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Note

Stabilization of 5-methyltetrahydrofolic acid and subsequent analysis by reversed-phase high-performance liquid chromatography

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The determination of food folates is a difficult problem because of the very low concentration of folic acids which differ in the oxidation/reduction state of the pteridine ring, the substituents at the 5- and 10-positions and the number of glutamyl units in the poly-γ-glutamyl side-chain. In the human organism, reduced folates play an important role in the transfer of one-carbon units; among these vitamers 5-methyltetrahydrofolic acid (5-CH_3H_4 folic acid) is the most naturally occurring folacin active substance. It is, however, susceptible to oxidation by air and degradation by thermal treatment[1–4]. Therefore, a serious problem exists in stabilizing 5-CH_3H_4folic acid and other labile folates within the different analysis steps to obtain the native pattern for folates in food.

Conventional microbiological assays are time-consuming and do not provide reliable results[5,6]. Only "free" and "bound" folic acids can be determined but exact differentiation between folacin vitamers possessing different biological activity and bioavailability is impossible[7]. Stabilization procedures have often been neglected so that most food tables still reveal contradictory values for folates in food[8].

An appropriate method for analysis of different folic acids seems to be high-performance liquid chromatography (HPLC), although up to now the sensitivity of UV-monitoring of folates in the eluate is proclaimed lower than that of microbiological assay. However, a previous study demonstrated that HPLC analysis with fluorimetric detection and post-column derivatization has a sensitivity comparable with that of the *Lactobacillus casei* procedure for folic acids[9].

Several HPLC methods have been employed for the analysis of folates: ion-exchange chromatography succeeds in separating folic acids, but this system has the disadvantages of inconsistency due to the influence of pH and temperature and long regeneration periods of the packing material[10]. Other investigators used ion-pair reversed-phase HPLC; however, they did not succeed in separating 5-CH_3H_4folic acid from folic acid (pteroylmonoglutamic acid)[11–14]. For this purpose more compli-

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cated methods have been developed, but with limited success[15–17]. Good resolution between the different folic acids can be achieved using ionic suppression reversed-phase HPLC, but the eluents had to be adjusted to pH 4.0[18] or 2.3[19–21]. These ascidic conditions, however, have a destructive effect on folates even in the presence of stabilizing agents[22]. Therefore, the isocratic HPLC methods of Branfman and McComish[11] and Briggs *et al.*[12] were modified so that sufficient separation of 5-CH_3H_4folic acid from folic acid could be achieved at neutral pH[23].

To protect 5-CH_3H_4folic acid from degradation by the thermal treatment usually applied during preparation of food samples in vitamin assay, we developed stabilizing procedures (elimination of oxygen by ultrasonification and flushing with nitrogen gas, addition of stabilizing agents) that were efficient even at a high temperature. In using stabilizing agents, *e.g.* antioxidants, the subsequent HPLC method had to be optimized to achieve sufficient separation of these substances from folates and their degradation products. Therefore, an ion-pair reversed-phase HPLC method with gradient elution was developed for the precise determination of folates and degradation products with high resolution, high recovery, low detection limit and good reproducibility.

EXPERIMENTAL

5-CH_3H_4folic acid (barium salt) and the other folates were purchased from Sigma (Taufkirchen, F.R.G.) and used without further purification. Standard solutions were made by dissolving 1 mg of folate (1.7 μmol) in 100 ml of either 0.05 *M* Tris–HCl buffer, pH 8.0 (analytical reagent grade; Merck, Darmstadt, F.R.G.) or in a mixture of methanol (HPLC grade; Roth, Karlsruhe, F.R.G.) and Tris–HCl buffer (30:70, v/v).

To stabilize folic acids, sodium ascorbate (analytical reagent grade; Merck) was added to the solutions in a final concentration of 1, 5, 10, or 15 m*M*. To eliminate oxygen, samples were subjected to ultrasonification for 5 min followed by flushing with nitrogen gas for 15 sec. All folate solutions used were stable for at least 8 days when stored at −24°C.

