Journal of Chromatography, 121 (1976) 100–103

CHROM. 9022

Note

Separation of folic acid derivatives by high-performance liquid chromatography

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(Received December 2nd, 1975)

Investigations of the distribution and metabolic role of naturally occurring folic acid derivatives (pteroylmonoglutamates) have relied largely on thin-layer chromatography or on classical ion-exchange chromatography with DEAE-cellulose as separation methods[1,2]. These techniques are time-consuming and often provide poor resolution. We present here a method for the separation of several important, naturally occurring folates, which uses high-performance anion-exchange chromatography. High-performance liquid chromatography (HPLC) has been previously used to separate folic acid from a mixture of other water-soluble vitamins[3] but has not been employed to separate the reduced and the N^5- or N^{10}-substituted folates which represent the natural coenzyme forms.

EXPERIMENTAL

Liquid chromatography

The following equipment and procedures were employed: A Milton Roy 5000-p.s.i. minipump fitted with an LDC Model 709 pulse damper; an LDC UV monitor fitted with a 254-nm detector; a Honeywell Electronik 196 recorder; a Varian 1000-p.s.i. injector with septum seal; No. 316 stainless-steel columns, 3 mm I.D., (Tube Sales, Cranbury, N.J., U.S.A.); column packing of AL-Pellionex-WAX 37–53 μm (Reeve Angel, Clifton, N.J., U.S.A.) (packed dry); an ambient column temperature; an eluent of 0.025 *M* sodium dihydrogen phosphate, pH 4.8, or 0.006 *M* sodium dihydrogen phosphate, pH 4.0 — deaerated by bubbling nitrogen gas; and a flow-rate of 1–1.5 ml/min at pressures up to about 1000 p.s.i. Samples were injected with a 50-μl gas-tight syringe (Hamilton).

Standard compounds

Folic acid, 7,8-dihydrofolic acid, 5,6,7,8-tetrahydrofolic acid, 5-methyltetrahydrofolic acid, and *p*-aminobenzoylglutamic acid were purchased from Sigma (St. Louis, Mo., U.S.A.). 5-Formyltetrahydrofolic acid was purchased from Grand Island Biological (Grand Island, N.Y., U.S.A.).

5,10-Methenyltetrahydrofolic acid and 10-formyltetrahydrofolic acid were prepared by the method of Rabinowitz[4]. 5,10-Methylenetetrahydrofolic acid was prepared by the method of Huennekens *et al.*[5].

RESULTS AND DISCUSSION

AL-Pellionex-WAX, a pellicular, weak anion-exchange material, consists of an aliphatic matrix with amino functional groups; we used it in this study because it was specifically developed to extend the classical weak anion-exchange chromatographic separations, such as DEAE-cellulose, to modern HPLC.

We initially examined the three oxidation states of unsubstituted folic acid. Reduced derivatives of folic acid are extremely labile to oxidation in air; for this reason, 2-mercaptoethanol is normally added to all solutions containing 7,8-dihydro and 5,6,7,8-tetrahydro derivatives. This reductant, however, absorbs at 254 nm and would thus interfere with detection of chromatographic peaks. In order to overcome the problem of oxidation during chromatography, nitrogen gas was first saturated with water vapor and then bubbled through the eluent buffer before being pumped through the column. In this way the chromatographic system was anaerobic, and oxidative decomposition of reduced folates was minimized. Sample solutions were normally prepared with a small amount of mercaptoethanol, which gave a narrow peak at the solvent front but did not otherwise interfere in the chromatographic separation. Care was taken in making up standard solutions to ensure complete dissolution of the folate derivatives. They were first dissolved in basic solution and the pH was then quickly brought down to about pH 7.0 with hydrochloric acid to retard oxidative decomposition.

Using a 4½-ft. column, with an eluent of 0.025 *M* sodium dihydrogen phosphate adjusted to pH 4.8, we achieved a base-line separation of tetrahydrofolic acid, dihydrofolic acid and folic acid (Fig. 1). A small peak was always present just before the tetrahydrofolic acid peak. This peak was shown to have both a retention time and a UV spectrum identical to *p*-aminobenzoylglutamate; it probably represented a small amount of contamination or decomposition[6] of the standard compounds.

Standard curves were obtained by plotting peak area *versus* the amount of derivative injected onto the column. The standard curves were linear, at least over the range 1–20 μg. The column appeared to overload when amounts greater than 20 μg of folate and dihydrofolate were injected onto it. The tailing of the peaks may have been caused, however, by the low solubility of the derivatives under the conditions of elution. Tetrahydrofolate showed no distortion of peak shape up to 40 μg injected onto the column. An estimate of the minimum levels of the three derivatives that can be quantitated under these conditions is 0.02 μg each of tetrahydrofolic acid and folic acid and 0.1 μg of dihydrofolate.

We next investigated methods for the rapid separation of naturally occurring and biochemically significant derivatives of tetrahydrofolic acid. Fig. 2 shows the separation, again using AL-Pellionex-WAX, of 5,6,7,8-tetrahydrofolic acid, *p*-aminobenzoylglutamate, and 5-methyl-, 5-formyl- and 5,10-methenyltetrahydrofolic acid.

The mobile phase in this case was again sodium dihydrogen phosphate, but the concentration required for the separation was only 0.006 *M* and the pH was 4.0. Under these conditions, oxidized derivatives of the folic acid compounds were not eluted, and the column could occasionally be stripped of dihydro and oxidized compounds by eluting with 0.1 *M* sodium dihydrogen phosphate, pH 4.0.

