Combined affinity and ion pair liquid chromatographies for the analysis of folate distribution in tissues

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Overview

The cellular metabolism of folate is comprised of numerous reactions, including the synthesis and hydrolysis of polyglutamyl derivatives, the synthesis of tetrahydrofolates with and without one carbon substitution at various oxidation levels, and the transfer of these one carbon units for the synthesis of methionine, thymidylate, purines, and serine/glycine interconversion. These reactions result in a variety of intracellular folate compounds which differ in the pteridine ring structure as well as in the glutamate chain length. Changes in the rates of these reactions result in changes in the distribution of folates. Determining folate distribution in tissue therefore provides important information on the folate dependent metabolism of the cell.

The multiplicity of folate forms in tissue renders the analysis of folate distribution a difficult and cumbersome task.[1,2] The traditional method, which combines DEAE-cellulose chromatography with differential microbial assays,[3-5] includes lengthy chromatography and the collection of multiple fractions. These fractions are each treated with pteroylpolyglutamate hydrolases to cleave polyglutamyl folates to their respective monoglutamyl derivatives. These are then assayed microbiologically using two, and often three, assay organisms.

Other methods, including those that are based on the selective cleavage of the C_9-N_{10} bonds[6] and those that rely on ternary complexes of thymidylate synthase with ^{3}H-5-fluorodeoxyuridinemonophosphate (FdUMP) and 5,10-methylenetetrahydrofolates,[7,8] are also quite lengthy and involve many steps.

The method described herein is designed to analyze the intact folate molecule in a simple two-step procedure. First, folates in the tissue extracts are purified by affinity chromatography using immobilized milk folate binding protein as the affinity matrix.[9,10] In the following step, the

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purified folate mixture is resolved using ion pair high performance liquid chromatography in combination with a diode array detection system.[11]

Procedures

1. The high affinity folate binding protein (FBP) from milk is purified from commercial powdered whey by affinity chromatography.
2. The purified FBP is immobilized by reaction with CNBr-activated Sepharose 4B.
3. The FBP-Sepharose preparation is used as an affinity matrix for the purification of folates from tissue extracts.
4. The purified folate preparation is analyzed by ion pair high pressure liquid chromatography (HPLC) which is attached to a diode array UV detection system.

Purification of FBP

Preparation of folate Sepharose column

All work which is related to the activation of Sepharose and subsequent reaction with diaminohexane should be conducted using rubber gloves and in a well-ventilated hood. The door of the hood should be kept at maximum closure. Since the CNBr is highly poisonous, avoid breathing its fumes.

Place inside the hood the following:

1. A pH meter with a magnetic stirrer motor and a 50–100 ml biuret containing 5 M NaOH.
2. A 600-ml sintered glass funnel (coarse) over a two-liter suction flask. Attach the flask to a water aspirator.
3. A bucket of ice.
4. CNBr (kept in a closed jar in a dessicator) and 1,6 diaminohexane (both from the Sigma Chemical Co., St. Louis, MO).
5. Prepare at least 2 hours before 2–4 liters 0.1M $NaHCO_3$. Keep refrigerated (not in the hood).

Other reagents and equipment

Folic acid, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (both from the Sigma Chemical Co.), *Sepharose 4B* (Pharmacia LKB Biotechnology, Uppsala, Sweden), *6 liters 2M NaCl containing 0.05M potassium phosphate buffer, pH 7.0, 2 liters 0.2M acetic acid,* and *2 liters 1M potassium phosphate buffer, pH 7.0.* One 7 × 40 cm glass column with a sintered glass fitting (coarse) at the bottom.

Procedure

Sepharose 4B, 400 ml bed volume, is washed with 2 liters of water, suspended in 400 ml water, and placed inside a 1-liter beaker containing a magnetic stirrer. The beaker is placed in the hood over the magnetic stirrer motor under the pH electrode and the suspension is kept stirring.

