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Note

High-performance liquid chromatographic separation of clinically important folic acid derivatives using ion-pair chromatography

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Within the last ten years, there has been an increased clinical use of folate metabolites, particularly 5-formyltetrahydrofolic acid (5-formyl FH_4, citrovorum factor), in various rescue protocols associated with the use of high doses of antifolate cancer chemotherapeutic drugs such as methotrexate. At present, however, there are no satisfactory therapeutic guidelines for the use of citrovorum factor as a rescue agent. In theory, citrovorum factor should be administered immediately after circulating methotrexate has fallen below the effective level of tumor cell kill and be maintained until endogenous serum folates are maintained at normal levels[3]. The rapid qualitative and quantitative measurement of naturally occurring serum folates (pteroylmonoglutamates) would therefore be useful in determining the extent of the *in vivo* inhibition of dihydrofolate reductase, and the subsequent drug induced decrease and eventual recovery of the formation of the various tetrahydrofolate derivatives. In addition, the simultaneous determination of the pharmacokinetics of both 5-formyl FH_4 and 5-methyl FH_4, the major circulating reduced serum folate derivative[4], together with the actual measurement of antifolate drug levels may provide a new parameter for evaluation of the therapeutic effectiveness of high dose antifolate–citrovorum factor rescue combinations.

Identification of the various forms of folate in biological materials has been a problem due to inadequate separation techniques. Therefore, a method is required that is more rapid than microbiologic assays[5], more sensitive than traditional DEAE-cellulose column chromatography[6] and more convenient than presently available radioimmunoassay techniques which by design can determine only a single specific drug or metabolite with each assay procedure[7].

In this paper, we describe a high-performance liquid chromatography (HPLC) method developed for the qualitative and quantitative separation of folic acid, dihydrofolate, 5-formyl- and 5-methyltetrahydrofolates and *p*-aminobenzoyl-L-glutamic acid (PABG), a degradation product of the folates.

MATERIALS AND METHODS

Chromatographic apparatus

A Spectra-Physics Model 8000, microprocessor-controlled high-performance

liquid chromatograph was equipped with a Shoeffel Model 770 variable-wavelength UV detector, set at 285 nm, and a manual injector with a 10 μl sample loop. The column used was a Spectra-Physics, ODS 5 μm column (4.6 mm × 25 cm) and was eluted with a degassed, Millipore-filtered (0.45 μm) solution of methanol and water, each containing 0.005 *M* tetrabutylammonium phosphate (PIC A; Waters Assoc., Milford, Mass., U.S.A.) using the gradient system described in Table I at a flow-rate of 1.0 ml/min. A guard column (7 × 0.2 cm), packed with Co:Pell ODS 25–37 μm (Whatman, Clifton, N.J., U.S.A.) was installed to protect the 5 μm column.

TABLE I

SOLVENT GRADIENT FOR COLUMN ELUTION

Time (min)	*% Water**	*% Methanol**
0.0	85.0	15.0
10.0	65.0	35.0
15.0	60.0	40.0
20.0	30.0	70.0
23.0	30.0	70.0

* Contains 0.005 *M* tetrabutylammonium phosphate (PIC A).

Chemicals

PABG, folic acid (FA) and 5-methyl FH_4 were purchased from Sigma (St. Louis, Mo., U.S.A.). 5-Formyl FH_4 was a gift from the National Cancer Institute. Dihydrofolate was prepared according to the method of Futterman[8]. Ascorbic acid and HPLC grade water and methanol were purchased from J. T. Baker (Phillipsburg, N.J., U.S.A.).

5-Formyl- and 5-methyltetrahydrofolate stock solutions were made by dissolving the compounds in HPLC grade water. FA and PABG stock solutions were made by suspending the compounds in HPLC grade water. Addition of base (NaOH) solubilized the compounds, and the pH of the resultant solution was brought to 7 by the addition of acid (HCl). Dihydrofolate was prepared as a suspension[8] and diluted in HPLC grade water. The concentration of dihydrofolate was estimated using Beer's law and the extinction coefficient of the compound at 282 nm[9]. Ascorbic acid was added to the folate solutions as an antioxidant, at a final concentration of 1 mg/ml. The stock solutions were stored at −70° and remained stable for several months. All dilutions of these stock solutions were carried out using HPLC grade water containing ascorbic acid at 1 mg/ml. The solutions of methanol and water containing PIC A were made according to the procedure described by Waters Assoc.[10].

