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Note

A study of serum folate by high-performance ion-exchange and ion-pair partition chromatography

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The term "folic acid" designates a family of related compounds that vary by functional group, degree of reduction, and number of glutamic acids. Identification of the various forms of folate in biological materials has been a problem due to inadequate separation techniques. Microbiological assays, although commonly used, are difficult to perform, are time-consuming, and show wide variation.

Standard N^5- and N^{10}-substituted pteroylglutamic and pteroylpolyglutamic acids have been separated by ion exchange with high-pressure liquid chromatography (HPLC) [1, 2], and a recent preliminary study described the use of this method to characterize polyglutamate folates from mammalian cells in culture [3]. To our knowledge however, HPLC techniques have not been used to study serum folate.

Pteroylglutamic acid is the compound used clinically to alleviate folic acid deficiency, but there is confusion concerning the identity of the folate present in the serum after the oral administration of this compound. The present work describes a preliminary study utilizing high-performance ion-exchange and ion-pair partition chromatography to characterize the predominant serum folate in subjects pretreated with pteroylglutamic acid.

MATERIALS AND METHODS

Crystalline pteroylglutamic acid, N^5-methyltetrahydrofolic acid, and dihydrofolic acid were purchased from Sigma, N^5-formyltetrahydrofolic acid was a product of ICN—K & K Labs., and [^{14}C]-N^5-methyltetrahydrofolic acid-barium salt was a product of Amersham-Searle. Standards were made in 0.5% sodium ascorbate.

Ion-exchange separations were performed with a potassium perchlorate gradient on a column (1 m × 2.1 mm I.D.) prepacked with Permaphase AAX (DuPont), Ion-pair partition chromatography was performed with a 0.05 *M* Pic A (Waters Assoc.) methanol—water (30:70) mobile phase on a column (30 cm × 4 mm I.D.) prepacked with μBondapak C_{18} (Waters Assoc.). The columns were attached to a Model 4100 liquid chromatograph (Varian) equipped with a gradient elution device and a 280-nm detector.

Serum samples were taken from volunteers who had received 0.169 mg/kg Folvite (Lederle) 4 h prior to the experiment. For ion-exchange separations, the serum was mixed with sodium ascorbate to a final concentration of 35.4 m*M*, autoclaved for 3 min, and centrifuged at 1000 × *g* for 5 min. Fifty microliters of the serum supernatant fraction were chromatographed in the presence and in the absence of 100 ng of [^{14}C]-N^5-methyltetrahydrofolic acid (0.0125 μCi). For ion-pair chromatography, the serum samples were mixed with 5 mg/ml ascorbic acid and filtered through a 0.45-μm Millipore filter (HAWPO 1300). Recovery experiments with standard folate did not show significant adsorption on Millipore filters of this pore dimension.

The eluted samples were collected in sodium ascorbate and assayed with *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043), and *Pediococcus cerevisiae* (ATCC 8081). The microbiological assays were performed to the methods of Herbert et al. [14] and Scott et al. [5]. Pteroylglutamic acid was used in standards curves for the *L. casei* and the *S. faecalis* assays; N^5-formyltetrahydrofolic acid was used in standard curves for the *P. cerevisiae* assay. Ascorbate assays were performed by the method of Bessey et al. [6].

In isotope experiments, the eluted samples were counted in a Beckman LS-100 scintillation counter in 15 ml of a scintillation cocktail which contained 4.5 g of 2,5-diphenyloxazole, 1.5 g of 1,4-bis-2-(5-phenyl-oxazolyl) benzene, 1.5 l of toluene, and 0.5 l of Triton X-100.

RESULTS AND DISCUSSION

Fig. 1A shows the elution pattern for the reference folate compounds separated by ion exchange. The large peak at the solvent front can be identified as the ascorbate which was added to the standard solutions to prevent oxidation. After the ascorbate was eluted, the $KClO_4$ gradient was started and all three reference compounds were separated. The chromatographic identities of N^5-formyltetrahydrofolic acid (peak I), N^5-methyltetrahydrofolic acid (peak II), and pteroylglutamic acid (peak III) were confirmed by microbiological assay of the eluted peak fractions.