Cyanogen bromide crystals (30 g) were then added to the suspension. Following the addition of CNBr, the NaOH solution in the biuret is used continuously for the next 15 to 30 minutes to maintain the pH between 10–11. During this time, handfuls of ice are added to the stirred suspension to maintain the temperature around 20°C.

Completion of the activation is evident when the CNBr crystals are totally dissolved and the pH becomes stable at around 10. When this occurs, the suspension is filtered over vacuum in the sintered glass funnel and washed with 1.5–2 liters of the cold $NaHCO_3$ solution. The activated Sepharose is then reacted with 1,6-diaminohexane as follows: The diaminohexane, 1.8 g, is dissolved in 400 ml 0.1M $NaHCO_3$ and the pH is ad-

justed to 9.0. This solution is then mixed with the activated Sepharose cake, removed from the hood into a cold room, and stirred for 48 hours. The suspension is filtered through a sintered glass filter, washed with 0.1 M $NaHCO_3$, and resuspended at room temperature in 400 ml of the bicarbonate solution.

Folic acid, 4 g, is suspended in 50 ml water and dissolved by the dropwise addition of 1 N NaOH. This solution is then added to the Sepharose suspension. The suspension is stirred, adjusted to pH 8.5 and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl, 4 g, is then added over a period of 45 minutes (at room temperature). Stirring is continued for 3 hours at room temperature, and then 48 hours in the cold room. The suspension is then washed, to remove non-bound folate, with 4 liters 2 M NaCl containing 0.05 M potassium phosphate buffer, pH 7.0, 2 liters water, 1 liter 0.2 M acetic acid, and 2 liters water. The Sepharose matrix is resuspended in an equal volume of water, poured into a 7 × 40 cm column, washed in 1 liter 1 M potassium phosphate buffer, pH 7.0, and then with 1 liter water. This column is now ready for use.

Purification of folate binding protein (FBP) from dried whey

Reagents

Dried whey

Procedure

Whey is a product of the cheese industry and is available as a dried powder in two forms, acid whey and sweet whey. Either form is suitable. For the size of the folate column described above, 4 Kg of the dried whey is suspended in 8 liters water and the pH is adjusted to 7.0 with 5 M NaOH. The suspension is centrifuged for 10 minutes at 20,000*g* and the supernatant fraction is applied onto the folate-Sepharose column at room temperature. After application of the supernatant, the column is washed with 2 liters 1M NaCl containing 0.05 M Tris HCl, pH 7.4, followed by 1 liter water. The FBP adsorbed to the column is then eluted with 0.2 M acetic acid. During the acid wash, the pH of the effluent is monitored and when it becomes acidic, a fraction of 300 ml is collected separately, an 0.2 ml aliquot is saved to determine FBP activity (see below), and the remainder is lyophilyzed to dryness.

The folate Sepharose column is regenerated by washing with 400 ml 1 M potassium phosphate buffer, pH 7.4, and then with 1 liter water. This procedure is repeated using 9 additional batches of whey.

Determination of FBP activity

Reagents

[^{3}H]folic acid (1 nmol/ml, 0.5–1.0 μCi/nmol). Is prepared by mixing [^{3}H]folic acid (20–40 μCi/nmol, Amersham, Arlington Heights, IL) with unlabeled folic acid to the desired specific radioactivity.
Dextran-coated charcoal. Dissolve 1 g Dextran T20 (Pharmacia) in 100 ml water. Add 2.5 g acid washed charcoal (Norit A), stir for 1 hour, and store refrigerated.
1M potassium phosphate buffer, pH 7.4.
Bovine serum albumin (50 mg/ml).

Procedure

In a 12 × 75 mm test tube, mix 0.1 ml of the potassium phosphate buffer, 0.05 ml of the [^{3}H]folic acid solution, 0.1 ml of the bovine serum albumin, sufficient FBP to bind 2–30 pmol folic acid and water to a 1 ml final volume. After 15 minutes at room temperature, 0.25 ml of a well-stirred dextran-coated charcoal suspension is added to the incubation mixture. The latter

is mixed for about 30 seconds in a vortex and centrifuged for 10 minutes at 2,000*g*. An 0.5 ml of the supernatant is counted in a liquid scintillation counter. Blank incubations without added FBP should be carried out each time that this assay is performed.

