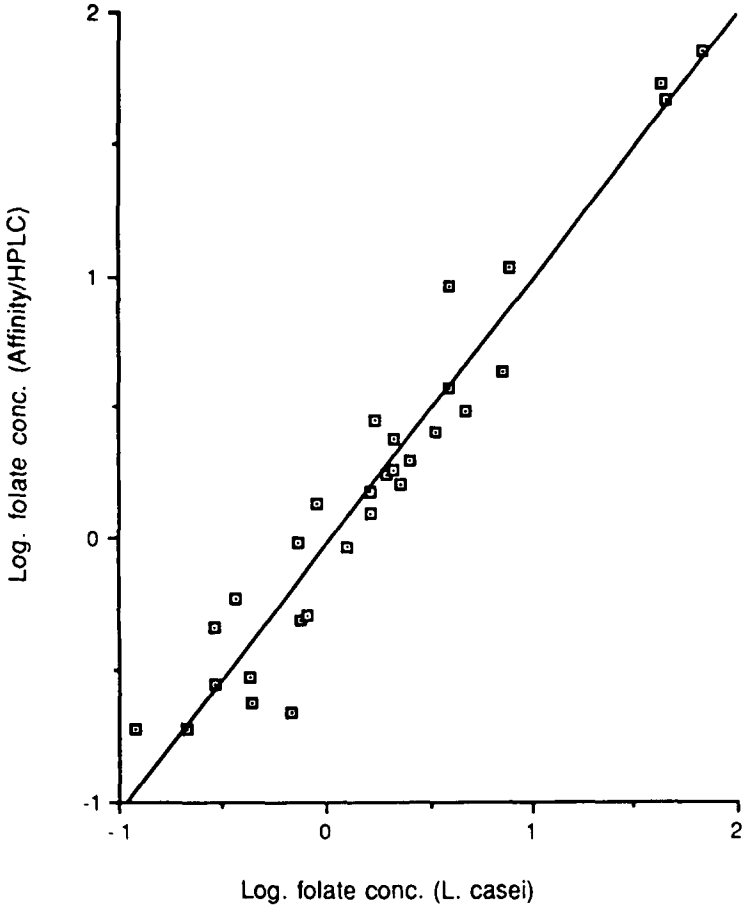

Figure 2 Comparison of the affinity/HPLC method with *L. casei* for estimating total folate concentrations. Data representing total folates determined in the 10 food products described in Methods. Three separate batches of each product were analyzed.

to folate distribution pattern for the individual food products tested. Egg yolk contained only one form of folate, thawed frozen cow liver contained three monoglutamyl folate derivatives, extracts of lima beans contained mostly pentaglutamate derivates, while extracts from baker's yeast contained mostly hexa- and heptaglutamyl folates. Within the same food product, however, the reproducibility was not the same for the various derivatives. In yeast extract (and egg yolk) 5-methylatedtetrahydrofolates, which are more abundant, have lower coefficients of variation and were in the range of 5–8.7%. Less abundant forms, i.e., unsubstituted tetrahydrofolates (T) and 10-formyltetrahydrofolates (F) have higher coefficients of variation and were in the range of 8.2–29.7%. The observed high CV for folates in cow liver and lima beans probably reflects changes that occurred prior to folate extraction such as differential thawing characteristics of various pieces of cow liver, which were allowed to thaw at room temperature and then individually extracted for folate analysis. The lima bean samples were prepared from dried purchases. Intra-sample reproducibility was excellent: if the liver extract was repeatedly analyzed the coefficients of variation for the various folates were 10% or less, and when all the 10 food products are considered, the estimate for coefficients of variation averaged 10% (data not shown).

A recent study by Gregory et al. used deuterium-lableled monoglutamyl folates to show in humans that different forms of monoglutamyl folates have different bioavailability.[16] Also, available information indicates lower availability of folate polyglutamates compared to monoglutamyl folates.[9,17] Thus, both ends of the folate molecule may be important determinants of

folate bioavailability. The method described herein for the analysis of food folates is potentially important in the studies of food folate biovavailability. Other factors that may have important roles in determining food folate bioavailability, such as stability during food processing and during passage through the stomach, may also be easily studied with this method. With our method one person can perform as many as eight analyses in a single working day. Hence it would be possible within a relatively short period to gather information on the folate distribution in a variety of food items.

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