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Note

Separation of folate derivatives by *in situ* paired-ion high-pressure liquid chromatography

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Methodology for the determination of the folate content of foods, biological fluids, and tissues is lengthy and often non-specific. Physiological levels of folacin are in the nanogram range, and until recently the microbiological assay[1] has been the method of choice since it has demonstrated the required sensitivity. The microbiological procedure suffers from a variety of shortcomings, particularly the length of time required to complete the assay, poor reproducibility of separate assays, and the extreme care that must be taken to obtain accurate values[2]. Moreover, this assay does not distinguish between the various folate forms present in biological samples. It is necessary to separate the various folate forms because there is evidence that they differ in biological activity and bioavailability[3,4].

Determination of the various biochemical forms of folate can be accomplished by the use of chromatographic procedures. Several methods using high-pressure liquid chromatography (HPLC) for the separation of folate derivatives have recently appeared[5-9]. Only one such method was applicable to biological samples[9]. However, it employed an anion-exchange column with the inherent difficulties of short column life, pH sensitivity, and lengthy column regeneration time.

We describe a new approach to the separation of folates which is particularly suited to biological systems. The method reported herein employs *in situ* generation of the paired-ion derivatives of folate to separate folic acid, 7,8-dihydrofolic acid, 5,6,7,8-tetrahydrofolic acid, 5-methyltetrahydrofolic acid, and *p*-aminobenzoyl-glutamic acid after prior removal of interfering substances in biological samples on the same column. Although there is an increase in retention time for all the folates, separation efficiency is not sacrificed because the folates are retained on the column as ion pairs. Many of the problems associated with folate lability are overcome by minimal sample preparation.

EXPERIMENTAL

Liquid chromatography

An HPLC system composed of a Tracor Model 980A solvent programmer,

Tracor Model 950 chromatographic pump, Rheodyne Model 7120 injector, and Waters Assoc. Model 440 absorbance integrator was used. The column (30 cm × 3.9 mm I.D.) was packed with μBondapak phenyl (10 μm) (Waters Assoc., Milford, Mass., U.S.A.). In addition, a precolumn (12.5 × 3.0 mm, I.D.), packed with μBonda-pak phenyl/Corasil (30 μm), was used (Water Assoc.). Injections were made at ambient temperature from the 20-μl loop of the Rheodyne injector. Two consecutive isocratic eluents were employed. Initially the column was equilibrated with mobile phase containing a phosphate buffer and composed of: 0.10 M potassium dihydrogen phosphate, 0.082 M sodium hydroxide, 0.005 M tetrabutyl ammonium phosphate (PIC A), and 1.2% methanol, pH 7.2. Immediately upon injection, the second mobile phase was employed. It had the following composition: 0.036 M sodium perchlorate, anhydrous (G. Frederick Smith Co., Columbus, Ohio, U.S.A.), 0.00013 M potassium dihydrogen phosphate, 0.0009 M potassium hydroxide, 0.5% methanol, pH 7.2, adjusted with 1.0 M potassium hydroxide. Mobile phases were filtered through a 0.22-μm filter prior to use. All water used in preparation of mobile phases was treated to remove organic and inorganic impurities using a Nanopure water purification system (Barnstead Sybron Corp., Boston, Mass., U.S.A.). A flow rate of 1.0 ml/min was maintained through the column with a resultant pressure of about 1000 p.s.i.

Standard compounds

Folic acid (FA), 7,8-dihydrofolic acid (FH$_2$), 5,6,7,8-tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid (5-CH$_3$THF), and *p*-aminobenzoylglutamic acid (PABG) were purchased from Sigma (St. Louis, Mo., U.S.A.). Standard solutions were prepared by dissolving each compound in 0.01 M potassium dihydrogen phosphate, pH 7.5, containing 0.003% ascorbic acid and a trace of mercaptoethanol to inhibit oxidative decomposition. A trace of ammonium hydroxide was added to facilitate dissolution of 5,6,7,8-tetrahydrofolic acid. Standards were prepared immediately prior to use, protected at all times from light, and were kept cold.