FBP activity estimation

$$\text{Units of FBP/ml protein} = 2.5\,(\text{dpm-blank})/V(SA) \quad (1)$$

where V refers to the volume in ml of the original FBP protein solution added to the incubation mixture and, SA, the specific activity in dpm/pmol of the [^{3}H]folic acid solution used.

A unit of FBP activity is equivalent to an amount of protein that will bind 1 pmol folic acid.

Preparation of FBP-Sepharose matrix

Reagents

Purified FBP (5,000–7,000 Kilounits); CNBr; Sepharose 4B; 1 liter 0.1 M $NaHCO_3$; 2 liters 2 M NaCl containing 0.05 M Tris HCl, pH 7.4; 2 liters 0.02 M trifluoroacetic acid; 2 liters 0.05 M potassium phosphate buffer pH 7.4; $NaNO_3$.

Procedure

It is important that the FBP-Sepharose column be of a capacity of at least 25–50 Kilounits (30–60 nmol) per ml bed volume. The dried whey contains about 250 units per gram of which 50% is recovered in the purified fraction. The purified FBP preparation from the 10 batches of whey should contain approximately 5,000 Kilounits. This amount is sufficient for the preparation of 50–60 ml FBP-Sepharose matrix.

First the purified FBP preparations are combined by suspending the lyophilized powder in 250 ml 0.1 M $NaHCO_3$. The cloudy suspension is left at room temperature ready to use. An amount of Sepharose 4B not exceeding 1 ml bed volume per 50–70 Kilounit of FBP is activated in a well-ventilated hood with CNBr using the same conditions of activation and subsequent washing with cold $NaCHO_3$ as outlined above. The activated Sepharose cake is suspended in 50 ml $NaCHO_3$, and then mixed with the FBP solution. The suspension is stirred for 2 hours at room temperature, and then 48 hours at 4°C. The Sepharose matrix is then transferred into a coarse sintered glass funnel and washed with 2 liters 2 M NaCl containing 0.05 M Tris HCl, pH 7.4, 1 liter water, 2 liters 0.02 M trifluoroacetic acid, 2 liters 0.05 M potassium phosphate buffer pH 7.4, and finally with 1 liter water. The Sepharose cake is suspended in an equal volume of 0.3% sodium azide and stored in the refrigerator.

Purification of folates from tissue extracts

Reagents

Extraction buffer, freshly prepared, consists of 2% sodium ascorbate, 10 mM 2-mercaptoethanol in 0.1M Bis-Tris, pH 7.85; boiling water bath; 10 ml vacutainers; 10 ml plastic syringes.

Tissue folate extraction

The extraction of folate from tissue should be conducted under conditions which will ensure the integrity of the extracted folates and will avoid losses due to oxidative cleavage or alterations due to polyglutamyl folate hydrolysis by the action of endogenous pteroylpolyglutamate hydrolases. For these reasons, the tissue must be fresh and not frozen. Thawing frozen tissue is associated with extensive hydrolysis of the folate polyglutamates irrespective of the conditions used.

Extraction is normally carried out using a ratio of 10 volumes buffer per gram tissue. First the buffer is dispensed in glass test tubes, placed in a boiling water bath, and allowed to reach the temperature of the bath. Next, freshly minced tissue of a known weight is dropped into the hot buffer and boiling is continued for an additional 15 to 25 minutes. The extract is cooled in an ice water bath, dispersed by homogenization, and the volume adjusted to make a final suspension that corresponds to 10 volumes per gram wet tissue. This extract is then centrifuged at 36,000*g* for 15 minutes, and the supernatant fraction is injected into vacutainers and stored as such at −70°C until used.

