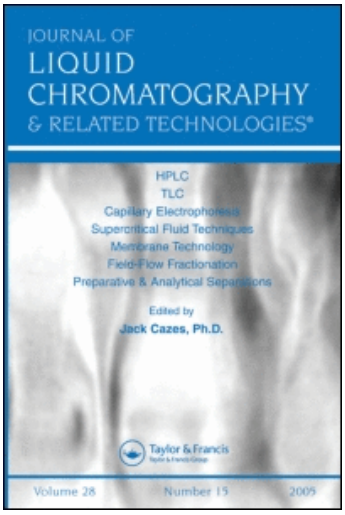

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DETERMINATION OF FOLIC ACID IN COMMERCIAL DIETS BY ANION-EXCHANGE SOLID-PHASE EXTRACTION AND SUBSEQUENT REVERSED-PHASE HPLC

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ABSTRACT

Folic acid at the μg/g level is determined in total nutritional diets by concentration on disposable commercial anion exchange columns followed by elution with a concentrated salt solution and subsequent reversed-phase HPLC with absorbance detection at 365 nm. The method is specific for folic acid with respect to some known degradants and electrochemically generated oxidation products.

INTRODUCTION

Although folic acid (pteroylmonoglutamic acid) represents only a small percentage of the naturally occuring folates, its stability and availability account for its widespread use in the fortification of foods and the preparation of vitamin supplements and total nutritional diets.

Reversed-phase HPLC is generally the method of choice for folic acid determinations replacing microbiological, photometric,

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and ion-exchange HPLC techniques whenever possible (1,2). The reversed-phase HPLC methods are sensitive and, for stability-indicating purposes, selective with respect to known folic acid degradants such as the oxidation products pterine (I) and pterin-6-carboxylic acid (II)(3) and the cleavage product N-(p-aminobenzoyl)-L-glutamic acid (III)(4).

However, when an assay is sought for low levels of folic acid in complex preparations such as total nutritional diets, which characteristically contain proteins, amino acids, saccharides, lipids, and minerals, the sample must be cleaned up prior to HPLC.

Folic acid and other folates in food and human milk were solute-focused at the head of a microparticulate phenyl column equilibrated with eluent containing tetrabutylammonium ion-pairing agent (2). After elution of interfering substances, the column was reequilibrated with eluent free of ion-pairing agent, eluting the folates. Folic acid in infant formula was extracted by enzymatic digestion followed by concentration on DEAE cellulose anion-exchange columns packed in the laboratory (5). After elution from the column, the extract containing folic acid was injected on a microparticulate octyldecylsilane column with an acetonitrile-acetate gradient.

This report extends the anion-exchange clean-up technique to the selective determination of folic acid at the μg/g level in total nutritional diets. Sample handling is greatly facilitated by use of the newly introduced solid-phase extraction

system comprising small, disposable, prepacked columns of 40-60 μm bonded phase packing that are eluted under vacuum with a special manifold system.

MATERIALS

Reagents and Materials

Pterine (I), pterin-6-carboxylic acid (II), N-(p-aminobenzoyl)-L-glutamic acid (III), folic acid (FA), dihydrofolic acid (IV), dl-L-tetrahydrofolic acid (V), dl-N-5-methyltetrahydrofolic acid (VI), and p-aminobenzoic acid (VII) were purchased from Sigma Chemical Co., St. Louis, MO.

In addition to folic acid at 1.6 and 2.2 μg/g, respectively, powdered diets A and B contained maltodextrin, modified starch, L-leucine, L-glutamine, L-arginine acetate, magnesium gluconate, L-valine, L-isoleucine, calcium glycerophosphate, safflower oil, L-lysine acetate, L-aspartic acid, L-alanine, L-phenylalanine, L-proline, glycine, L-threonine, L-methionine, sodium glycerophosphate, L-histidine monohydrochloride monohydrate, L-serine, potassium chloride, potassium citrate monohydrate, L-tryptophan, sodium citrate dihydrate, potassium sorbate, L-tyrosine, choline bitartrate, ascorbic acid, ferrous gluconate, polyoxyethylene sorbitan monooleate, zinc acetate dihydrate, niacinamide, α-tocopherol acetate, calcium pantothenate, manganese glycerophosphate, riboflavin phosphate sodium salt, cupric citrate, pyridoxine hydrochloride, thiamine hydrochloride, ascorbyl palmitate, Vitamin A palmitate, biotin, sodium selenite, sodium molybdate anhydrous, potassium iodide,

chromic acetate monohydrate, Vitamin K_1, Vitamin B_{12}, and Vitamin D_3. Diet C contained glucose oligosaccharides in place of maltodextrin and modified starch, a higher proportion of amino acids, and folic acid at 0.55 µg/g.

All other reagents were reagent grade.