RESULTS AND DISCUSSION

Fig. 1 illustrates the separation obtained when a mixture of FA, FH_2, PABG, 5-formyl FH_4 and 5-methyl FH_4 was injected onto the column and eluted under the conditions described in Table I. Standard curves were obtained for folic acid and its reduced derivatives by plotting peak height (cm) *versus* the amount of the compound injected onto the column (μg). These relationships were shown to be linear over a ten-fold concentration difference, with excellent correlation coefficients (see Fig. 2).

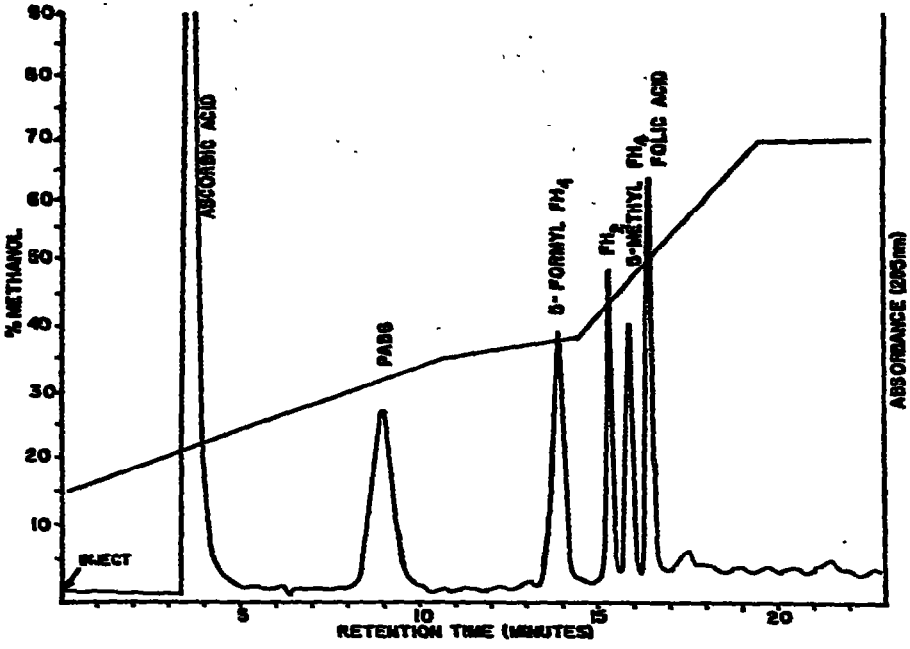

Fig. 1. Chromatographic separation of folic acid compounds on ODS (C_{18}) column. Conditions: column, 25 cm × 4.6 mm I.D.; mobile phase, methanol–water–PIC A as in Table I; flow-rate, 1.0 ml/min; column temperature, ambient; detector, Schoeffel 770 variable-wavelength UV (285 nm) at 0.1 a.u.f.s. Sample: PABG (50 ng); 5-formyl FH_4 (100 ng); FH_2 (34 ng); 5-methyl FH_4 (40 ng); folic acid (40 ng).

The minimum levels detectable at a detector setting of 0.01 a.u.f.s. (absorbance units full scale) and a recorder attenuation of 1.0 are listed in Table II along with previously published results[5,11,12]. The microbiological assay listed is much more sensitive than the HPLC technique, however, at present the quantitation of 5-methyl FH_4 by microbiologic technique must be done by differential assay utilizing at least two strains of *Lactobacillus casei*[5]. Not only is this a time-consuming method, but it also precludes the simultaneous determinations of the various reduced folate derivatives that can be easily achieved by HPLC technique.

TABLE II

SENSITIVITY OF VARIOUS METHODS FOR DETECTION OF FOLATES

Data represent the minimum levels (μg) detectable under the conditions described in each paper.

Compound	*This work*	*Ref. 11*	*Ref. 12*	*Ref. 5*[a]
Folic acid	0.025	0.02	0.05	0.005
Dihydrofolate	0.015	0.10	0.10	—
Tetrahydrofolate	—	0.02	0.20	—
5-Methyl FH_4	0.025	—	—	0.005
5-Formyl FH_4	0.05	0.10	—	0.001
PABG	—	—	0.02	—

[a] Microbiologic assay; data in μg/ml.

