TWO IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF BIOLOGICALLY SIGNIFICANT FORMS OF FOLATE*

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SUMMARY

Two new separations of folate derivatives by *in situ* paired-ion high-performance liquid chromatography are described. The first utilizes two C_{18} phenyl columns and the second utilizes a C_8 radial compression cartridge. Folic acid, dihydrofolic acid. folinic acid, tetrahydrofolic acid. 5-methyltetrahydrofolic acid and a decomposition product. *p*-aminobenzoylglutamic acid are separated. A comparison is made between these two methods and with the standard microbiological *Lactobacillus casei* assay for folates. The first method offers improved resolution and *in situ* sample clean-up. The second method accomplishes this in one-fifth the time. Both methods provide information not obtainable by the microbiological assay.

INTRODUCTION

Interest in a satisfactory method for the determination of folacin in biological samples stems from the critical role that this B-vitamin plays in the biosynthesis of nucleic acids. Accordingly, it is a prime requisite for cellular division and growth. Methodological problems arise from the fact that folacin actually comprises a large family of natural products of the parent folic acid (pterolylglutamic acid). At least three reduced states of the pyrazine ring, six different one-carbon substituents present in the position N^5 and/or N^{10}, and the glutamyl residue linked in gamma peptide linkage to a poly-γ-glutamyl side-chain of unknown and likely, varied length, exist in nature[1].

The method of choice of folacin assay has been microbiological[2]. using *Lactobacillus casei* because it allows measurement of total folates at the nanogram level, the typical physiological concentration. This method, however, has several drawbacks, most notably the length of time required to complete the assay, poor reproducibility of separate assays, the extreme care that must be taken to avoid erroneously high or low values. and the lack of specificity for the various folate forms that are present in

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biological samples[3-5]. The necessity to separate the forms of folate is central because of the evidence that they differ in biological activity and bioavailability.

We recently reported a technique to separate folate forms by *in situ* paired-ion high-performance liquid chromatography (HPLC)[6]. Although we are able to apply this method to some biological samples, it became clear that there was still a great deal of chromatographic interference from other sample components. To facilitate the removal of interfering sample components, we have developed two improved techniques. These both employ *in situ* generation of the paired-ion derivatives of folate to separate folic acid, folinic acid, 7,8-dihydrofolic acid, 5,6,7,8-tetrahydrofolic acid and 5-methyltetrahydrofolic acid. The first of these methods utilizes two C_{18} phenyl columns as a direct extension of our previous separation. The second method uses a C_8 radial compression cartridge. A comparison is made between these methods and with the microbiological assay.

Those forms of folate, regardless of the state of reduction or nature of the single carbon substituent, which contain more than two additional glutamyl residues, are not reliably detected by any of the standard microbiological procedures[1]. In addition, those folylpolyglutamates which support *L. casei* growth do not do so uniformly. Previously, it was assumed that chromatographic separations of the folate forms was a function of the pteridine ring, independent of the number of glutamic acid residues[7]. Thus, it was assumed that a complete chromatographic assay could be done on the native folates in biological samples. We have, however, separated folyltriglutamate and folylpentaglutamate from folic acid by both HPLC methods reported herein. Therefore, it is clear that biological samples containing a mixture of conjugated folates must be treated with conjugase to cleave the glutamyl residues prior to the determination of the total folate content.

EXPERIMENTAL

Two C_{18} phenyl column separation

An HPLC system composed of a Tracor Model 980A solvent programmer, Tracor Model 950 chromatographic pump, Rheodyne Model 9120 injector, Waters Associates Model 440 absorbance detector, and Hewlett-Packard Model 3380A integrator recorder was used. The two columns (each 30 cm × 3.9 mm I.D.) were packed with μBondapak phenyl (10 μm) (Waters Assoc., Milford, MA, U.S.A.). In addition, a precolumn (12.5 cm × 3.0 mm I.D.), packed with μBondapak phenyl/Corasil (30 μm), was used (Waters Assoc.). Injections were made at ambient temperature from a 20-μl or 100-μl loop of the Rheodyne injector. Two consecutive isocratic eluents were employed. Initially, the columns were equilibrated with a mobile phase containing a phosphate buffer and composed of 0.10 *M* potassium dihydrogen phosphate, 0.082 *M* sodium hydroxide, 0.005 *M* tetrabutylammonium phosphate (PIC A), and 1.2 %, methanol, pH 7.0. Six minutes after injection, the second mobile phase was initiated. It had the following composition: 0.036 *M* sodium perchlorate, anhydrous (G. Frederick Smith Co., Columbus, OH, U.S.A.), 0.0013 *M* potassium dihydrogen phosphate, 0.0009 *M* potassium hydroxide, 0.5 % methanol, pH 7.0 adjusted with 1.0 *M* potassium hydroxide. Mobile phases were filtered through a 0.22-μm filter prior to use. All water used in the preparation of mobile phases was treated to remove organic and inorganic impurities using a nanopure water purification system (Barnstead

