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Note

Isocratic separation of food folacin by high-performance liquid chromatography

DAVID R. BRIGGS*, GWYN P. JONES and PANADDA SAE-EUNG
Department of Human Nutrition, Deakin University, Victoria 3217 (Australia)
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Naturally occurring folacin derivatives are a group of chemically related pteroylglutamates which differ in the oxidation/reduction state of the pteridine ring, the number of glutamyl units in the poly-γ-glutamyl side chain and substituents at the 5- and 10-positions[1]. Most studies for determining folacins have utilized a microbiological assay procedure[2]. Although these procedures have high sensitivity they are time-consuming and do not provide information about individual folacins.

High-performance liquid chromatography (HPLC) has been applied to the separation of some monoglutamyl forms of folacin. Good resolution of a number of folacin derivatives has previously been achieved using paired-ion chromatography but requiring non-linear gradient[1,3] and multi-solvent elevation[4,5]. A previous report of an isocratic system did not succeed in separating tetrahydrofolic acid and *p*-aminobenzoylglutamate[6]. We describe here a simple paired-ion isocratic procedure for the separation of the monoglutamyl forms of folacin derivatives most commonly occurring in food[7].

EXPERIMENTAL

Chromatographic system

A Waters Assoc. Model 6000A chromatograph fitted with Model U6K injector and a UV detector set at 280 nm was used. The column was an Alltech C_{18} (250 × 4.6 mm; particle size 10 μm) protected by a guard column (25 mm × 4.6 mm) containing C_{18}/Corasil (particle size 37–50 μm). The column was enclosed in a water jacket maintained at 28 ± 1°C. The solvent for the isocratic system comprised degassed, filtered (Millipore filter; porosity 0.45 μm) methanol (28 %) in water containing 0.005 *M* tetrabutylammonium phosphate (PIC A; Waters Assoc.). The solvent flow-rate was 1.0 ml/min at a pressure of 1500 p.s.i. Injections (10–45 μl) were made using a 50-μl SGE syringe.

Chemicals

Folic acid, 7,8-dihydrofolic acid, 5,6,7,8-tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5-formyltetrahydrofolic acid (folanic acid) and N-(*p*-aminobenzoyl)-L-glutamic acid (a folacin decomposition product) were obtained from Sigma (St. Louis, MO, U.S.A.). Solutions were freshly prepared by accurately weighing (Mettler

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H 20 balance) about 1 mg of the compound of interest and dissolving it in 50.0 ml of 0.1 *M* phosphate buffer (pH 7.0) containing 0.3% mercaptoethanol, which was then degassed. During determinations the solutions were protected from the light and maintained at ice temperature.

RESULTS AND DISCUSSION

Oxidative deterioration of the folacin solutions was minimized using mercaptoethanol. 5,6,7,8-Tetrahydrofolic acid has been reported as unstable in the presence of ascorbic acid[3]. Using mercaptoethanol the solutions were stable over the period required for chromatography.

As shown in Fig. 1, separation of the main forms of folacin known to occur in food was achieved. Folic acid and 5-methyltetrahydrofolic acid were not completely resolved, but folic acid has not previously been reported to be naturally present in food[7]. In addition the degradation product common to the folacins, N-(*p*-aminobenzoyl)-L-glutamic acid, was successfully separated. This procedure has the advantage over previously reported systems in that separation is achieved uninterrupted, using a single pump.

An additional advantage of our procedure is that fixed-wavelength detection at 280 nm can be used. This wavelength is close to the maximum absorption for each