Folate purification by affinity chromatography

Reagents

FBP-Sepharose columns; tissue extracts; 1M potassium phosphate buffer, pH 7.0; 0.02 M trifluoroacetic acid containing 10 mM dithioerythritol (DET); [^{3}H]folic acid tracer (20–40 Ci/mmol); 1M piperazine.

Procedure

The supernatant fraction in the vacutainers is thawed and an aliquot with approximately 2–15 nmol total folate is mixed with 0.2 μCi[^{3}H]folic acid tracer. This preparation is applied onto an FBP-Sepharose column. The latter (affinity column) is made of a Pasteur pipette fitted with a glass wool at the lower neck and filled with a 1 ml bed volume of the FBP-Sepharose matrix. Before use, the column is washed with 5 ml 0.02 M trifluoroacetic acid, 5 ml water, and 5 ml 1 M potassium phosphate buffer, pH 7.4. After sample application, the column is washed with 10 ml of the same phosphate buffer followed by 10 ml water. Adsorbed folate is eluted from the column with 0.02 M trifluoroacetic acid containing 10 mM dithioerythritol (DET) as follows. The column is first washed with 1 ml of the trifluoroacetic acid/DET solution. This fraction is discarded. The next 1.5 ml which includes the acid front is collected in a separate test tube and promptly neutralized to pH 7.5 by the addition of 0.02 ml 1M piperazine (Sigma). A 0.05 ml aliquot is used to determine folate recovery based on radioactivity counts. A recovery less than 85% is unacceptable and the purification should be repeated with a lower amount of the supernatant fraction.

Note

The [^{3}H]folate is often not pure and undergoes oxidative cleavage with time. Products of the oxidative cleavage are not retained by the affinity column. This principle can be used to determine % purity of the tracer, or use a column to specifically purify a quantity of the tracer to add to the supernatant from the tissue extract.

Analysis of folate distribution

Reagents

Acetonitrile; DET; NaCl; HPLC grade water; tetrabutyl ammonium phosphate (TBAP, from Sigma). A mixture containing 10 nmol/ml each of $PteGlu_{1\text{-}7}$.

Procedure

The analysis of folate distribution in the affinity purified folate samples are carried out using ion pair high pressure liquid chromatography (HPLC) in combination with a diode array detection system.

The HPLC system consists of a C-18 Econosphere (5μ, 4.6 × 100 mm) column from Alltech and a gradient which is made from two solutions. These two solutions contain 5 mM tetrabutylammonium phosphate

(TBAP), 0.5 mM dithioerytritol, and 25 mM NaCl. Solution A is made in water. Solution B is made in 65% acetonitrile and 35% water. The column is equilibrated with a mixture that contains 90% A and 10% B. After sample application, the column is washed for 5 minutes with the equilibration buffer at a flow rate of 1 ml/min. Thereafter, the proportion of B in the wash is increased linearly with time to reach 36% at 15 minutes, 50% at 35 minutes, and 60% at 52 minutes. The flow rate is maintained at 1 ml/min. A diode array detector (Hewlett Packard) is used to monitor folate activity in the column effluent. This detector is set to simultaneously determine absorption at 280, 350, and 258 nm.

