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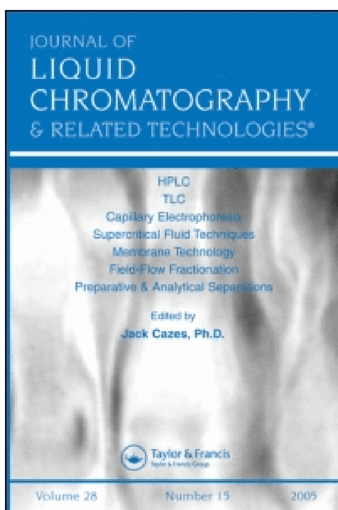

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THE DETERMINATION OF FOLIC ACID (PTEROYLMONOGLUTAMIC ACID) IN FORTIFIED PRODUCTS BY REVERSED PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reversed phase HPLC method was developed for the separation and determination of pteroylglutamic acid (PGA) in fortified foods. Extraction was carried out by heating with phosphate-citrate buffer, pH 8.0 containing ascorbate, and incubation with papain at 40°C for 4 hrs. The extracts were purified and concentrated on a short DEAE column which was rinsed with phosphate buffer, pH 7.0, of increasing molarity. PGA was eluted with 0.1M phosphate buffer, pH 7.0, containing 0.5M NaCl. The eluants were chromatographed on a Spherisorb ODS 10 µm column (250 x 4.6 mm) using a 30 min linear gradient of 2% to 30% acetonitrile in 0.1M acetate buffer, pH 4.0, at 1 ml/min and an absorbance detector at 280 nm. The coefficients of variation on analysis of 8 replicate samples of a milk and soy protein based infant formulas were 5.9% (at 4.6 ng/50 µl inject) and 6.8% (at 1.8 ng/50 µl inject) respectively.

INTRODUCTION

The various naturally occurring forms of folic acid (pteroylmnoglutamic acid, PGA) function as essential coenzymes in the synthesis of proteins and nuclei acids (1). The derivatives differ in the substitution at N^5 and N^{10} positions, the state of oxidation and the number of glutamic acid residues (2). Determination and identification of the various forms in biological samples has been carried out using a combination of ion-

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exchange chromatography on DEAE-cellulose and DEAE-Sephadex and differential microbiological assays (3,4).

Since the introduction of high-pressure liquid chromatography (HPLC), several methods using HPLC for the separation of folate derivatives, based on ion-exchange or ion-pair partition chromatography, have appeared (5-12). Furthermore, it has been shown that reversed phase chromatography with microparticulate octadecyl-silica (ODS) as the stationary phase provides high resolution and fast analysis and it can be used in place of ion-exchange chromatography for microanalysis of folate derivatives (13,14). However, only a few of the HPLC methods have been applied to biological samples (7,10,11,12). While microbiological assays possess the required sensitivity and specificity, they do not distinguish between the various folate derivatives present in a biological sample. Although folate derivatives in standards can be separated by HPLC and measured quantitatively, difficulties have been encountered in obtaining the same sensitivity and specificity with biological samples (7,10,11,12). This points out the need for clean-up and concentration of folate derivatives in some biological samples.

Many studies of folate absorption have used PGA, a form which does not occur naturally. It has been utilized, because of its stability and availability, for therapeutic treatment, in standards for microbiological assays, in pharmaceutical preparations and in the fortification of foods and infant formulas. The reversed phase HPLC method reported here is specific and sensitive for the determination of added P.G.A. in all types of samples. It is especially suitable for foods and infant formulas enriched with PGA which require sample purification and concentration.

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MATERIALS

Folic acid (PGA) was purchased from Sigma, St. Louis, Mo., U.S.A. A stock solution was prepared by dissolving 20 mg of dried crystalline PGA in ca. 75 ml of distilled water and adjusting to pH 9.5 with 0.1N NaOH. After adding 20 ml of ethanol, the pH was adjusted to 7.0 with 0.05N HCl and the final volume was mde up to 100 ml with distilled water. The concentration of PGA was measured from the U.V. absorption spectrum of folic acid in 0.1N NaOH as described by Strohecker and Henning (13); the final stock solution contained 200 μg of folic acid per ml. Further standard solutions of 10 μg/ml in 20% ethanol and 1 μg/ml in distilled water were prepared weekly and daily, respectively.