Two major peaks were evident in the gradient-elution pattern of serum samples taken from subjects pretreated with folate (Fig. 1B). The first peak had a retention time similar to that of N^5-formyltetrahydrofolic acid, but the results of microbiological assays were not confirmatory. Only a small and variable growth response was observed with the three organisms. It has been demonstrated that autoclaving in a neutral environment leads to the chemical conversion of N^{5-10}-methenyltetrahydrofolate and N^{10}-formyltetrahydrofolate to N^5-formyltetrahydrofolate [7, 8]. If the serum contained significant quantities

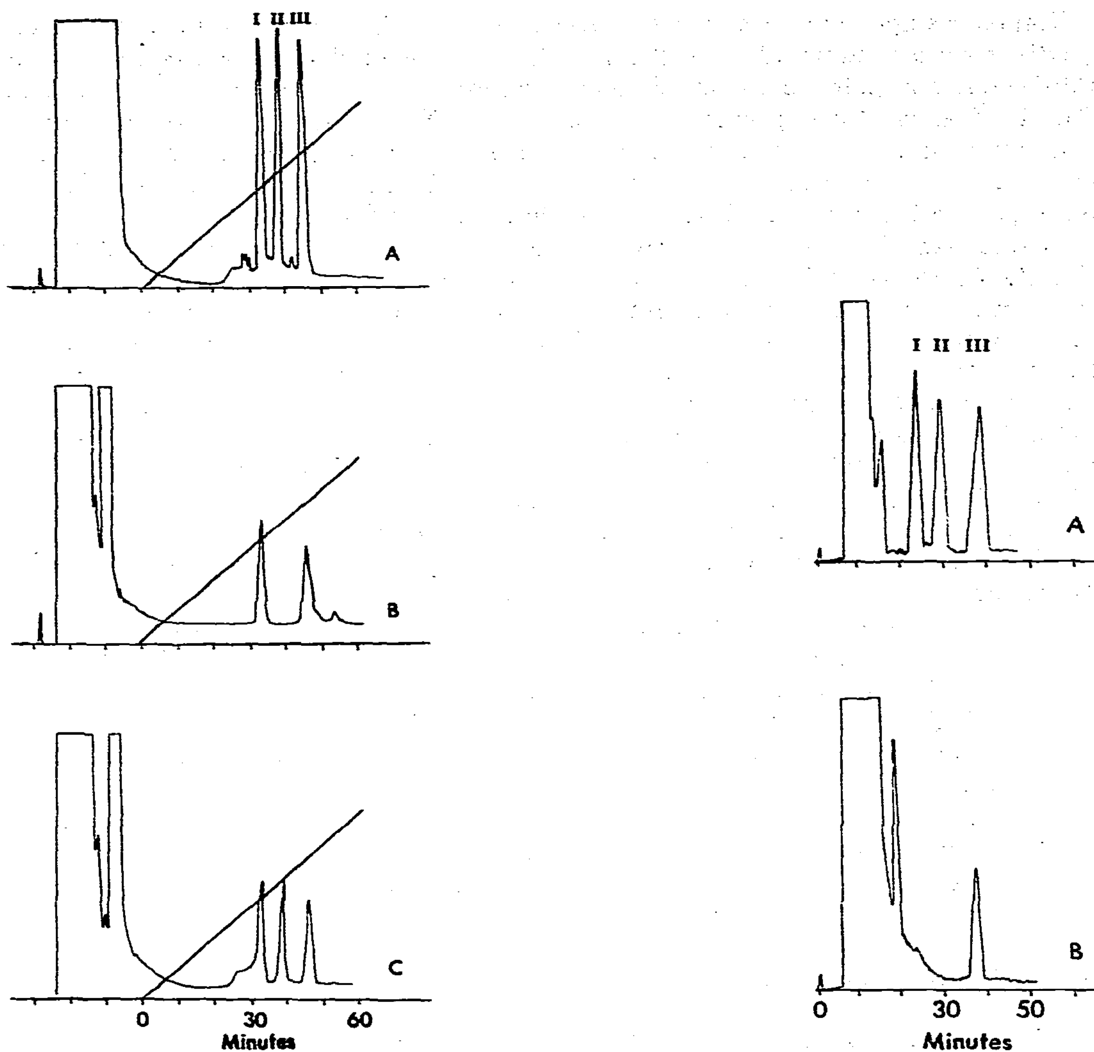

Fig. 1. Ion-exchange separations of folic acid compounds. Standards and serum samples were chromatographed on a Permaphase AAX column as described in Materials and methods. Conditions: mobile phase, potassium perchlorate gradient (0—0.006 *M* at 1%/min); flow-rate, 30 ml/h; column temperature, ambient; detector, UV photometer (280 nm). (A) Chromatography of reference compounds; I = N^5-formyltetrahydrofolic acid (100 ng); II = N^5-methyltetrahydrofolic acid (100 ng); III = pteroylglutamic acid (100 ng). (B) Chromatography of serum samples from subjects pretreated with folate. (C) Chromatography of serum samples to which [^{14}C]-N^5-methyltetrahydrofolic acid has been added.

Fig. 2. Ion-pair partition chromatography of folic acid compounds. Compounds were chromatographed on a μ-Bondapak C_{18} column as described in Materials and methods. Conditions: mobile phase, 0.005 *M* Pic A reagent (pH 7.5) in methanol—water (30:70); flow-rate, 30 ml/h; column temperature, ambient; detector, UV photometer (280 nm). (A) Chromatography of reference compounds; I = dihydrofolic acid (100 ng); II = N^5-formyltetrahydrofolic acid (100 ng); III = pteroylglutamic acid or N^5-methyltetrahydrofolic acid (100 ng). (B) Chromatography of serum samples from subjects pretreated with folate.

of these convertible forms, the resultant N^5-formyltetrahydrofolate peak would have produced a greater growth response than we observed. The identity of this peak is still in question.

The second peak had a retention time similar to that of our pteroylglutamic acid standard and its identity as pteroylglutamic acid was confirmed by microbiological assay. The eluted material supported the growth of *L. casei* and of *S. faecalis*, but not of *P. cerevisiae*. To confirm that our material was pteroylglutamic acid and not N^5-methyltetrahydrofolic acid, a sample of [^{14}C]-N^5-methyltetrahydrofolic acid was added to serum samples prior to our chromatographic separation. The counts were recovered in the elution fraction representing a peak (Fig. 1C) which did not exist with serum alone.