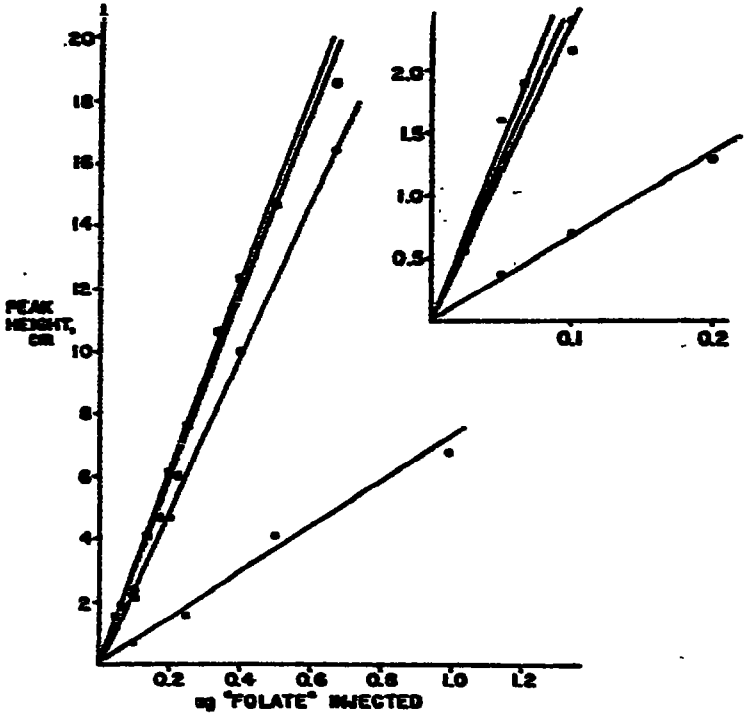

Fig. 2. Calibration curves for folic acid compounds: ■—■, folic acid ($r = 0.998$); ●—●, 5-methyl FH_4 ($r = 0.999$); □—□, dihydrofolic acid ($r = 0.997$); ○—○, 5-formyl FH_4 ($r = 0.993$). Each point represents one sample injected onto the column. The r values were calculated using the least-squares regression line. Inset: sensitivity of the method.

The reproducibility of the HPLC method was checked by injecting six samples of 5-formyl FH_4 of the same concentration, and then measuring the retention time and heights of the eluted peaks. 5-Formyl FH_4 was chosen as its standard curve had the lowest correlation coefficient. The standard error of the retention time was found to be less than 0.5% of the mean (14.5 ± 0.07 min), and the standard error of the peak height was less than 2.5% (7.4 ± 0.10 cm).

Ascorbic acid rather than mercapthoethanol was used as an antioxidant, because variation in the concentration of the latter has been reported to affect the relative retention times of the early peaks[13]. We also found that the relationship between peak height and the concentration of folate injected was not linear at lower folate concentrations and led to erroneous results.

The wavelength setting of 285 nm was chosen on the basis of the absorption maxima of the folates investigated. The compounds of interest all exhibit a UV maximum of 285 ± 10 nm[9].

The elution of tetrahydrofolate (FH_4), methotrexate (MTX) and an investigational lipid-soluble antifolate drug, metoprine [2,4-diamino-5-(3', 4'-dichlorophenyl)-6-methylpyrimidine; DDMP], under these conditions was also investigated. The FH_4 was found to be unstable even in the presence of antioxidants, giving several peaks occurring between 10 and 18 min in the chromatogram. Methotrexate and DDMP do

not show any peaks under the conditions described in this paper. These compounds may be bound to the column, however, the column may be flushed daily with 10–15 volumes of 100% water followed by a similar volume of 100% methanol, thereby extending the useful life of the column to several months.

The application of this method to the determination of serum levels of reduced folates in rats and humans, before and after receiving the folate analogs DDMP or MTX is now under investigation in this laboratory. Research on the effect of administration of folic acid or 5-formyl FH_4 on serum reduced folate levels is also in progress. Preliminary studies have shown that the serum may be injected onto the columns without prior extraction and that the elution of the folate compounds from the serum sample is identical to that obtained with the water-based standard samples.

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