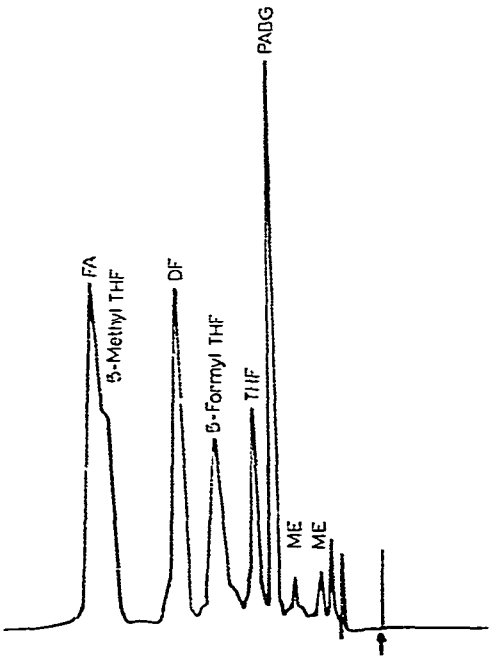

Fig. 1. Chromatographic separation of folacin on Alltech C_{18} column. Conditions: mobile phase, methanol–water (28:72) + 0.005 *M* tetrabutylammonium phosphate; flow-rate, 1 ml/min; detector, UV detector at wavelength 280 nm; column temperature, 28°C. PABG = *p*-aminobenzoylglutamic acid; THF = tetrahydrofolic acid, 5-Formyl THF = 5-formyltetrahydrofolic acid; DF = dihydrofolic acid; 5-Methyl THF = 5-methyltetrahydrofolic acid; FA = folic acid; ME = mercaptoethanol.

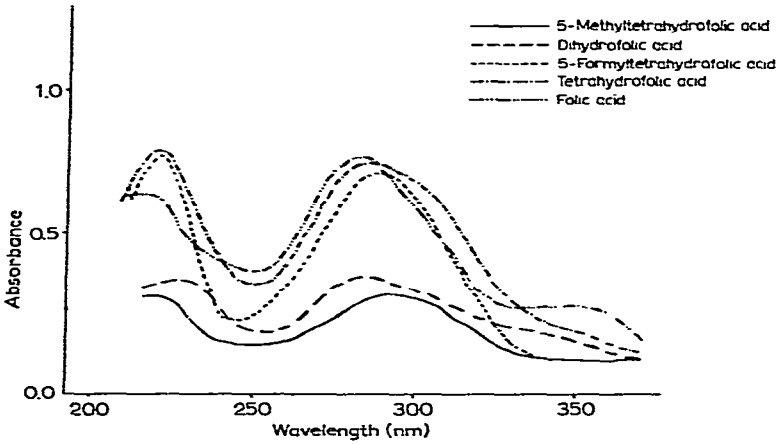

Fig. 2. Spectra of folacin compounds.

compound in 28% methanol in water containing 0.005 *M* tetrabutylammonium phosphate (Fig. 2). Calibration curves for each compound showed a linear response for peak height *versus* concentration over the range 0.05 μg to 1.0 μg. The correlation coefficients, *r*, are high and are shown in Table I.

TABLE I

QUANTITATIVE CHROMATOGRAPHIC PARAMETERS OF FOLACIN

Folacin compounds	*0.1–1 μg of standard compounds*		*0.01–0.05 μg of standard compounds*	
	Linear regression equation	*r*	*Linear regression equation*	*r*
Folic acid	$y = 18.67x + 0.50$	0.99	$y = 241.79x + 0.97$	0.99
Dihydrofolic acid	$y = 20.80x + 0.20$	0.99	$y = 492.16x + 0.34$	0.99
Tetrahydrofolic acid	$y = 14.96x + 0.36$	0.99	$y = 446.74x + 0.47$	0.99
5-Formyltetrahydrofolic acid	$y = 21.64x + 0.67$	0.99	$y = 412.93x + 1.11$	0.99
5-Methyltetrahydrofolic acid	$y = 14.54x + 0.12$	0.99	$y = 364.51x + 0.12$	0.99
p-Aminobenzoylglutamic acid	$y = 68.48x + 0.14$	0.99	$y = 1828.04x + 0.60$	0.99

In applying this procedure to food folacin we intend to use ion-exchange chromatography prior to HPLC. This will allow both an initial clean-up of the sample extract and a concentration of the low levels of folacin naturally present in food.

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