The peaks of the reduced derivatives showed no distortion up to about 50 μg of each compound injected onto the column. Minimum detectable levels varied

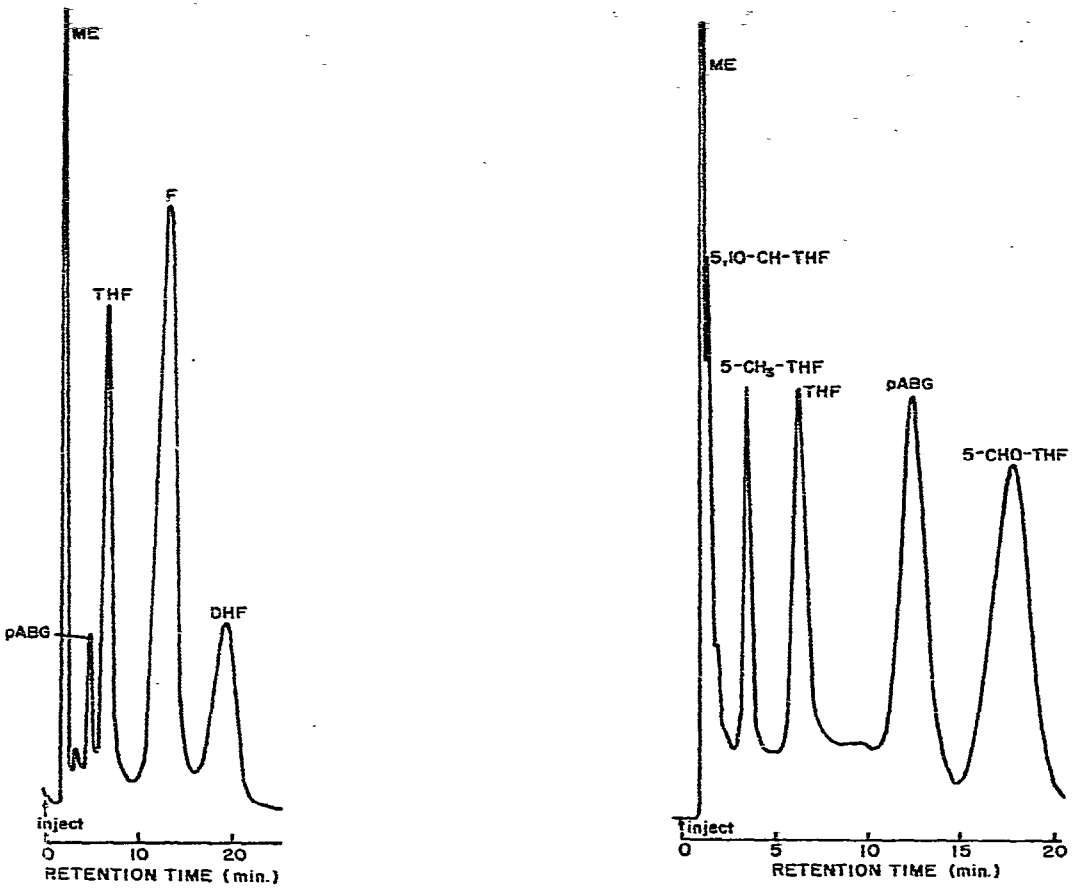

Fig. 1. Separation of three oxidation states of folic acid on AL-Pellionex-WAX. Chromatographic conditions: column, 4½ ft. × 3 mm I.D.; mobile phase, 0.025 *M* sodium dihydrogen phosphate, pH 4.8; pressure, 1000 p.s.i.; flow-rate, 1.3 ml/min; column temperature, ambient; detector, UV photometer (254 nm); attenuation, ×8. ME = 2-Mercaptoethanol; pABG = *p*-aminobenzoylglutamate; THF = 5,6,7,8-tetrahydrofolic acid (5 μg); F = folic acid (5 μg); DHF = 7,8-dihydrofolic acid (5 μg).

Fig. 2. Separation of derivatives of tetrahydrofolic acid on AL-Pellionex-WAX. Chromatographic conditions: column, 4½ ft. × 3 mm I.D.; mobile phase, 0.006 *M* NaH_2PO_4, pH 4.0; pressure, 1000 p.s.i.; flow-rate, 1.2 ml/min; column temperature, ambient; detector, UV photometer (254 nm); attenuation, ×8. ME = 2-Mercaptoethanol; 5,10-CH-THF = 5,10-methenyltetrahydrofolic acid (1 μg); 5-CH_3-THF = 5-methyltetrahydrofolic acid (2.5 μg); THF = 5,6,7,8-tetrahydrofolic acid (10 μg); *p*ABG = *p*-aminobenzoylglutamate (2.5 μg); 5-CHO-THF = 5-formyltetrahydrofolic acid (10 μg).

according to the peak width and absorptivity at 254 nm, but the estimates ranged from 0.02 μg for 5,10-methenyltetrahydrofolate to 0.1 μg for 5-formyltetrahydrofolate.

5,10-Methylenetetrahydrofolate was not retained on AL-Pellionex-WAX and eluted at the solvent front under all the investigated conditions of pH and ionic strength. Under the chromatographic conditions described for separating the other

tetrahydrofolate derivatives, 10-formyltetrahydrofolate eluted at the same retention time as tetrahydrofolate. It could be quantitated by acidification, however, in a mixture of other tetrahydro derivatives. Under acidic conditions, 10-formyltetrahydrofolate is quantitatively converted into 5,10-methenyltetrahydrofolate[7]. Re-chromatography of this acidified sample then yields a pure tetrahydrofolate peak, and 10-formyltetrahydrofolate can be estimated by subtraction.

These chromatographic methods will be useful tools in research on naturally occurring folic acid derivatives. Application of the methods to biological material and development of the chromatographic systems to include polyglutamate derivatives are currently in progress in this laboratory.

ACKNOWLEDGEMENT

This work was supported by Grant No. 493 from the Nutrition Foundation Inc., New York, N.Y.

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