Sybron, Boston, MA, U.S.A.). A flow-rate of 1.0 ml/min was maintained through the columns with a resultant pressure of *ca.* 2000 p.s.i.

Radial compression cartridge separation

The HPLC system was composed of a Rheodyne Model 5302 3-way slider valve, Tracor Model 950 chromatographic pump, Rheodyne Model 7125 injector, Waters Assoc. Model 440 abosrbance detector, and Hewlett-Packard 3380A integrator recorder. A radial compression cartridge (either 5 mm I.D. or 8 mm I.D.) packed with C_8 (10 μm) in a radial compression module model RCM-100 (Waters Assoc.) was used with a precolumn (5.0 cm × 3.0 mm I.D.), packed with μBondapak phenyl/-Corasil (30 μm). Injections were made at ambient temperature from a loop-column fitted with a C_{18} cartridge on the Rheodyne injector (Rheodyne, Cotati, CA, U.S.A.). Two consecutive isocratic eluents were used. Initially the cartridge was equilibrated with the phosphate–PIC A buffer used with the two C_{18} phenyl column separation. Three minutes prior to injection, the second mobile phase was used, of the following composition: 0.042 M sodium perchlorate, anhydrous (G. Frederick Smith Co.), 0.0015 M potassium dihydrogen phosphate, 0.001 M potassium hydroxide, 1.6% methanol, pH 7.0 adjusted with 1.0 M potassium hydroxide. Mobile phases were filtered as described above. A flow-rate of 2.0 ml/min for the 5 mm I.D. cartridge or 3.0 ml/min for the 8 mm I.D. cartridge was maintained through the cartridge with a resultant pressure of *ca.* 700 p.s.i.

Standard compounds

Folic acid (FA), 7,8-dihydrofolic acid (FH_2), folinic acid (CHO-FA), 5,6,7,8-tetrahydrofolic acid (THF), and 5-methyltetrahydrofolic acid (5-CH_3THF) were purchased from Sigma (St. Louis, MO, U.S.A.). Folylpentaglutamate (PG_5), and folyltriglutamate (PG_3) were purchased from the laboratory of Carlos L. Krumdieck, University of Alabama, Birmingham, AL, U.S.A. Standard solutions were prepared by dissolving each compound in 0.01 M potassium dihydrogen phosphate, pH 7.5, containing 0.003% sodium ascorbate and a trace of 2-mercaptoethanol to inhibit oxidative decomposition. A trace of ammonium hydroxide was added to facilitate the dissolution of 5,6,7,8-tetrahydrofolic acid. Standards were prepared immediately prior to use, protected at all times from light, and kept cold.

Conjugase treatment

Chicken pancreas (Difco Labs., Detroit, MI, U.S.A.) was purified by the method of Iwai *et al.*[8]. An aliquot of this purified conjugase was then added to each sample to be analyzed for total folate content. The contents of the tubes were evenly dispersed mechanically on a "Vortex" mixer and then incubated at 37°C for 24 h. After centrifugation, aliquots of the supernatant were frozen for analysis by HPLC or *L. casei*.

Folate analysis by L. casei

The method of folate assay of Scott *et al.*[2] which employs *L. casei* as the test organism was used to determine the amount of free and total folates present in the samples (prepared above) for HPLC analysis.

Response of L. casei growth to various folate forms

Aliquots of the standard FA, FH₂, CHO-FA, THF, 5-CH₃THF, PG₅ and PG₃ solutions were analyzed by the *L. casei* method[2] to determine the relative microbiological activity of the various folate forms on an equal molar basis. Folic acid was used as the standard, its activity placed at 100%.