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Mobile Phases

The mobile phases were prepared from sodium acetate buffer, 0.1M, pH 4.0, and acetonitrile (Fisher, HPLC grade). The buffer was filtered through a Millipore type HA 0.45 μm filter (Millipore Corp., Bedford, Mass., U.S.A.) prior to mixing with acetonitrile. The starting eluant (solvent A) consisted of 0.1M, pH 4.0, sodium acetate buffer containing 2% (v/v) acetonitrile and the same buffer containing 30% acetonitrile (v/v) was used as the gradient former (solvent B). After mixing, each mobile phase was degassed by sonication. Distilled deionized water was used throughout.

Analytical HPLC

A Waters system (Waters Assoc. Inc., Milford, Ma., U.S.A.) consisting of dual 6000 A pumps, a 660 solvent flow programmer and a U6K loop injector, was used with a prepacked reversed phase column (4.6 mm x 250

mm) of 10 μm Spherisorb ODS (Spectraphysics, Santa Clara, Ca., U.S.A.). The effluent was monitored at 280 mm with a Waters 440 UV absorbance detector connected to a Spectraphysics Minigrator and pen recorder for quantitation.

METHODS

Extraction of Samples

Pharmaceuticals, being the least difficult to prepare, were initially extracted with 0.05M, pH 8.0 sodium phosphate-citrate buffer containing 0.5 mg of ascorbate per ml and further diluted with distilled water to a concentration of 1-0.1 μg PGA per ml. An aliquot was filtered through a Millipore type FH (.45 μm) membrane filter and appropriate volumes were injected directly into the HPLC.

Extraction of PGA from samples of infant formula, both milk and soy protein type, proved the most difficult. Enzyme digestion with papain was found to be efficacious. Three g of powder was dispersed in 10 ml of distilled water plus 10 ml of 0.05M, pH 8.0, sdium phosphate-citrate buffer containing ascorbate. Liquid formula (10 ml) was diluted with 10 ml of the same buffer and thoroughly vortexed. After adding 0.2 g papain (Matheson, Coleman and Bell, Norwood, Ohio), the samples were again well mixed and incubated in a water bath at 40°C for a minimum of 4 hrs and usually overnight. On completion of the enzyme digestion, the samples were mixed and centrifuged for 15 min at 12000 rpm in a Beckman J-21C centrifuge. The supernatant was decanted through glass-fibre paper (Whatman GF/A) taking care to avoid the transfer of floating material to the filter paper. This step was repeated after washing the precipitate with

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10-20 ml of 0.005M, pH 8.0 sodium phosphate-citrate buffer. The filter paper was rinsed with another 10 ml of diluted buffer and the extract was then reconcentrated and purified on a DEAE column as described.

For samples in which proteinaceous material could be precipitated by holding in a boiling water bath for 10 min, and which sedimented distinctly on centrifugation, the extraction with papain and decantation through filter paper, respectively, could be avoided.

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Purification of the Extract

The efficient binding of PGA by DEAE cellulose was utilized for purification and reconcentration of PGA in the extracts. The column was prepared from a 0.2 g DEAE cellulose (Cellex D; Bio. Rad Laboratories, Richmond, Calif.) water slurry in disposable "Chromaflex" polypropylene columns (8 mm i.d.) (Kontes Glass Co., Vineland, N.J.) plugged with a pledget of glass wool. The column was washed with 10 ml of 0.005M, pH 7.0, phosphate buffer. As soon as this solution drained to the column surface, the extract was allowed to pass through slowly followed in sequence by a 10-20 ml wash with 0.05M, pH 7.0, phosphate buffer. Finally, a 10 ml wash with 0.1M, pH 7.0, phosphate buffer containing 0.5M NaCl was collected in graduated 10 ml flasks or tubes, filtered and 50 µl was injected in the HPLC. This injection volume was chosen so as to contain a suitable amount of PGA when approximately 1 µg of PGA was applied to the DEAE column. When eluted in 10 ml this would correspond to a concentration of approximately 0.1 µg/ml or 5 ng of PGA per HPLC injection.