Figure 1 shows the results of a chromatography of a mixture of Pte-

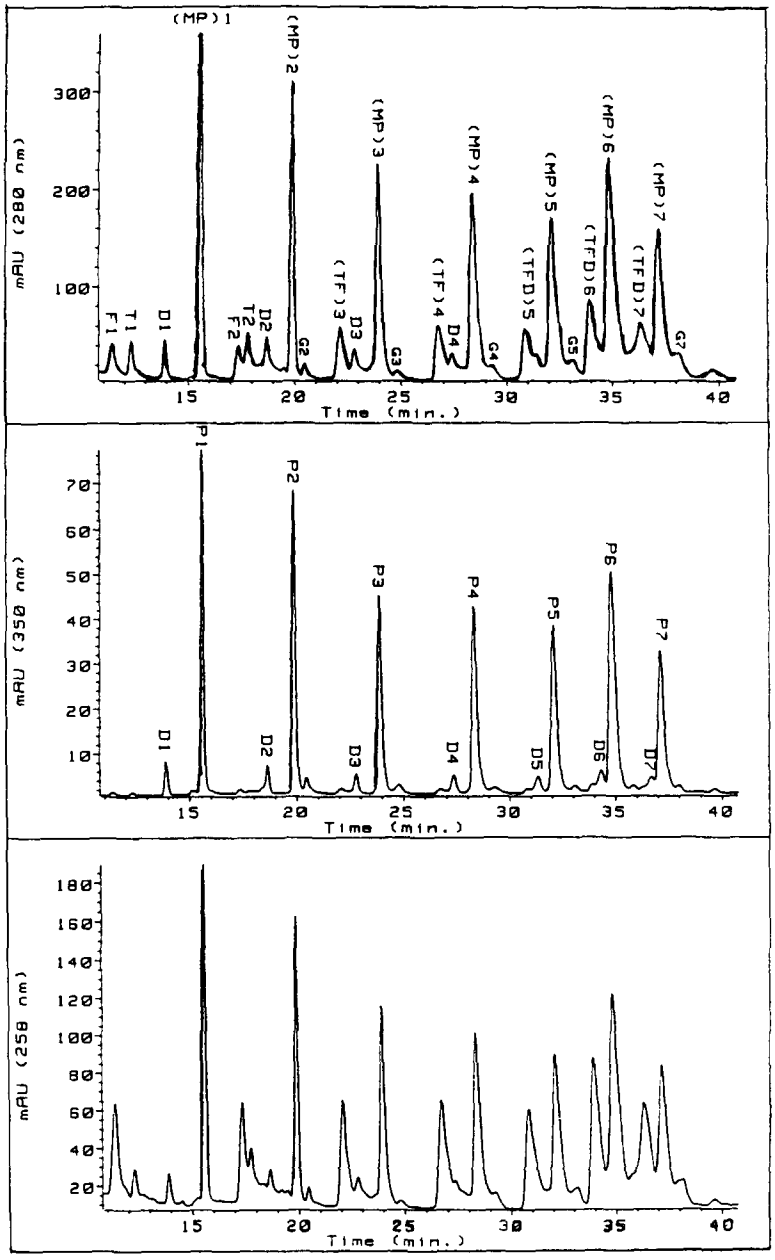

Figure 1 Chromatography of a mixture that contained $PteGlu_{1-7}$ and its various reduced forms. A total of 35 derivatives. Activities shown in the top panel represent absorption values at 280 nm; those at middle panel, 350 nm; and those in the bottom panel, 258 nm. Symbols and concentrations are as follow: F, 10-formyl$H_4PteGlu_{1-7}$ (1.8-2.0 nmol); T, $H_4PteGlu_{1-7}$ (0.9–1.2 nmol); D, $H_2PteGlu_{1-7}$ (0.4–0.6 nmol); M, 5-methyl$H_4PteGlu_{1-7}$ (1.0–1.2 nmol); and P, $PteGlu_{1-7}$ (2.4–2.7 nmol). Numbers following symbols refer to numbers of glutamate residues (from reference 11).

Glu_{1-7} (P1–P7) and their corresponding dihydro- (D1–D7), tetrahydro- (T1–T7), 10-formyltetrahydro- (F1-F7), and 5-methyltetrahydro- (M1-M7) derivatives.

The chromatographic pattern obtained under these conditions is in the form of seven clusters each composed of those folates which have the same number of glutamate residues and each differing by its neighbor by one residue. In clusters containing mono and diglutamyl folates, there is a separation between the 10-formyl- (F), the tetrahydro- (T), and the dihydro- (D) derivatives. In clusters containing a higher number of residues, these three folates tend to elute in the same fraction.