Determination of the amount of chicken pancreas conjugase required to convert folyl-pentaglutamate to folic acid

Folylpentaglutamate was prepared as described above onder *Standard compounds*. An aliquot of 50 μl containing 0.005 μmole of PG₅ was combined with extracts of chicken pancreas in purified water (see *Conjugase treatment*). Extracts of 10–60 mg per ml of water were used. 100-μl aliquots were added to the reaction mixture, and the volume was brought to 200 μl with purified water. The reaction mixture was then incubated at 37°C for 24 h and frozen for later HPLC analysis on the two C₁₈ phenyl column system.
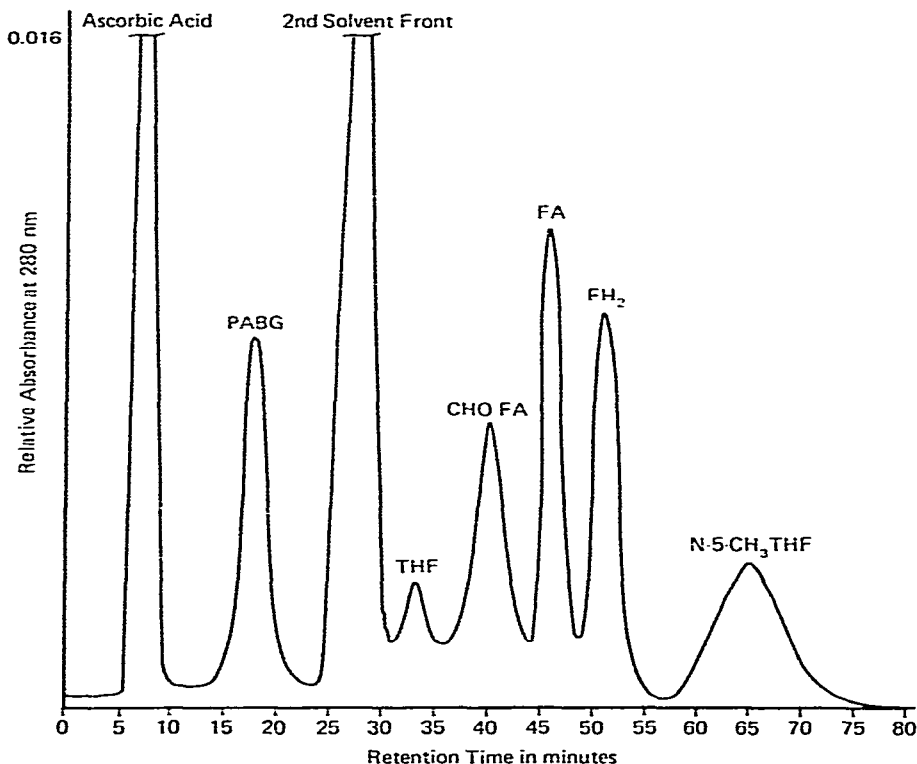

Fig. 1. Chromatogram of six folate forms obtained by isocratic elution at pH 7.0 on the two C₁₈ phenyl column system pre-equilibrated with PIC A phosphate buffer. Elution, after 6 min delay, with 0.036 *M* sodium perchlorate, 0.0013 *M* potassium dihydrogen phosphate buffer, pH 7.0, 0.5% methanol; flow-rate, 1.0 ml/min; inlet pressure, 2000 p.s.i.; ambient temperature; UV detection at 280 nm; 0.5 nmole each of *p*-aminobenzoylglutamate (pABG), 5,6,7,8-tetrahydrofolic acid (THF), folinic acid (CHOFA), 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.

RESULTS AND DISCUSSION

The approach we use here to separate folic acid, folinic acid, 7,8-dihydrofolic acid, 5-methyltetrahydrofolic acid and 5,6,7,8-tetrahydrofolic acid is a modification of our previously reported single C_{18} phenyl column separation[6]. By equilibrating the column or columns with PIC A prior to injection, ion-pairs of the various folate forms are prepared *in situ* and retained on the column or columns while endogenous interfering sample components are removed. The decision was made to use two C_{18} phenyl columns instead of one because of the extra time margin gained to remove these chromatographically interfering substances without appreciable loss in peak resolution or broadening of folate peaks. This is because the ion-pairs are tightly held on the column for 5–10 min as the PIC A buffer is being used. Beyond this time, broadening of the folate peaks is seen; thus, we switch to the second mobile phase 6 min after injection to begin the elution of the folate ion-pairs. The entire separation requires 75 min with *ca.* 15 min for re-equilibration of the column between separations.