Conditions of HPLC

Sufficiently pure extracts and purified DEAE eluants were chromatographed on the Spherisorb O.D.S. 10 μm reversed phase column 4.6 mm x 250 mm) using a 30 min linear gradient (curve No. 6, Waters 660 solvent flow programmer) from 0% solvent B (100% solvent A) to 100% solvent B flowing at 1 ml/min. The effluent was monitored at 280 nm and retention times and peak areas were calculated automatically by the minigrator. All analyses were carried out at ambient temperature. Linear response for PGA was confirmed by plotting peak area versus the amount of standard PGA injected. The response was linear over the range of 0-100 ng PGA. In subsequent analysis the results were calculated from the peak areas using factors derived by intermittent chromatography of standards.

Initially the column was first equilibrated with solvent A and a blank run was performed. Depending on the relative "purity" of the sample extracts, the column was washed at the end of the day or periodically with 100% acetonitrile and left overnight. When the system was not operated for more extended periods of time the column was washed with distilled water and stored in 50% (v/v) acetonitrile in water.

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Microbiological Assay

"Free and total" folacin activities were determined microbiologically, using L. casei and Difco folic acid casei assay medium (16). The extracts were prepared as previously reported (17), using ascorbate-phosphate buffer and Difco chicken pancrease conjugase; the assay tubes were incubated at 37°C for 22 hr, and turbidity was measured at 660 mμ.

RESULTS AND DISCUSSION

In ion exchange chromatography, which traditionally has been used to separate folic acid derivatives, retention is determined by coulombic interactions between the fixed charges of the stationary phase and the oppositely charged solute molecules. In reversed phase chromatography, the stationary phase is non-polar and retention is achieved by a hydrophobic effect that can be changed by increasing or decreasing the charge on the solute molecule, i.e. controlling the pH of the buffer eluent. Recently, it has been shown that use of both techniques in combination for the separation and identification of folate derivatives and polyglutamates yields high resolution and fast analysis (13,14). In the present method, an acetate buffer, pH 4.0, eluant with increasing linear acetonitrile concentration provided adequate retention, peak shape, buffering capacity and detection ability for repetitive HPLC of PGA in fortified samples. Furthermore, the reversed phase column was less prone to accumulate materials than ion exchange columns and it was relatively easy to wash and keep clean. A typical working standard curve ranging from 0-25 µg PGA is shown in Fig. 1, and a separation of 50 µl of a 10 µg/ml PGA standard or 5 ng is shown in Fig. 2.

The initial extraction of the samples was carried out in pH 8.0, 0.05M phosphate-citrate buffer because it has been reported (16) that PGA is less stable in the presence of phosphate and that this stability can be improved by the simultaneous presence of citrate. If dilution of PGA standards with buffer is desired, 0.05M, pH 7.0 sodium phosphate-citrate buffer should also be used.

