Determination of Folacin Derivatives in Selected Foods by High-Performance Liquid Chromatography

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A reverse-phase high-performance liquid chromatographic (LC) method was developed for the separation and quantitation of the principal folacin derivatives of foods and other biological materials. Pterin-6-carboxylic acid (PT-6-COOH) and the monoglutamate forms of 5,6,7,8-tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid (5-CH_3-THF), 7,8-dihydrofolic acid (DHF), folic acid (FA), and 5- and/or 10-formyltetrahydrofolic acid (5-CHO-THF and/or 10-CHO-THF), were isocratically separated by using coupled octadecylsilica and phenylsilica columns and an acidic phosphate–acetonitrile mobile phase. Detection was performed by monitoring ultraviolet (280 nm) absorption or by a postcolumn fluorogenic oxidative derivatization method which enhanced the specificity and sensitivity for THF, DHF, and FA. A preparative chromatographic method was devised by using a hydrophobic resin (Bio-Beads SM-2) to provide sample extract purification. The LC procedure was successfully applied to the determination of total folates in beef liver, fortified breakfast cereal, and a fortified liquid infant formula. This acidic phosphate reverse-phase technique provided excellent chromatographic efficiency and resolution of the folates and compared favorably with recently reported ion-exchange and ion-pair LC methods.

The folacin groups constitutes a large family of biologically active compounds which differ from each other in regard to the number of glutamate residues present, the state of oxidation of the pteridine nucleus, and the nature of any one-carbon substituents (i.e., methyl, methylene, methenyl, formyl, etc. groups at N-5 and/or N-10 positions).

Several nutrition surveys have indicated that a dietary deficiency of the folacin group may be common in the American population (Chung et al., 1961; Ten-State Nutrition Survey, 1968–1970; Bailey et al., 1979). These findings demonstrate the need for further research concerning the nutritional adequacy of foods with respect to the biologically active folates and further assessment of folacin nutriture. The problems associated with existing analytical methods for the determination of folates in foods and biological samples have served to limit research in these areas.

The most widely used methods for folacin assay are the microbiological procedures employing *Lactobacillus casei*, *Pedicoccus cerevisiae* and *Streptococcus faecalis*. These methods are very sensitive but are lengthy, imprecise, and lack reproducibility. Also, the various folacin forms are not differentiated, and there is evidence that the various folacin forms differ in biological activity and bioavailability (Perry and Chanarin, 1968; Blakely, 1969).

Radioisotopic methods have been employed for blood tissue and food analysis (Waxman et al., 1971; Rothenberg et al., 1972; Tigner and Roe, 1979; Graham et al., 1980), but recent data (McGown et al., 1978; Shane et al., 1980; Waxman et al., 1978) question the accuracy of these procedures. Fluorometric assay procedures also have been developed (Allfrey et al., 1949; Stepanova et al., 1974); however, complexity, susceptibility to interference and lack of detection of all folates limit their usefulness.

The various folacin forms have been determined by using traditional paper, thin-layer, ion-exchange, and molecular-size chromatography; however, such techniques are time consuming and lack resolution of all the forms.

Several methods using high-performance liquid chromatography (LC) have been recently reported for the separation of folacin derivatives. These methods have employed either anion exchange (Stout et al., 1976; Clifford and Clifford, 1977; Chapman et al., 1978; Reed and Archer, 1980; Cashmore et al., 1980) or ion-pair separations (Chapman et al., 1978; Branfman and McComish, 1978; Reingold et al., 1980a,b; Allen and Newman, 1980). Cashmore et al. (1980) recently described a reverse-phase LC separation of folacin polyglutamates employing gradient elution at pH 5.5. The methods of Clifford and Clifford (1977) and Reingold et al. (1980a) have been the only LC procedures to be applied to food folacin analysis to date; however, neither is universally suitable for the assay of naturally occurring folates.

This communication reports a new approach for the high efficiency reverse-phase LC separation of folates and its application to the determination of naturally occurring and added folates in selected foods. The separation was achieved by using coupled octadecylsilica and phenylsilica columns, an acidic (pH 2.3), phosphate–acetonitrile mobile phase, and UV detection at 280 nm. A postcolumn derivatization procedure was developed to enhance the detection selectivity and fluorescence detectability of THF, DHF, and FA. The isocratic conditions were selected to eliminate the need for reequilibration associated with gradient elution while maintaining a high efficiency separation of the principal folate monoglutamates. Simplicity and long column life were other distinct advantages of this procedure.