The two C_{18} phenyl columns were used to separate five forms of folate (see Fig. 1) and a decomposition product of the folates, *p*-aminobenzoylglutamic acid (pABG). A reproducible and quantitative analysis of FA, FH_2, 5-CH_3THF, THF and pABG can be obtained with this approach (Table I). Standard curves were obtained for each folate form by plotting peak area *versus* the amount of compound injected on the column. The curves for all five compounds were linear over the following ranges: FA, 0.008–0.906 nmole, $r = 0.99$; FH_2, 0–0.902 nmole, $r = 0.99$; PABG, 0–1.500 nmole, $r = 0.98$; 5-CH_3THF, 0–0.672 nmole, $r = 0.95$; THF, 0.092–0.462 nmole, $r = 0.82$. In the case of THF, the amount injected was adjusted for the presence of FA and FH_2 in the sample, as determined from the chromatograph.

TABLE I

QUANTITATIVE DETERMINATION OF FIVE FOLATE FORMS USING TWO C_{18} PHENYL COLUMNS

Quantitative determination of five folate forms obtained by isocratic elution at pH 7.0 on the two C_{18} phenyl column system pre-equilibrated with tetrabutylammonium phosphate buffer. Eluent, 0.036 *M* sodium perchlorate, 0.0013 *M* potassium phosphate buffer, pH 7.0; flow-rate, 1.0 ml/min; inlet pressure, 2000 p.s.i.; ambient temperature.

Folate form	Range of linearity (nm)	Regression*	
		Equation	Coefficient (r)
FA	0.008–0.906	$y = 1207.510x - 9.714$	0.99
FH_2	0–0.902	$y = 1063.796x + 3.144$	0.99
pABG	0–1.500	$y = 872.132x + 19.218$	0.98
5-CH_3THF	0–0.672	$y = 1133.689x + 60.503$	0.95
THF**	0.092–0.462	$y = 527.430x - 48.463$	0.82

* y = area count/1000 as reported on Hewlett-Packard Model 3380A integrator recorder; x = nmoles of folate form injected.

** The amount injected for tetrahydrofolic acid has been adjusted for the presence of folic acid and 7,8-dihydrofolic acid in the sample.
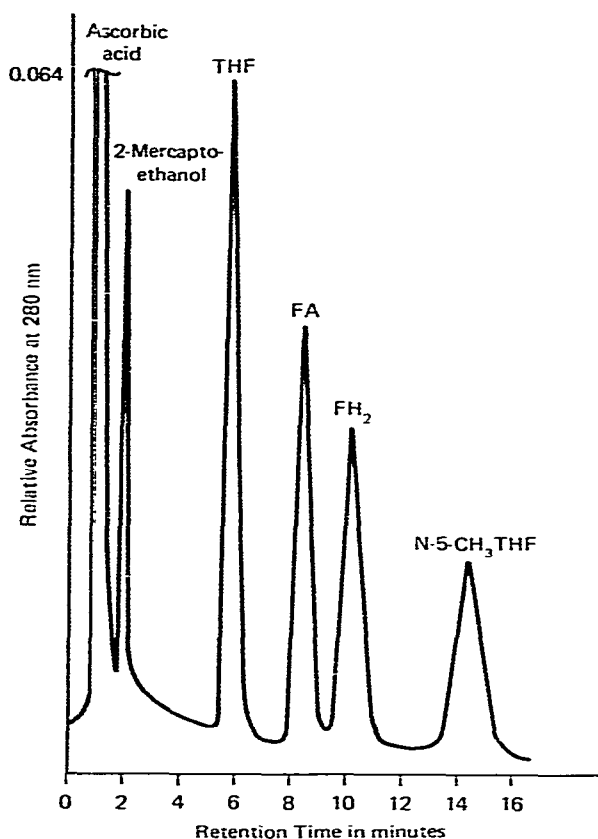
Fig. 2. Chromatogram of four folate forms obtained by isocratic elution at pH 7.0 on the radial compression C_8 cartridge. 5 mm I.D., pre-equilibrated with PIC A phosphate buffer until 3 min prior to injection. Eluent. 0.042 M sodium perchlorate. 0.0015 M potassium dihydrogen phosphate. 1.6% (v v) methanol buffer. pH 7.0; flow-rate, 2.0 ml min; inlet pressure. 680 p.s.i.; ambient temperature: UV detection at 280 nm; 0.5 nmole each of folic acid (FA). 7,8-dihydrofolic acid (FH_2). and 5-methyltetrahydrofolic acid (5-CH_3THF) and 1.0 nmole of 5,6,7,8-tetrahydrofolic acid (THF). containing 0.003% ascorbic acid and traces of 2-mercaptoethanol and ammonium hydroxide.