The HPLC equipment (Waters Assoc., Milford, MA, U.S.A.) consisted of two solvent delivery systems (Model 6000 A), an injector (Model U6K), a solvent programmer (Model 660) and a UV device (Pye Unicam, Cambridge, U.K.) set to 280 nm (0.04 a.u.f.s.). An analytical column with a guard column was used (250 × 4.6 mm I.D.; 30 × 4.6 mm I.D.; Knauer, Bad Homburg, F.R.G.), each factory-packed with LiChrosorb RP-18 (particle size, 10 μm; Merck).

Two solvents were used: solvent A consisted of 5 m*M* tetrabutylammonium phosphate in double-distilled water, pH 7.4 (PIC A®; Waters Assoc.); solvent B consisted of 5 m*M* tetrabutylammonium phosphate in a mixture of methanol (HPLC grade; Roth) and double-distilled water (30:70, v/v). Prior to use, solvents were filtered through 0.5-μm Fluoropore® filters and degassed by heat, vacuum and ultrasonification.

For gradient elution, a linear continuous gradient over 45 min was employed, from 50:50 A:B to 0:100 A:B at a flow-rate of 1 ml/min. Isocratic elution was performed using solvent B.

The injection volume was 50 μl. Quantification of eluted folates was accomplished by estimation of peak height.

RESULTS AND DISCUSSION

Since thermal treatment and incubation procedures are necessary for extraction of folates from food samples, considerable losses of unstable folic acids will occur[5,9]. To protect the principal naturally occurring folacin vitamer, 5-CH_3H_4folic acid, from oxidative and thermal destruction we therefore looked for stabilizing agents that do not interfere with HPLC analysis. Folic acids were dissolved in Tris–HCl buffer and methanol, and antioxidants were used as stabilizing agents to prevent the destruction of 5-CH_3H_4folic acid. Dithiothreitol (Cleland's reagent), 2-mercaptoethanol and sodium ascorbate were tested. Sodium ascorbate proved to be the most suitable antioxidant: a concentration of 10 m*M* sodium ascorbate in 0.05 *M* Tris–HCl buffer stabilized 5-CH_3H_4folic acid during 60 min of heating (100°C). In a mixture of methanol and 0.05 *M* Tris–HCl buffer (30:70, v/v), a concentration of 5 m*M* sodium ascorbate was sufficient to prevent any degradation of 5-CH_3H_4folic acid. The other antioxidants tested, which had to be used in much higher concentrations to have the same stabilizing effect as sodium ascorbate, interfered with the chromatographic separation.

In contrast to Wilson and Horne[16], who used a ten-fold concentration of antioxidant, we did not detect any interconversions of folates when sodium ascoarbate was added. Furthermore, the neutral pH of the eluents protected the folic acids from degradation during the separation procedure.

After modifying the methods of Branfman and McComish[11] and Briggs *et al.*[12] in initial studies (without application of antioxidants), we were able to determine 5-CH_3H_4folic acid, 5-formyltetrahydrofolic acid, folic acid and the degradation products pterin-6-carboxylic acid and *p*-aminobenzoylglutamic acid. With the isocratic elution system described above (flow-rate, 0.5 ml/min)[23], a good resolution of 5-CH_3H_4folic acid from folic acid, a low detection limit and a high recovery for 5-CH_3H_4folic acid (Table I) could be achieved.

This reversed-phase HPLC technique with an ion-pairing agent resulted in a

TABLE I

CHARACTERISTICS OF ISOCRATIC AND GRADIENT ELUTION HPLC FOR SEPARATING FOLIC ACIDS

	Isocratic elution	*Gradient elution*
Time required for one separation	40 min	50 min
Detection limit for 5-CH_3H_4folic acid*	3.6 ng (6.1 pmol)	4.8 ng (8.1 pmol)
Column recovery (n = 2) for 5-CH_3H_4folic acid**	104%	81%
Resolution between 5-CH_3H_4folic acid and folic acid***	1.6	1.2

* The linearity of peak height amounts detected was tested in a range between 1.5 nmol and 6.1 pmol of folate. Comparison between sample amounts determined by the integral method and by peak height measurements led to the same results.