EXPERIMENTAL SECTION

Liquid Chromatography. An Altex liquid chromatography system (Altex Scientific, Inc.) was used which consisted of a Model 110A solvent metering pump, Model 905-42 sample injection valve, and a Model 153 ultraviolet absorption detector with a 280-nm filter and a 20-μL analytical optical unit. The coupled columns used were an Altex Ultrasphere IP (4.6 mm × 25 cm), followed by a μBondapak phenyl (3.9 mm × 30 cm; Waters Associates). A precolumn (4.6 mm × 25 cm) packed with 37–53 μm of silica (Whatman, Inc.) was employed prior to the injection valve to provide saturation of the mobile phase with silica, thus prolonging the life of the analytical columns (Rabel, 1980). Injections were made at ambient temperature using a 100-μL sampling loop.

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An isocratic mobile phase of 0.033 M potassium phosphate with 9.5% v/v acetonitrile (pH 2.3) was utilized. The mobile phase was filtered through a 0.45-μm filter (Gelman Instrument Co.) and deaerated by sonication under vacuum. A flow rate of 0.7 mL/min was normally maintained which yielded an inlet pressure of 1500 psi.

Postcolumn Derivatization. This procedure was a modification of the method developed by Moye and St. John (1980). A calcium hypochlorite oxidative cleaving agent consisting of 0.005% w/v HTH dry chlorine (Olin Co.) in 0.1 M dibasic potassium phosphate and 0.2 M sodium chloride, was employed to convert THF, DHF, and FA to their highly fluorescent pterin fragments. This oxidant was metered into the LC column eluate at 0.23 mL/min by using a Technicon AutoAnalyzer II proportioning pump via a three-way manifold (Dionex) which was installed after the ultraviolet absorption detector. The combined stream of LC eluate and oxidant was passed through 10 m of coiled Teflon tubing (0.8-mm i.d.) which gave a reaction residence time of ~2 min. The tubing was maintained at 60 °C in a constant-temperature water bath to enhance the cleaving reaction before fluorescence detection by an Aminco FluoroMonitor (American Instrument Co.). The FluoroMonitor was equipped with a General Electric blacklight lamp (Model F4T4-BL), a Corning 7-65 excitation filter (365-nm transmission maximum), and a Wratten 2-A emission filter (>415-nm transmission). The ultraviolet absorption and fluorescence of the separated folacin monoglutamates were recorded on a Linear Model 385 dual-channel strip chart recorder.

Folate Standards. PT-6-COOH, THF, 5-CH_3-THF, DHF, and Fa were purchased from Sigma Chemical Co. 5-CHO-THF was purchased from ICN Pharmaceuticals, K and K Labs Division. 10-CHO-THF was prepared by the method of Rabinowitz (1963), with modifications as suggested by Robinson (1971). Standards of Pt-6-COOH, DHF, and FA were initially dissolved in 5% w/v dibasic potassium phosphate (pH 9.1) before quickly diluting in a protective 0.1 M potassium phosphate buffer containing 0.25% w/v ascorbate (pH 7.0). Standards of THF, 5-CH_3-THF, 5-CHO-THF, and 10-CHO-THF were dissolved directly in the phosphate–ascorbate buffer. Standards were prepared immediately prior to use, protected from light, and stored at 4 °C.

Extraction and Preparative Chromatography. Fresh beef liver (20 g) was homogenized in 100 mL of 0.1 M potassium acetate containing 0.25% w/v ascorbate (pH 4.0). The homogenate was incubated at 37 °C for 2 h to allow the endogenous γ-glutamyl carboxypeptidase (conjugase) to cleave polyglutamates to their monoglutamate derivatives (Silink et al., 1975; Tigner and Roe, 1979), heated to 100 °C for 10 min, and centrifuged at 25000*g* for 10 min. The resulting supernatant was applied to a column (0.7 × 20 cm, ~11-cm bed height) containing 20–50-mesh Bio-Beads SM-2 (Bio-Rad Laboratories) which had been previously equilibrated in the pH 4.0 acetate–ascorbate buffer. Under these conditions the folates are retained, thus providing on-column concentration in addition to extract purification. The column was washed with 20 mL of the pH 4.0 acetate–ascorbate buffer. The folates were eluted with 20 mL of 0.1 M potassium phosphate containing 0.25% w/v ascorbate and 9.0% v/v acetonitrile (pH 7.0). The column eluates were filtered (0.45 μm) and then analyzed as described.