Apparatus

The extraction clean-up step was accomplished with disposable, bonded-phase, quaternary amine anion-exchange solid phase extraction columns having a 500 mg sorbent mass and a 2.8 ml column volume (Bond Elut®, Analytichem International, Harbor City, CA). A vacuum manifold (Vac Elut®, Analytichem International) capable of holding up to 10 disposable columns was used to greatly facilitate elution. The loss of collected eluent due to splattering was prevented by use of a vacuum controller/ release and an additional needle valve. Detachable 75-ml plastic solvent reservoirs were used to increase the solvent capacity of the columns.

A high-performance liquid chromatograph (ALC/GPC 204, Waters Associates, Milford, MA) equipped with a 250 µl (diets A and B) or 500 µl (diet C) fixed-loop injector (Model 7125, Rheodyne, Berkeley, CA), a variable wavelength detector set at 365 nm (Model III, Laboratory Data Control, Riviera Beach, FL), a reciprocating pump (Model 6000A, Waters Associates), and a prepacked microparticulate reversed-phase column (µ-Bondapak® C_{18}, Waters Associates) was used. The column was pumped isocratically at 2.0 ml/min with a 6% acetonitrile 94% 0.1 *M*

sodium acetate buffer adjusted to pH 5.7. A diode array detector (HP 1040A, Hewlett Packard, Palo Alto, CA) and a precolumn coulometric cell (6) were used for the specificity study. Peak heights were measured manually.

METHODS

Procedure

Ten g of diet was mechanically shaken with 100.0 ml of a 0.01 *M* pH 7.4 phosphate buffer until a homogeneous dispersion resulted (about 20 min). The dispersion was filtered through Whatman GF/A glass fiber paper and 10.0 ml of the filtrate was loaded onto a disposable anion-exchange column that had been pretreated with one column-volume each of hexane, methanol, and water, respectively. The solution was pulled through the column under vacuum and the eluate was discarded. After washing with two column volumes of water, the folic acid was eluted from the disposable column with approximately 4.5 ml of a 10% sodium chloride, 0.1 *M* sodium acetate solution. The eluate was collected in a 5 ml volumetric flask, which was then diluted to volume with the sodium chloride-acetate solution. The sample was injected directly into the LC.

A reference standard stock solution was prepared by dissolving 15 mg of folic acid in a small volume of phosphate buffer and then diluting to 100 ml. This stock solution was further diluted with the phosphate buffer to yield a concentration of 1-5 μg/ml. Finally, 1.0-5.0 ml aliquots of the stock solution were diluted to 100.0 ml with sodium chloride-

acetate solution to prepare a standard response curve. Since folic acid standards were completely recovered from the anion-exchange column, a "method standard" was not needed.

The diet was also assayed using a standard addition technique. In this case, 20.0 g of diet was leached with 200.0 ml of phosphate buffer. Zero to 10.0 ml of a 1.5 μg/ml folic acid standard solution was added to 50.0 ml aliquots of the filtered leachate. The solutions were diluted to 100.0 ml with phosphate buffer, and extracted with the disposable columns as described above.

Specificity Studies

Solutions containing known degradation products and folate forms other than folic acid were prepared by dissolving 0.5 to 1 mg of each compound in 50 ml 0.01 *M* pH 7.4 phosphate buffer containing 0.3% mercaptoethanol. The solutions were injected directly and capacity factors were examined. In addition, specificity with respect to electrochemically generated oxidation products was examined with a precolumn coulometric cell (6) maintained at 2.0 V with respect to a silver/silver chloride (0.1 *M* in KCl) reference electrode.

RESULTS AND DISCUSSION

Initially, a detector wavelength of 280 nm, near the absorption maximum for folic acid, was chosen. However, a strongly absorbing excipient eluted just following the folic acid peak. To avoid potential interference that might be observed with some columns, a wavelength of 365 nm, at which the excipient

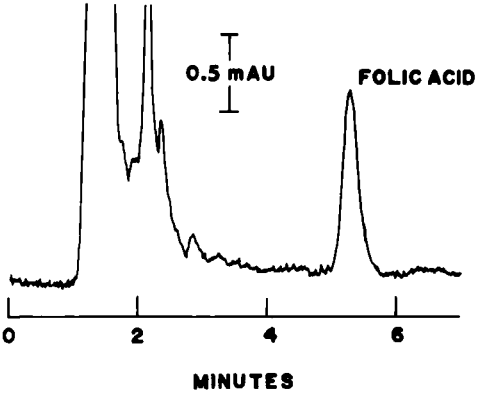

Figure 1. HPLC of a commercial diet extract. 2.0 ml/min flow rate. 250 µl injection volume. 365 nm detector wavelength.

was completely transparent, was chosen, yielding the chromatogram shown in Figure 1.