A very similar technique was used to separate the folate forms on a C_8 radial compression cartridge (Fig. 2). Modifications were required in the composition of the perchlorate-phosphate mobile phase and in the injection-buffer change sequence to accommodate the characteristics of the C_8 cartridge. Separation of the four folate forms requires 15 min with *ca.* 6 min of re-equilibration time between separations. The quantitative chromatographic parameters are presented in Table II. Standard curves were obtained as in the two C_{18} phenyl column system. The curves were linear over the following ranges: FA, 0–1.36 nmole. $r = 0.99$; FH_2, 0–0.90 nmole. $r = 0.99$; 5-CH_3THF, 0–1.34 nmole. $r = 0.99$; THF, 0.02–0.81 nmole. $r = 0.89$. As before. the amount of THF injected was adjusted for the presence of FA and FH_2 in the sample.

Folylpentaglutamate, folyltriglutamate and folic acid are separated on either the two C_{18} phenyl column system or the C_8 cartridge system. Although this was not

TABLE II

QUANTITATIVE DETERMINATION OF FOUR FOLATE FORMS USING A RADIAL COM-
PRESSION C_8 CARTRIDGE

Quantitative determination of four folate forms obtained by isocratic elution at pH 7.0 on the C_8 radial compression cartridge system pre-equilibrated with tetrabutylammonium phosphate buffer. Eluent, 0.042 *M* sodium perchlorate, 0.0015 *M* potassium phosphate buffer, pH 7.0; flow-rate 2.0 ml/min; inlet pressure, 680 p.s.i.; ambient temperature.

Folate form	Range of linearity (nm)	Regression*	
		Equation	Coefficient (r)
FA	0–1.36	$y = 745.113x + 5.291$	0.99
FH$_2$	0–0.90	$y = 644.786x + 12.833$	0.99
5-CH$_3$THF	0–1.34	$y = 599.558x + 3.706$	0.99
THF**	0.02–0.81	$y = 375.129x - 7.706$	0.89

* y = area count/1000 as reported on Hewlett-Packard Model 3380A integrator recorder; x = nmoles of folate form injected.

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

previously seen in HPLC separations of folates[7], the folylpolyglutamates are readily separated by conventional chromatography on DEAE-cellulose chloride[1]. In both of the HPLC separations presented here, the order of elution is PG$_5$, PG$_3$, FA.

Folylpentaglutamate was incubated with chicken pancreas conjugase which hydrolyzes the folate polyglutamates to monoglutamate forms. Using varying amounts of conjugase, one can then determine the minimum amount of conjugase necessary to completely hydrolyze the folylpentaglutamate to folic acid (see Fig. 3). The peak areas corresponding to PG$_5$, PG$_3$ and FA were monitored as a function of the amount of conjugase used. The chicken pancreas conjugase must first be purified by the method of Iwai et al.[8]. Both the *L. casei* assay and the HPLC separation show amounts of folate present in the unpurified extracts comparable with that in foods and biological fluids.

A comparison was made of the *L. casei* response to the various forms of folate used as standards on the HPLC system. The growth response of *L. casei* to FA was set at 100 %, giving a relative response to FH$_2$, THF, CHOFA, and 5-CH$_3$THF of 75.4 %, 43.9 %, 54.8 % and 87.1 %, respectively. These relative values are comparable with those reported previously[9,10]. Because the *L. casei* response to the different folates is not uniform, any assay using this microorganism will give spurious results for a sample containing a mixture of folates. Thus, the need for another method of analysis, e.g. the HPLC separation, is even more apparent.