The following procedures were developed for folic acid in cornflakes (Kellogg Co.) and liquid infant formula (Enfamil, Mead Johnson & Co.). Cornflakes (20 g) were homogenized in 200 mL of pH 7.0, 0.1 M KH_2PO_4–0.25% w/v ascorbate buffer. The homogenate was centrifuged at 25000*g* for 10 min and the supernatant filtered (0.45 μm) before LC injection. The infant formula (20 mL) was diluted with 20 mL of pH 7.0 0.1 M KH_2PO_4–0.25% w/v ascorbate buffer. The pH was adjusted to 4.5 with concentrated HCl and centrifuged at 25000*g* for 10 min. The supernatant was filtered (0.45 μm) before LC injection. FA was the only detectable form of folacin in these products; therefore, the mobile phase flow rate was increased to 1.0 mL/min to shorten the LC analysis time.

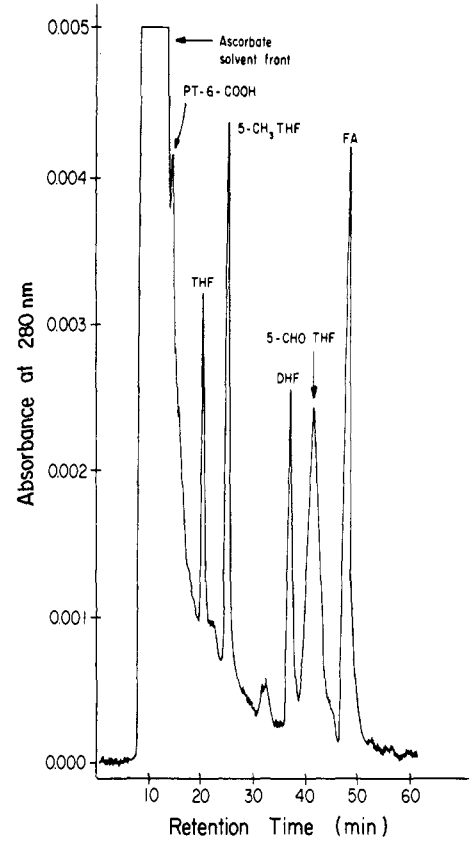

Figure 1. Chromatographic separation of folic acid derivatives. Conditions: coupled columns, Ultrasphere IP (4.6 mm i.d. × 25 cm) and μBondapak phenyl (4.6 mm i.d. × 25 cm); mobile phase, 0.033 M phosphoric acid and 9.5% acetonitrile (pH 2.3); flow rate, 0.7 mL/min; column temperature, ambient; detector, Altex 153 (280 nm) at 0.005 absorbance unit full scale. Sample: PT-6-COOH (50 ng); THF (100 ng); 5-CH_3-THF (100 ng); DHF (100 ng); 5-CHO-THF (200 ng); FA (100 ng).

RESULTS AND DISCUSSION

The reverse-phase LC separation of six folacin vitamers is shown in Figure 1. Under these conditions, 10-CHO-THF coeluted with 5-CHO-THF. PT-6-COOH could be completely resolved by decreasing the mobile phase flow rate to 0.5 mL/min or decreasing the acetonitrile concentration to 9.0% v/v; however, the total analysis time was increased to ~90 min. The use of coupled columns provided complete separation of DHF and 5-CHO-THF. These columns have been used for over 1 year with no detectable loss of efficiency. Standard curves were obtained by plotting peak height vs. the amount of standard folacin injected onto the column. These relationships were found to be reproducible and linear over a 100-fold concentration range. The minimum detectable levels by ultraviolet absorption detection were 2 ng (PT-6-COOH), 5 ng (THF), 3 ng (5-CH_3-THF), 5 ng (DHF), 10 ng (5-CHO-THF), 3 ng (FA), and 40 ng (10-CHO-THF, assuming 100% conversion from 5,10-methenyltetrahydrofolic acid in synthesis).