In addition, a sample volume of 20.0 ml was initially eluted through the ion-exchange columns. However, a plot of peak height versus sample weight yielded a negative deviation from linearity at weights greater than about 6 g, presumably due to elution of some of the folic acid from the column by the relatively high ionic strength sample matrix. When the sample aliquot was reduced to 10.0 ml, a plot of peak height versus sample weight yielded linearity (correlation coefficient 0.998 for n=5) to at least 14 g of sample.

An eight-point plot of peak height versus nanogram of standard folic acid injected yielded a straight line from about 40 to 200 ng injected (100 ng nominal) with a correlation coefficient of 0.9995, a % intercept [(y-intercept/$\bar{y}$)x100, where $\bar{y}$ is the average y] of 0.9%, and a % variation [($S_{y/x}/\bar{y}$)x100,

TABLE 1

Results for the Assay of Folic Acid in Three Commercial Diets.

g sample	µg/g	% theory	g sample	µg/g	% theory
	Diet A			Diet B	
5.0	2.03	90.6	7.5	1.68	102.4
7.0	2.02	90.2	10.0	1.58	96.3
7.0	1.98	88.4	10.0	1.71	104.3
9.0	2.10	93.8	10.0	1.69	103.0
10.0	1.99	88.8	Av.	1.66	101.5
10.0	2.01	89.7	RSD	3.5%	
10.0	2.12	94.6			
10.0	1.99	88.8		Diet C	
10.0	2.07	92.4			
10.0	2.10	93.8	5.0	0.55	100.5
11.0	2.05	91.5	8.0	0.54	97.7
Av.	2.04	91.1	11.0	0.51	93.3
RSD	2.4%		14.0	0.52	95.6
			Av.	0.53	96.8
			RSD	3.2%	

where $S_{y/x}$ is the standard error of estimate] of 1.9%. Assays of three commercial diets yielded the data listed in Table 1.

Four-point standard addition yielded 2.03 µg/g for diet A, 1.64 µg/g for diet B, and 0.57 µg/g for diet C, in good agreement with the standard curve values listed in Table 1. Because of the large number of components in each diet, recoveries determined by using a diet specially prepared without folic acid would be difficult. Therefore, recoveries for each diet were estimated by calculating the ratio of the standard additions plot slope to the slope of the standard response curve (8). Recovery values of 101.4%, 102.6%, and 100.2% were obtained for diets A, B, and C respectively.

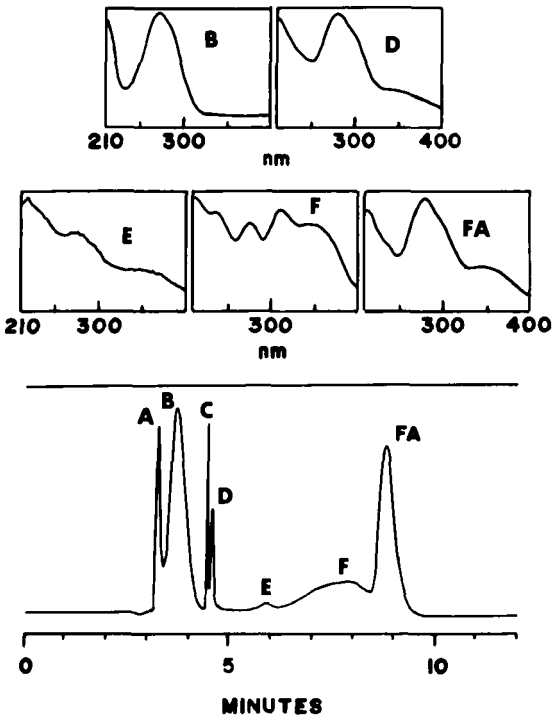

Figure 2. HPLC and UV spectra of electrochemically generated folic acid (FA) oxidation products. 1.0 ml/min flow rate. 250 µl injected. 280 nm detector wavelength. 80% coulometric yield. A yielded a spectrum nearly identical to that for B; C yielded a featureless spectrum.

Specificity

If an assay is to be stability-indicating, known or postulated degradation products must not interfere with the determination of the analyte. Oxidation products I and II were found to elute just after the solvent front and do not interfere. The cleavage product III eluted just after the folic acid peak but is transparent at 365 nm. VII was not observed at all, and

VI eluted after folic acid and did not interfere. IV and V, which represent metabolites of folic acid and other folate forms present in foods but not in the total nutritional diet, did interfere; however, separation of these folates from folic acid was not deemed necessary for the present method. In any case, there are other reversed-phase systems capable of separating these compounds from folic acid (2,7).

Forced degradation was accomplished by *in situ* electrochemical oxidation (6) of 80% of the folic acid to yield the peaks shown in Figure 2. UV spectra of some of the peaks are also shown. No further attempt was made to identify the oxidation products although product F was observed at about 0.5 area % in the folic acid standard. Decreasing the flow rate to 0.4 ml/min resulted in an observable 99% decrease in the folic acid peak, indicating no interference from coeluting oxidation products.

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