RESULTS AND DISCUSSION

Folic acid, 7,8-dihydrofolic acid, 5-methyltetrahydrofolic acid, 5,6,7,8-tetra-hydrofolic acid, and *p*-aminobenzoylglutamic acid were easily separated with a mobile phase consisting of a perchlorate–phosphate buffer on a μBondapak phenyl column. However, while working with this system in complex biological matrices (in our case, food and human milk samples) numerous endogenous sample components co-chromatographed with the various folate forms. To overcome this problem, the column was equilibrated with PIC A–phosphate buffer prior to injection. Ion-pairs of the various folate forms were formed, *in situ*, and retained on the column, until interfering endogenous substances were removed with the initial mobile phase. Since the nature and quantity of interfering substances will vary for each sample type, the elution of the folate ion-pairs from the column may be adjusted by delaying the onset of the perchlorate–phosphate buffer. As can be seen in Fig. 1, even though the elution time of the components is increased by this method, the relative separation or reso-lution of the components is not impaired. Retention times for the various folate forms are increased from 6–12 min to 9–36 min or more depending on the time of onset of the perchlorate phosphate mobile phase. This delay, however, does not impair peak
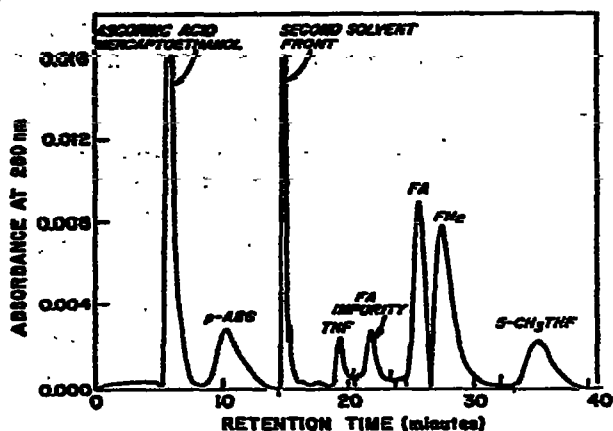
Fig. 1. Chromatogram of 5 folate forms obtained by isocratic elution at pH 7.2 on μBondapak phenyl column preequilibrated with PIC A–phosphate buffer. Eluent: 0.036 *M* sodium perchlorate, 0.001 *M* sodium phosphate buffer pH 7.2; flow-rate, 1.0 ml/min; inlet pressure, 740 p.s.i.; ambient temperature, 200 ng each of *p*-aminobenzoylglutamate (*p*-ABG), 5,6,7,8-tetrahydrofolic acid (THF), folic acid (FA), 7,8-dihydrofolic acid (FH₂), and 5-methyltetrahydrofolic acid (5-CH₃THF), containing 0.003% ascorbic acid and traces of 2-mercaptoethanol and ammonium hydroxide.

resolution (see Fig. 1) nor does it broaden the folate peaks because the ion-pairs are tightly held on the column as long as the PIC A–phosphate buffer is being used.

We have used this approach to separate and quantitatively determine five forms of folate. The quantitative chromatographic parameters are presented in Table I. Standard curves were obtained for each folate by plotting peak area *versus* the amount of compound injected on the column. The curves for all four compounds were linear over the following ranges: FA, 2–400 ng, $r = 0.99$; FH₂, 0–400 ng, $r = 0.99$; 5-CH₃THF 0–400 ng, $r = 0.95$; and PABG 0–400 ng, $r = 0.98$ (Table I). The standard tetrahydrofolic acid which was available was only 80% pure, thus the

TABLE I

QUANTITATIVE DETERMINATION OF FIVE FOLATE FORMS USING HPLC

Quantitative determination of 5 folate forms obtained by isocratic elution at pH 7.2 on μBondapak phenyl column preequilibrated with PIC A–phosphate buffer. Eluent: 0.036 *M* sodium perchlorate, 0.001 *M* sodium phosphate buffer pH 7.2; flow-rate, 1.0 ml/min; inlet pressure, 740 p.s.i.; ambient temperature.

Folate derivative	Range of linearity (ng)	Linear regression equation*	Regression coefficient (r)
Folic acid	2–400	$y = 2.073x - 4.574$	0.99
7,8-Dihydrofolic acid	0–400	$y = 2.399x + 3.144$	0.99
p-Aminobenzoylglutamate	0–400	$y = 3.275x + 19.218$	0.98
5-Methyltetrahydrofolic acid	0–400	$y = 1.906x + 60.503$	0.95
Tetrahydrofolic acid**	50–250	$y = 1.024x - 51.580$	0.82

 * y = area count as reported on Hewlett-Packard Model 3380A integrator recorder, x = ng of folate compound injected.

 ** The amount injected for tetrahydrofolic acid has been adjusted for the presence of folic acid and 7,8-dihydrofolic acid in the sample. ·

standard curve was obtained by plotting peak area *versus* amount injected minus amounts of FA and FH_2 as determined from the chromatograph. This was linear over the range 50–250 ng, $r = 0.82$ (Table I).

The method reported here enables one to observe the integrity of the sample or standard, by observing the area of the peak at 4.54 min which decreases proportionately as the area of the peak at 14.33 min increases when the folates involved become oxidized. In the analysis of biological samples, protected from oxidative decomposition by the addition of ascorbic acid, sample quality can be readily determined.

Preliminary clean up procedures used on biological samples destroy or interconvert the various folate forms which are labile to heat, light, oxygen, and changes in $pH^{10,11}$. Traditional clean-up procedures are far more time consuming. The present method offers a clear advantage in the elimination of external clean-up procedures.

We are presently using this technique to quantify the folacin content of various foods. We are also making comparisons between this technique and both the standard microbiological assay[1] and the recently proposed radiometric assay[12].

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