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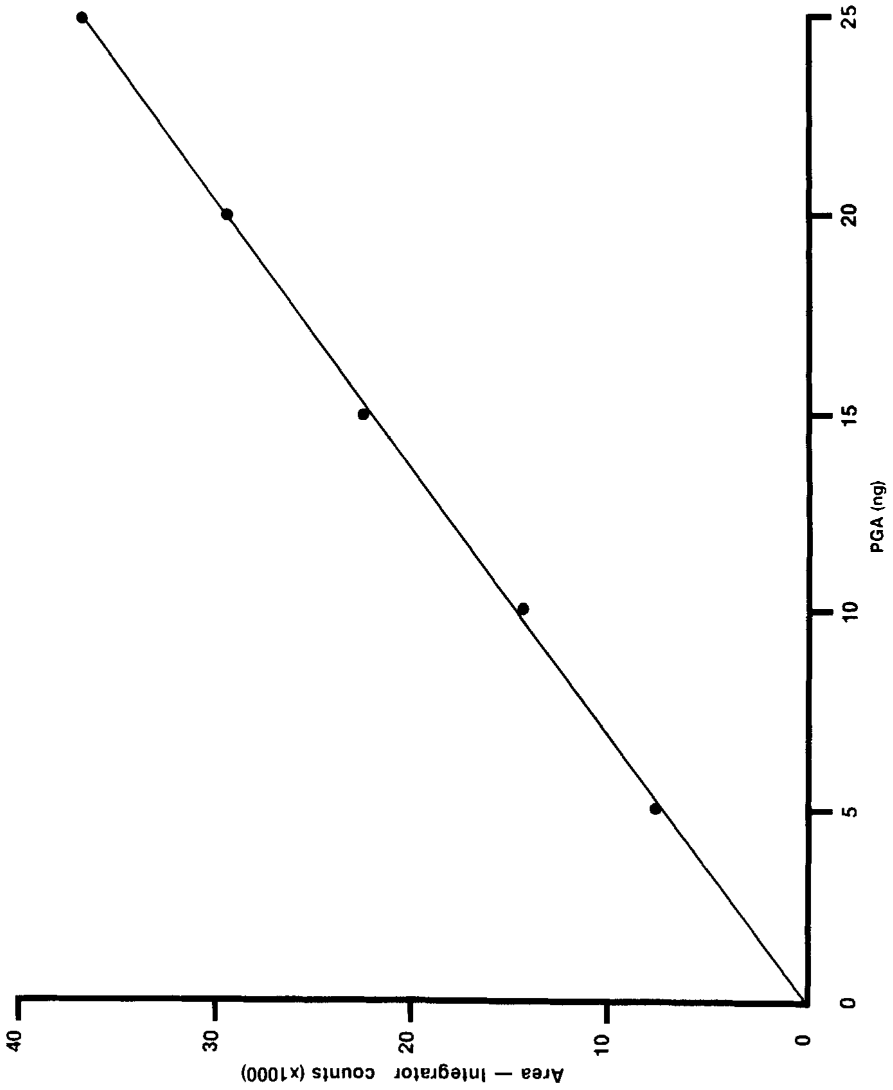

FIGURE 1. Standard curve for analysis of PGA by HPLC.

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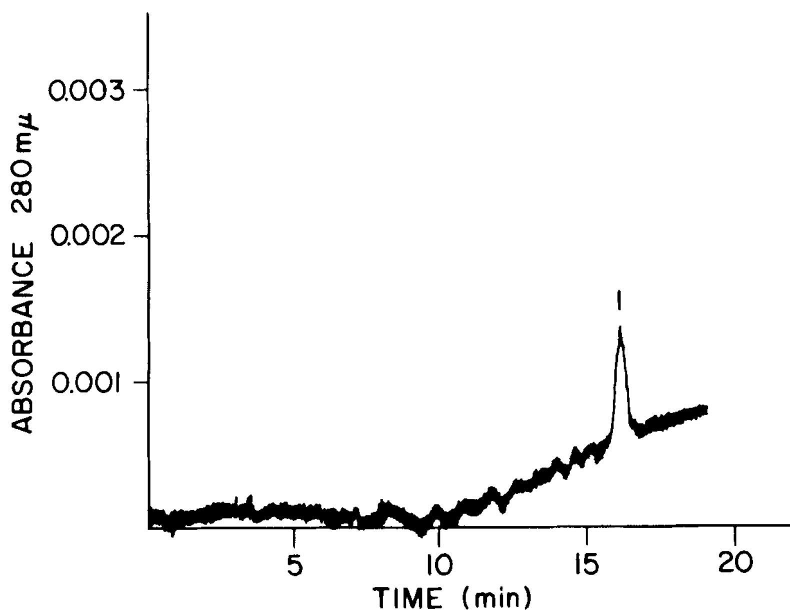

FIGURE 2. HPLC of PGA standard on Spherisorb ODS 10. Gradient A-B (0-100%) in 30 min. (A = 2%; B = 30% acetonitrile in 0.1M, pH 4.0, acetate buffer), 1 ml/min. (1) 50 μl PGA standard (1 μg/10 ml).