In the course of our ion-exchange separations, we discovered that dihydrofolic acid eluted in a position identical to that of pteroylglutamic acid. Both folate forms support the growth of *L. casei* and *S. faecalis*. To confirm the identification of the serum peak as pteroylglutamic acid and not dihydrofolic acid, we chromatographed our materials on an ion-pair partition system.

Fig. 2A shows the elution pattern for dihydrofolic acid (peak I), N^5-formyltetrahydrofolic acid (peak II) and pteroylglutamic acid or N^5-methyltetrahydrofolic acid (peak III) in the partition system. N^5-Methyltetrahydrofolic acid and pteroylglutamic acid cannot be separated by this technique, but they can be differentiated by microbiological assays.

When the sera from the subjects pretreated with pteroylglutamic acid were chromatographed in this system, the results in Fig. 2B were obtained. The peak eluting after ascorbic acid has a retention time comparable to that of either N^5-methyltetrahydrofolic acid or pteroylglutamic acid, but not to that of dihydrofolic acid. Results from microbiological assays suggest that the material is pteroylglutamic acid and not N^5-methyltetrahydrofolic acid; the peak fraction supported the growth of *L. casei* and *S. faecalis* but not *P. cerevisiae*.

In summary, our experiments were designed to determine the feasibility of using HPLC techniques to study serum folate and to resolve the controversy concerning the identity of the folate appearing in the serum after the oral administration of pteroylglutamic acid. Some reports have suggested that pteroylglutamic acid is absorbed unchanged [9, 10, 11]; some suggest that it appears as N^5-methyltetrahydrofolic acid [12]; some have measured a combination of both pteroylglutamic acid and N^5-methyltetrahydrofolic acid [13, 14]; and some have suggested that there is a gradual conversion to N^5-methyltetrahydrofolic acid 3 h after the administration of folate [15, 16].

Our preliminary investigations show that liquid chromatographic techniques can be used to study serum folates after the oral administration of folic acid; our results with both ion-exchange and ion-pair partition chromatography suggest that most of the administered folate appears unchanged in the serum 4 h later. The presence of smaller amounts of other folate forms is not excluded since the lower limit of detection in our system is 3 ng per 50 μl.

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