In summary, the need to supplement or replace the *L. casei* assay for folates is greatest because of the lack of specificity for the various folate forms. This specificity is obtained in a reliable and reproducible manner by either of the HPLC methods presented here. The two C_{18} phenyl column method is readily adapted to any HPLC system. Two shortcomings are inherent, however, in this system compared to the radial compression system. First, the relative cost of column replacement is four times that of the radial compression cartridge beyond the initial investment for the system.
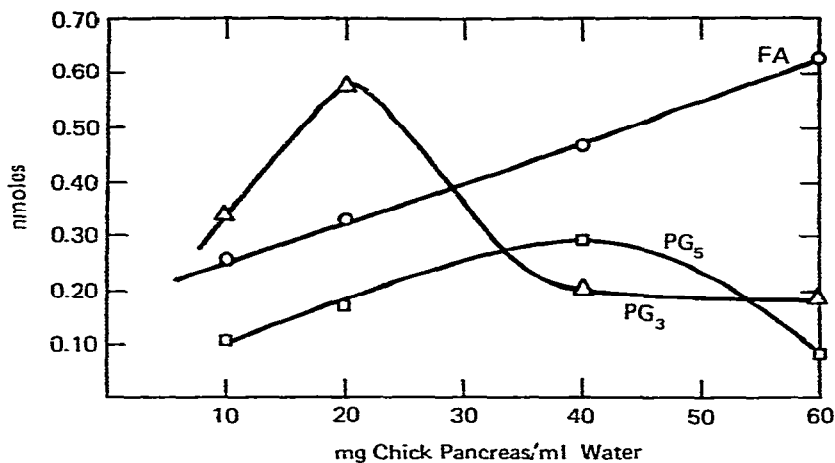
Fig. 3. Products of varying amounts of conjugase in incubation with folylpentaglutamate. Folylpentaglutamate (0.005 μmole) was incubated for 24 h at 37°C with varying amounts of chicken pancreas conjugase. At the end of the incubation period. the reaction mixture was frozen. Aliquots (20 μl) were subsequently thawed and injected for HPLC separation and quantification on the two C_{18} phenyl system: O, FA; △, PG₃; □. PG₅.

Secondly. the time required for separation of the folates is five times greater on the two C_{18} phenyl column system than on the radial compression system. The re-equilibration time is twice as long on the two C_{18} phenyl column system compared with the radial compression cartridge. However, it has not been possible to obtain a good separation of folinic acid from folic acid on the radial compression cartridge. Also. one must consider the initial investment necessary for a radial compression system.

Recently. Allen and Newman[11] have separated folic acid, dihydrofolic acid. folinic acid. 5-methyltetrahydrofolic acid and p-aminobenzoylglutamate using a tetrabutylammonium phosphate containing water–methanol gradient on an ODS column. This system was developed for direct serum analysis. The folate in serum is in a non-conjugated form. We have developed our systems for more complex biological fluids and tissues. for example. human milk. In such samples the folates are conjugated and typically the samples contain many more interfering substances. Our separations are specifically designed to overcome the problems involved with the more complex sample matrix. allowing removal of interfering substances before elution of the folate forms. The two systems reported herein also include separation and quantification of tetrahydrofolic acid. In addition, these improved separations have much lower detection limits and a two- to three-fold increase in the range of linearity.

The absolute necessity to determine the total folate content of biological samples by treatment with conjugase is shown here. We have been able to apply this method to some infant foods in our laboratory, but we are still working on problems arising from sample preparation. The *in situ* formation of folate ion-pairs facilitates the removal of most chromatographically interfering sample components. Some interference is observed, however, from by-products of conjugase treatment. One very promising approach is the rapid filtration of the biological sample using immersible 10,000-dalton filters (Millipore, Bedford, MA, U.S.A.) prior to conjugase treatment.

We have used the HPLC methods and the *L. casei* assay in tandem. For standard compounds the HPLC methods agree completely with the *L. casei* assay and also supplement this technique by identifying the forms of folate. For biological samples the HPLC determinations, although not fully refined, provide critical information which is obscured by the microbiological assay. To obtain these data microbiologically, one would need simultaneous differential analyses using several organisms. Using a single sample injection, the HPLC technique is clearly more direct, rapid and offers the opportunity to analyze for many more forms of folate.

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