In infant formula and fortified samples other than pharmaceuticals, enough interfering material coextracted to prevent direct HPLC of the initial extract and detection of small amounts of PGA. Therefore, prior purification on a short DEAE column was necessary. This column bound PGA from the extract and most of the interfering substances were separated before PGA was eluted in a small volume of solvent. The first wash with 0.005M, pH 7.0, phosphate buffer was sufficient to rinse the DEAE. The second wash with 0.05M, pH 7.0, phosphate buffer removed most of the interfering material which originated in the extract and, according to tests with standards, also removed 5 CHO, and 5 CH_3 tetrahydrofolate. PGA was the only monoglutamate derivative left on the

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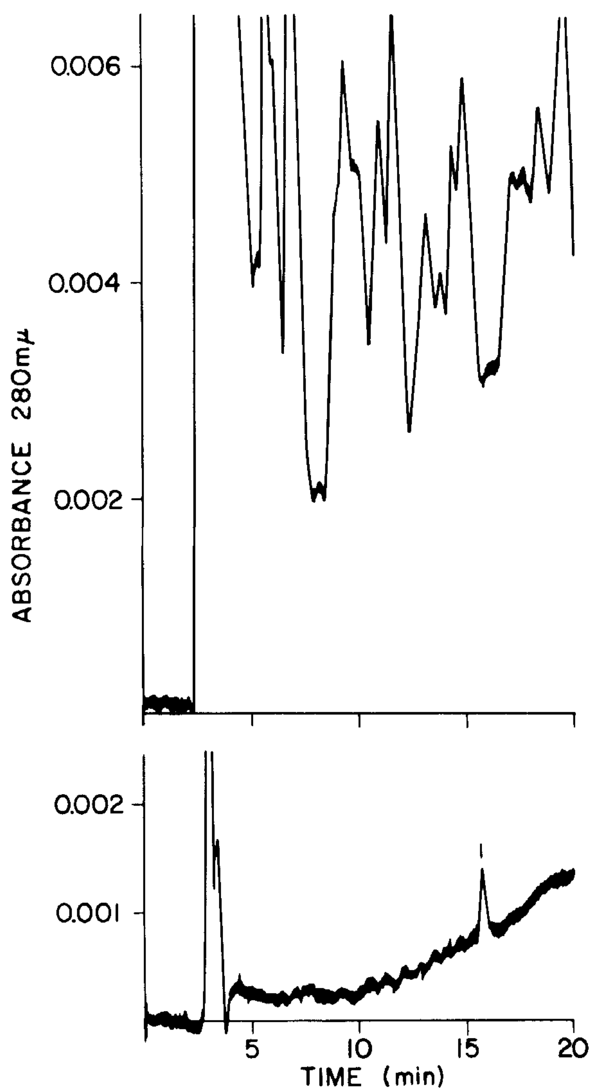

FIGURE 3. HPLC of milk protein based infant formula (3 g). (Upper) 20 µl of initial extraction volume of 30 ml before DEAE clean-up. (Lower) 50 µl of final elution volume of 10 ml after DEAE clean-up, (1) PGA.