In each cluster, $PteGlu_n$ (P) and corresponding 5-methyl$H_4PteGlu_n$ (M) elute in same peak activity separate from the above three folates.

The peak activities eluting immediately after each cluster with the designation "G_n" represent p-aminobenzoylglutamates ($PABAGlu_n$). These peaks have an absorption maximum at 270 nm and increase in concentration when strict anaerobic conditions are not maintained. Pteridine fragments elute with the void volume of the column and cannot be detected because of ascorbate which elutes in same fractions.

The 5-formyltetrahydrofolate derivatives (which are designated as L for leucovorin) elute with the corresponding clusters at positions slightly preceding the methyltetrahydrofolate/folic acid peaks.

Spectral analysis

An elution pattern of the type described in *Figure 1* is most suited to identify the number of glutamate residues in any eluting folate by determining which cluster this elution corresponds to. The position of the eluting folate within the cluster provides a first screening for determining the pteridine ring structure. If this position is in the area where the members of the cluster elute as separate peaks (e.g., F1, D1, T1, or F2, D2, and T2), then retention time alone would be sufficient for identification of the pteridine ring structure of the eluting folate. If, however, the position of the eluting folate is in the area where more than one member of the cluster elute, then identification requires in addition specific spectral features which are provided by the diode array detector. In *Figure 1*, the top panel represents UV absorption monitoring determined at 280 nm. Absorption values determined at this wavelength serve to determine folate activity indiscriminately. The middle panel of *Figure 1* represents UV absorption monitoring at 350 nm which serves to identify dihydropteroylglutamates (D) and pteroylglutamates (P), since reduction to tetrahydrofolate is associated with the abolishment of absorption in this region. As seen, this panel contains only seven pairs of activity peaks.

The bottom panel of *Figure 1* shows UV absorption monitoring at 258 nm. Absorption at this wavelength serves to identify 10-formyltetrahydrofolate derivatives. At 258 nm, absorption is maximum for these forms of folates unlike other derivatives where absorption maxima are around 280–300 nm.[11]

Quantitative determination

Table 1 shows the quantitative relationships between UV absorption signals at the three wavelengths mentioned above and folate concentrations, determined for the various reduced and oxidized derivatives. This table was constructed from [^{3}H]PteGlu which was used to prepare Tritium-labeled reduced folates with the same specific radioactivity.[11] These were then subjected to ion pair chromatography under the conditions described above. Fractions collected from these chromatographies were analyzed for [^{3}H] contents to estimate the amount of folate under each corresponding peak area. This relationship expressed as molar peak coefficient is defined in integrated peak area units per nmol folate. These are arbitrary units and are subject to change depending on the flow rate and cross section of the

Table 1 Molar peak coefficients expressed as integrated peak area of PteGlu and its derivatives at various wavelengths

Folate tested	Molar peak coefficient and molar coefficient ratios		
	280 nm	350 nm	258 nm
PteGlu	875 (1)	210 (0.24)	472 (0.54)
H_2PteGlu	787 (0.899)	152 (0.173)	321 (0.367)
H_4PteGlu	682 (0.779)	7 (0.008)	266 (0.304)
10-FormylH_4PteGlu	323 (0.369)	0	513 (0.586)
5-methylH_4PteGlu	787 (0.899)	0	314 (0.314)
5-FormylH_4PteGlu	812 (0.928)	0	323 (0.370)

The data were obtained using [^{3}H]PteGlu before and after conversion to the various reduced forms. Aliquots containing about 1,3, and 10 nmol were each injected into the column and the column was subsequently eluted under the same conditions described above. Fractions of 0.5 ml were collected to determine folate contents based on radioactivity counts. The molar peak coefficient (unit area/nmol) was calculated from the amount of folate which was eluted and the corresponding peak area determined at the wavelength indicated in the Table. Numbers in parentheses represent molar peak coefficient ratios (R) of the various folates at the various wavelengths versus the molar peak coefficient of PteGlu at 280 nm (from reference 11).

flow cell. Values in *Table 1* were found to double and quadruple when flow rates were reduced to 0.5 and 0.25 ml/min, respectively (data not shown).