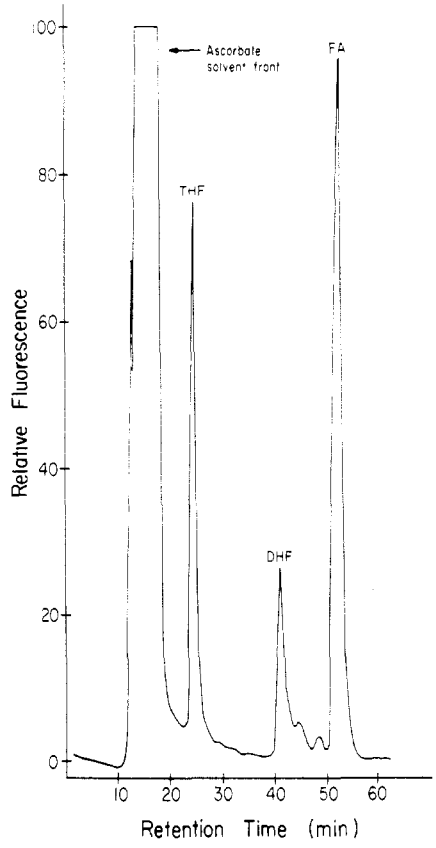

Figure 2. Chromatographic separation of the three oxidation states of folic acid. Conditions: same as for Figure 1 plus postcolumn oxidant, 0.1 M K_2HPO_4, and 0.2 M NaCl with 0.005% HTH chlorine; oxidant flow rate, 0.23 mL/min; mixing coil temperature, 60 °C; detector, Aminco FluoroMonitor (360-nm excitation; >415-nm emission); photomultiplier fine adjust, 3. Sample: THF (100 ng); DHF (100 ng); FA (100 ng).

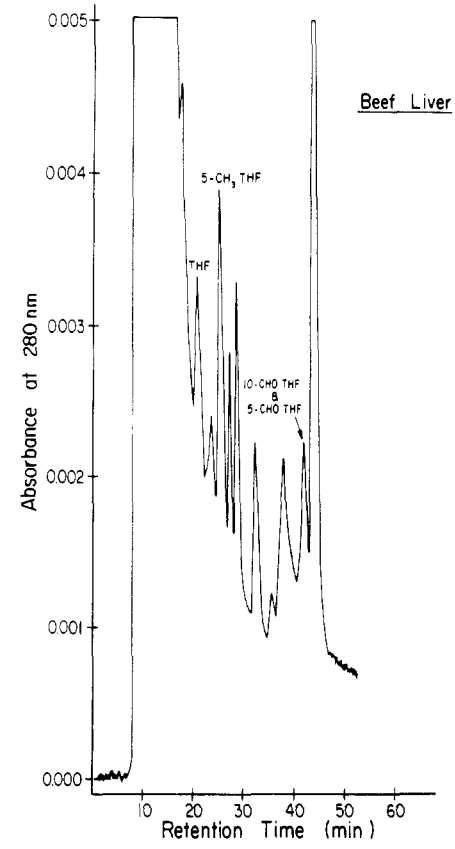

Figure 3. Chromatographic analysis of folic acid derivatives in beef liver. Conditions: same as for Figure 1. Sample: THF (0.78 μg/g); 5-CH_3-THF (0.76 μg/g); 5-CHO-THF and/or 10-CHO-THF (1.38 μg/g, calculated as 5-CHO-THF).

The LC separation and fluorometric detection of THF, DHF, and FA after postcolumn derivatization are shown in Figure 2. 5-CH_3-THF, 5-CHO-THF, and 10-CHO-THF were resistant to this oxidative cleaving reaction (Blakely, 1969; Maruyama et al., 1978). Calcium hypochlorite was found to be more effective than sodium nitrite (Reed and Archer, 1979), potassium ferricyanide (Chippel and Scrimgeour, 1970), potassium bromate, and potassium iodate for the oxidative cleavage of THF, DHF, and FA and was found to be reproducible and linear over a 100-fold concentration range for these folates. The minimum detectable levels by the fluorometric detection method were improved to 0.3 ng (THF), 0.9 ng (DHF), and 0.2 ng (FA) which are comparable to minimum levels detectable with the microbiological assay (Baker et al., 1971). No attempt was made to identify the fluorescing pterin product(s) in this study. Initial attempts to employ *o*-phthalaldehyde as a postcolumn fluorogenic labeling reagent were unsuccessful because of the low reactivity of this compound toward the folates.