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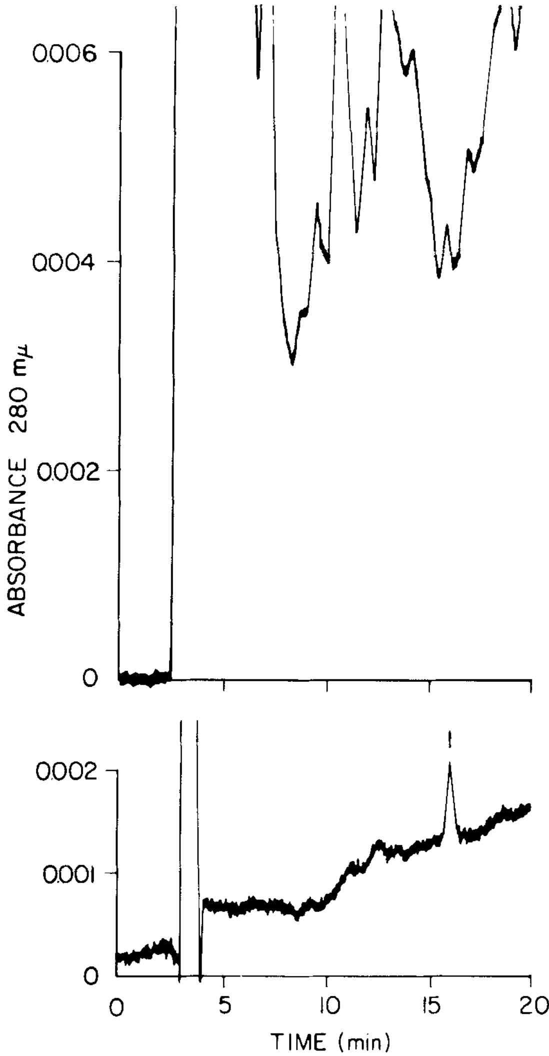

FIGURE 4. HPLC of soy protein based infant formula (10 ml). (Upper) 20 μl of initial extraction volume of 30 ml before DEAE clean-up. (Lower) 100 μl of final elution volume of 10 ml after DEAE clean-up, (1) PGA.

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TABLE 1

Pteroylmonoglutamic acid (PGA) in some fortified products

	L. casei assay		Label	HPLC	
Sample	Free Folate	Total Folate	Claim	PGA	Sample size
Infant formula, milk protein, µg/100 ml standard dilution	5.8	6.1	5.0	3.9	3 g
Infant formula, soy protein, µg/100 ml standard dilution	3.2	7.1	5.5	2.0	10 ml
Meal Replacer, µg/100 ml	24.3	26.9	22.0	20.3	5 ml
Egg Beater, µ/100 ml	48.0	51.2	added*	34.7	5 ml
Vitamin tablet, µg/tablet	118.0		100.0	117.0	1 tablet

* Amount not stated.

column and it was eluted in the final 0.1M, pH 7.0, phosphate buffer containing 0.5M NaCl. The purification of crude extracts of a milk and soy protein based infant formula on DEAE is illustrated in Figs. 3 and 4. In the case of soy protein based sample, Fig. 4, the amount of PGA contained in 10 ml represented the lower limit for quantitation.

To test the recovery of PGA during extraction and purification, 4 replicate samples of a milk (3 g) and a soy protein (10 ml) based infant formulae were analyzed before and after the addition of 1 μg of PGA. The recovery was 96% ± 3.9 and 98% ± 4.0 respectively. The reproducibility of the analysis was tested on 8 replicate samples of a milk and soy protein based formula and the coefficients of variation were 5.9% (at 4.6 ng/50 μl injection) and 6.8% (at 1.8 ng/μl injection) respectively.

The results of typical analysis are given in Table 1. In vitamin tablets, folic acid is present entirely as PGA. In infant formula the total folate consists of the natural derivatives from either milk or soy and PGA added in amounts to satisfy label requirements. Hence, in the sample of milk based formula, PGA represented aproximately 70% and, in the soy based formula, 25% of the total folate activity. In both the meal replacer and egg beater, PGA represented the major form of folate present. The HPLC method, therefore, can be used to determine PGA in samples to which it is added to supplement an already present source of natural folate derivatives. It is hoped that with refinements of the purification and reconcentration steps, this procedure can be extended to the detection and measurement of the natural methyl and formyl derivatives in biological materials.

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