Folate concentration in peaks containing single derivatives can be determined based on the observed integrated peak area and the coefficients given in *Table 1*. Peaks contianing overlapping activities are resolved based on the following expressions.

To resolve $PteGlu_n$ (P) and 5-methyl$H_4PteGlu_n$ (M), the expression is as follows:

$$A_{280} = A_p + A_m$$
$$A_p = (A_{350}) \times 4.17 \quad (2)$$

A_{280} and A_{350} represent observed absorption signal values at 280 and 350 nm, respectively. A_p and A_m represent the contributions of $PteGlu_n$ and 5-methyl$H_4PteGlu_n$ to the absorption signals at 280 nm, respectively. The number 4.17 is determined from a PteGlu absorption spectrum and represents the ratio of absorption at 280 nm versus that at 350 nm.

The same principles are applied for estimation of $H_2PteGlu_n$ which too absorb UV light at 350 nm and tend to coelute with $H_4PteGlu_n$ and 10-formyl$H_4PteGlu_n$. For $H_2PteGlu_n$, the ratio of absorption at 280 nm versus that at 350 nm is equal to 5.18.

The expression for resolution between $H_4PteGlu_n$ and 10-formyl$H_4PteGlu_n$, which tend to coelute in clusters with 3 or more glutamate residues, relies instead on absorption signals at 258 nm compared to those at 280 nm and is as follows:

$$A_{280} = A_f + A_t$$
$$A_{258} = 1.59A_f + 0.39A_t \quad (3)$$

A_{280} and A_{258} represents absorption signals at 280 and 258 nm, respectively; A_f and A_t represent the respective contributions of 10-formyl$H_4PteGlu_n$ and $H_4PteGlu_n$ to absorption activity at 280 nm. The numbers 1.59 and 0.39 represent ratios of absorption at 280 nm versus those at 258 nm, for 10-formyl$H_4PteGlu_n$ and $H_4PteGlu_n$, respectively.

Folate concentration in any eluting peak is then determined based on tables which depict the relationships between UV absorption signals at 280 nm (expressed in integrated area units) and folate concentration (*Table 1*). These integrated areas are reproducible if the flow rate of the mobile phase is maintained constant.

Column calibration

The development of this method required the preparation of many folate derivatives. In *Figure 1*, the folate mixture injected into the column consisted of 35 forms. In *Table 1*, radioactive PteGlu was used to prepare reduced folates with the same specific radioactivity. Methods for the preparation of reduced folates were developed for these purposes and were published elsewhere.[11] The preparation of these various folates would be helpful but not necessary. As shown in *Figure 1*, the outer boundary of each cluster is made of the corresponding $PteGlu_n$/5-methyl$H_4PteGlu_n$ pair. Column calibration can be attained therefore with the (oxidized) $PteGlu_{1-7}$ series alone. This calibration provides the outer boundary of each cluster. The diode array detector will provide the additional information to identify other folates within each boundary.

Molar extinction coefficients, as those presented in *Table 1*, can be constructed using PteGlu as the only standard. First known amounts of PteGlu are injected into the column and the resulting peak areas are used to calculate the molar extinction coefficients (in unit area/nmol) for PteGlu at the above-mentioned three wavelengths. The extinction coefficient value at 280 nm is then used to determine extinction coefficients for reduced folates at each of the three wavelengths using the following expression:

$$E_{x\lambda} = E_{p280}\,(R) \qquad (4)$$

where $E_{x\lambda}$ represents the molar extinction coefficient of any of the reduced folates in *Table 1* at the given wavelength, E_{p280} the extinction coefficient determined for PteGlu at 280 nm and (R) multiplication factor. Values for (R) are given in *Table 1* (in parentheses). They represent molar absorption coefficients of the various folates at each of the three wavelengths relative to the molar absorption coefficient of PteGlu at 280 nm. The authenticity of the calculated values can be tested with pure synthetic reduced folates.