The separation of THF, 5-CH_3-THF, and 5-CHO-THF and/or 10-CHO-THF in beef liver is shown in Figure 3. Recoveries for THF, 5-CH_3-THF, and 5-CHO-THF, added prior to extraction, were 65%, 82% and 73%, respectively. Quantitation indicated that THF (0.78 μg/g), 5-CH_3-THF (0.76 μg/g), and 5-CHO-THF and/or 10-CHO THF (1.87 μg/g, calculated as 5-CHO-THF) were the major folacin monoglutamates present. This is in general agreement with the findings of Shin et al. (1972), who reported almost equal proportions of 10-CHO-FA and/or 10-CHO-THF, 5-CH_3-THF, and THF pentaglutamates in rat liver.

This procedure has been applied to cabbage which also contains an active conjugase enzyme (Chan et al., 1973); however, interfering endogenous components coeluted with the folacin derivatives. Conjugase would have to be added to foods not naturally containing this enzyme. Further extract cleanup would be required for the analysis of cabbage or similar complex materials. We are currently investigating the use of a reverse-phase cartridge in place of the sample loop of the injector for additional sample concentration and purification, similar to that described for the LC determination of methotrexate in plasma (Lankelma and Poppe, 1978). We are also examining various ion-exchange techniques for their effectiveness in removing nonfolate compounds from plant and animal tissue extracts. Preliminary results indicate that strong cation-exchange chromatography is effective for extract purification.

Chromatograms from the analysis of folic acid in a fortified breakfast cereal and a fortified liquid infant formula are shown in Figure 4. The observed FA contents for the cereal and infant formula of 3.8 and 0.15 μg/g, respectively, agree closely with the manufacturers' claims of 3.5 and 0.11 μg/g. These initial results and the simplicity of the chromatograms suggest that this LC procedure would be well suited for the analysis of fortified products. For routine analysis of samples containing only folic acid, the flow rate and/or mobile phase acetonitrile content can be increased to reduce the retention time as needed. Analysis times for such products can be shortened to as little as 10 min/sample.

This LC and postcolumn derivatization procedure is presently being tested with other foods and selected animal tissues in this laboratory. The results will be correlated with those of the *L. casei* and radiometric assay procedures

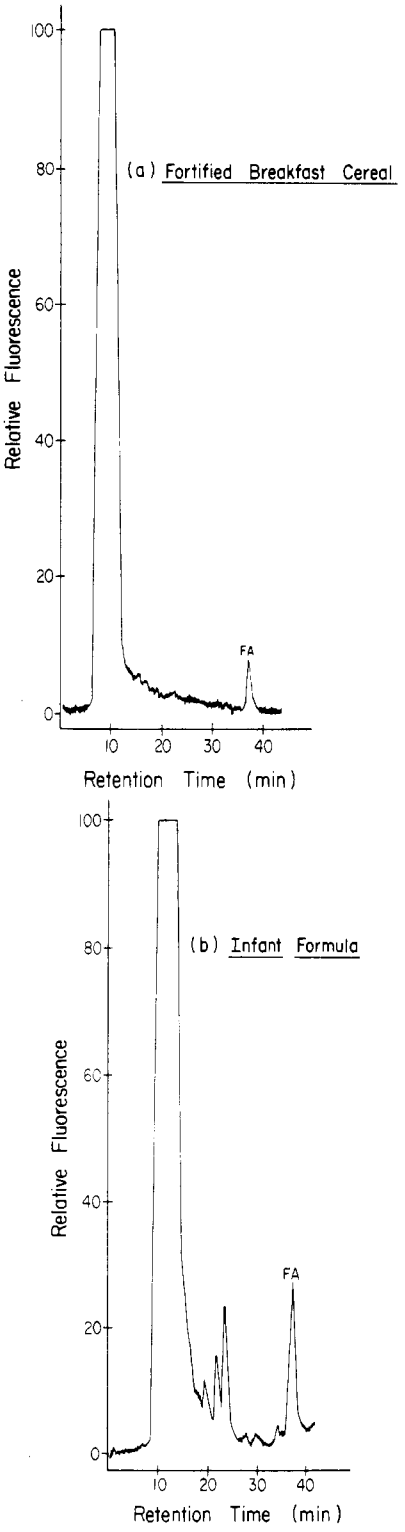

Figure 4. Chromatographic analysis of folic acid in fortified (a) breakfast cereal and (b) liquid infant formula. Conditions: same as for Figure 2 except mobile phase flow rate = 1.0 mL/min. (a) Photomultiplier fine adjust, 10; sample, FA (3.8 μg/g). (b) Photomultiplier fine adjust, 1; sample, FA (0.15 μg/g).

and will be reported, along with a refined extract purification procedure, in a subsequent publication.

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