** Recovery was calculated from $\frac{\text{amount of sample determined after rechromatography}}{\text{amount of sample determined originally}} \times 100$.

*** The resolution, R, was calculated from $R = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2}$, where t_{R2} and t_{R1} are the retention times, and w_1 and w_2 the peak base widths. The resolution between all folates and degradation products tested was greater than 1.5.

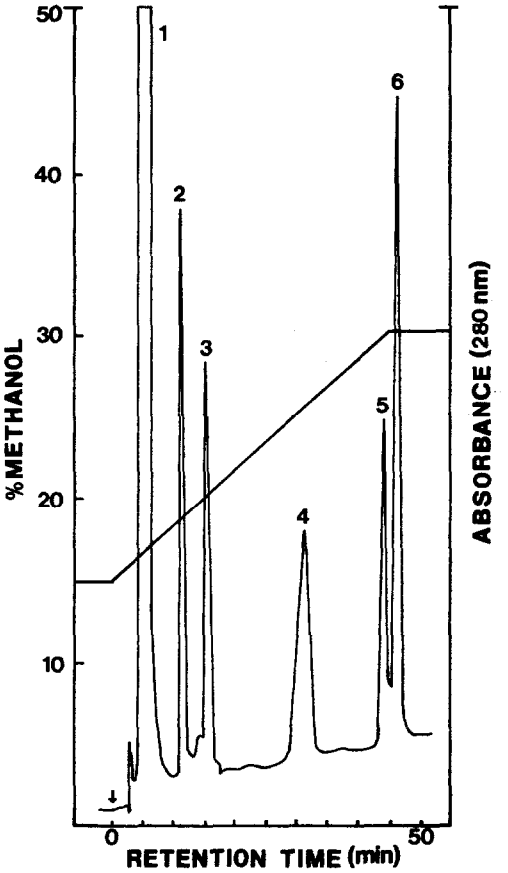

Fig. 1. Gradient elution HPLC of folates and degradation products. For chromatographic conditions see Experimental. Peaks: 1 = sodium ascorbate (250 nmol); 2 = pterin-6-carboxylic acid (1.2 nmol); 3 = *p*-aminobenzoylglutamic acid (0.99 nmol); 4 = 5-formyltetrahydrofolic acid (0.85 nmol); 5 = 5-methyltetrahydrofolic acid (0.85 nmol); 6 = folic acid (0.85 nmol).

better separation of folates and degradation products than a similar procedure with ionic suppression. When a 0.05 *M* ammonium acetate buffer (pH 4.5) was used instead of 5 m*M* tetrabutylammonium phosphate (pH 7.4) containing 6, 8, 10 or 12% (v/v) acetonitrile, 5-CH_3H_4folic acid was not detectable. This phenomenon is due to either complete destruction of this derivative at acidic conditions or to coelution with other substances.

Therefore, we optimized the ion-pairing reversed-phase HPLC method for determination of folic acids after stabilization. To obtain adequate separation of stabilizing substances from folates and degradation products, we tested several gradient elution methods using solvent B as organic modifier. With a linear gradient the resolution, recovery and detection limit were satisfactory (Fig. 1 and Table I). Compared with the isocratic elution system mentioned above, HPLC with gradient elution is superior with respect to the separation of stabilizing agents from folic acids and degradation products, but is time-consuming.

Both methods described are simple and efficient. For analysis of folates HPLC with isocratic elution is quite sufficient; if additional determination of degradation products is required and stabilizing agents are needed then HPLC with gradient elution is preferable. The linear continuous gradient is superior to step increment of the organic modifier during the separation procedure because of five-fold lower detection limits (*cf.* Allen and Newman[24]).

Preliminary studies have demonstrated that both methods described are suitable for fluorimetric detection of folates at lower detection limits. Therefore these methods are to be applied to food extract solutions where fluorimetric detection will be necessary.

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