Discussion

The method of folate analysis described herein is based on the use of affinity chromatography to obtain spectrally pure folates and ion pair HPLC combined with a diode array detector for quantitative analysis of folate distribution in the purified sample. As such, this method offers many advantages to preexisting methods which are laborious and often insufficient in providing details about the various forms in folate mixtures. The one disadvantage of this method is the relatively large amount of tissue which is needed. The lower limit of the diode array system is about 25 pmol folate/peak. We are currently investigating other means of detection that will provide higher sensitivity.

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Abbreviations

$PteGlu_{1-7}$, pteroylglutamates, the subscripts denote the number of glutamate residues. Folic acid or folates denote pteroylglutamates with unspecified number of glutamate residues. $H_2PteGlu_{1-7}$ and $H_4PteGlu_{1-7}$ refer to the dihydro and the tetrahydro folate derivatives. 5-methyl$H_4PteGlu_{1-7}$ and 10-formyl$H_4PteGlu_{1-7}$ refer to tetrahydrofolates with methyl and formyl substitutions, respectively.

References

1 Krumdieck, C.L., Tamura, T., and Eto, S. (1983). Synthesis and analysis of the pteroylpolyglutamates. *Vitam. Horm.* **40,** 45–104

2 Selhub, J. (1980). Analysis of tissue folate composition. In *Folic Acid Metabolism in Health and Disease* (M.F. Picciano, J.F. Gregory, and E.L.R. Stokstad, eds.), pp. 171–194, Wiley Liss, Inc., New York

3 Silverman, M., Law, L.W., and Kaufman, B. (1961). The distribution of folic acid active substances in leukemic cells of the house. *J. Biol. Chem.* **236,** 2530–2533

4 Noronha, J.M. and Silverman,M. (1962). Distribution of folic acid derivatives in natural material. I. Chicken liver folates. *J. Biol. Chem.* **237,** 3299–3302

5 Bird, O.D., McGlohon, V.W., and Vaitikus, J.W. (1965). Naturally occurring folates in the blood and liver of the rat. *Anal. Biochem.* **12,** 18–35

6 Eto, I. and Krumdieck, C.L. (1982). Determination of three different pools of reduced one-carbon substituted folates. III. Reversed-phase high performance chromatography of the azo-dye derivatives of p-aminobenzoylglutamates and its application to the study of unlabeled endogenous pteroylpolyglutamates of rat liver. *Anal. Biochem.* **120,** 323–329

7 Priest, D.G., Happel, K.K., and Doig, M.T. (1980). Electrophoretic identification of poly-γ-glutamate chain-lengths of 5,10-methylenetetrahydrofolate using thymidylate synthetase complexes. *J. Biochem. Biophys. Methods* **3,** 201–206

8 Priest, D.G., Happel, K.K., Magnum, M., Bednarek, J.M., Doig, M.T., and Baugh, C.M. (1981). Tissue folylpolyglutamate chain-length characterization by electrophoresis as thymidylate synthetase-fluorodeoxyuridylate ternary complexes. *Anal. Biochem.* **115,** 163–169

9 Selhub, J., Osman, A., and Rosenberg, I.H. (1980). Preparation and use of affinity columns with bovine milk folate binding protein (FBP) covalently linked to Sepharose 4B. *Meth. Enz.* **66,** 686–690

10 Selhub, J., Darcy-Vrillon, B., and Fell, D. (1988). Affinity chromatography of naturally occurring folates. *Anal. Biocehm.* **168,** 247–251

11 Selhub J. (1989). Determination of tissue folate composition by affinity chromatography followed by high pressure ion pair liquid chromatography. *Anal. Biochem.* **182,** 84–93