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DIRECT DETERMINATION OF FOLATE MONOGLUTAMATES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN AUTOMATIC PRECOLUMN-SWITCHING SYSTEM AS SAMPLE CLEAN-UP PROCEDURE

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SUMMARY

A simple and rapid technique for the simultaneous isolation and analysis of folate monoglutamates (folic acid, 7,8-dihydrofolic acid, 5,6,7,8-tetrahydrofolic acid and 5-formyl-, 5-methyl- and 10-formyl-5,6,7,8-tetrahydrofolic acids) was developed using reversed-phase high-performance liquid chromatography with an automatic precolumn-switching system. The plasma or the dissolved diet samples were directly injected onto a short precolumn flushed with 50 m*M* phosphate buffer. The folate vitamers absorbed on the precolumn were backflushed onto the analytical column with a 25 m*M* phosphate buffer containing 5% methanol and then detected by UV absorption at 280 nm. A linear response was found between the injected sample amounts and the integrated areas for all vitamers analysed. The detection limit was 1—10 pmol and the precision ranged from 1.6 to 10%, depending on the metabolite studied. The recovery rates of folates in plasma were 90–95%. Decomposition of the unstable folates was avoided. Our method was applied to the analysis of mouse plasma and animal diets.

INTRODUCTION

Folate vitamers (Fig. 1) are coenzymes in the biochemical pathways of nucleic acids and amino acids [1]. Folate deficiency due to malnutrition or drug therapy can cause various diseases [2–5], including birth defects [6, 7]. In order to lower the incidence of neural tube defects in man, folate and other vitamer supplementation was performed in clinical studies [8, 9]. We established an animal model to study the effects of folates on the incidence of neural tube defects caused by the antiepileptic drug valproic acid [10].

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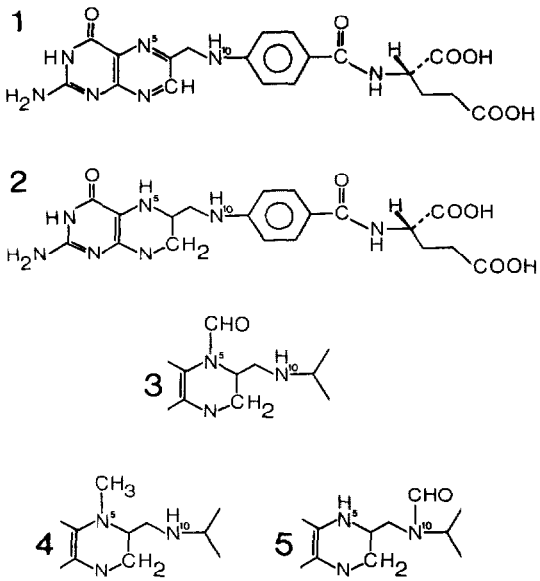

Fig. 1. Structures of several folate monoglutamates. 1 = PteGlu; 2 = H_4PteGlu; 3 = 5-formyl-H_4PteGlu; 4 = 5-methyl-H_4PteGlu; 5 = 10-formyl-H_4PteGlu.

These investigations included the measurement of the folate metabolic pattern in mouse plasma in order to investigate whether changes in the concentrations of some folate metabolites after application of valproic acid might occur.

The determination of folate vitamers is difficult because of their structural similarity, instability [11–13] and low concentrations in biological materials. *Lactobacillus casei* assay and radioimmunoassay provided high sensitivity, but did not discriminate between the various metabolites [14, 15]. Several methods have been developed for the assay of folates using high-performance liquid chromatography (HPLC) to overcome these problems [16–27]. In all methods, the proteins were first removed by physical (e.g. boiling) or chemical (e.g. trichloracetic acid) treatment. Our own results, in accordance with Wilson and Horne [11], showed that these procedures resulted in high decomposition rates of the labile folate vitamers. Therefore, we have developed a precolumn-switching system which removes the proteins and isolated folates without their interconversion from plasma samples within 50 s. This method does not require chemical or physical treatment of the plasma. The folate vitamers are therefore determined immediately after blood sampling by direct injection of plasma samples onto the HPLC system using an automatic precolumn-switching method, reversed-phase chromatography and UV detection.

EXPERIMENTAL

Materials

Folic acid (PteGlu), 5,6,7,8-tetrahydrofolic acid (H_4PteGlu), 7,8-dihydrofolic acid (H_2PteGlu), 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, pteridin, 6-COOH-pteridin and 6-OH-pteridin were obtained from Sigma (Munich, F.R.G.). 2-Mercaptoethanol, disodium hydrogen phosphate, sodium

dihydrogen phosphate and trichloroacetic acid were from Merck (Darmstadt, F.R.G.). From Phase Separation (Norwalk, CA, U.S.A.) we obtained Spherisorb ODS-2 and from Macherey & Nagel (Düren, F.R.G.) Nucleosil C_{18}. Both materials were 5 μm in diameter.

The folate compounds were dissolved in 25 m*M* phosphate buffer (pH 7.0) and stabilized with 0.1% 2-mercaptoethanol. They were stored for a maximum of one week under a nitrogen atmosphere at 4°C, exept for H_4PteGlu and H_2PteGlu, which were stored at −20°C. 5,10-Methenyl-H_4PteGlu and 10-formyl-H_4PteGlu were synthesized by the method of Rabinowitz [28].

Altromin 1324 diet was from Altromin (Lage, F.R.G.), and the diets with a controlled folic acid content were obtained from Ssniff (Soest, F.R.G.).

Minipumps with a volume of 241.0 ± 3.8 μl and a delivery rate of 7.6 ± 0.2 μl/h were a gift from Dr. J. Urquhart of Alza (Palo Alto, CA, U.S.A.). Sodium valproate was a gift from Dr. H. Schäfer of Desitin-Werke Karl Klinke (Hamburg, F.R.G.).

Animal experiments

NMRI mice were fed on an Altromin 1324 diet and obtained tap water ad libitum. 5-Formyl-H_4PteGlu was dissolved in water and injected intraperitoneally (4 mg/kg body weight) on gestational day 8, 1 h before, together with and 1 h after the injection of valproic acid. Valproic acid was dissolved in water and injected subcutaneously (500 mg/kg body weight) in the active period of the mice between 8 and 10 a.m.

Osmotic minipumps were filled with 12 mg of 5-formyl-H_4PteGlu in 240 μl of water and were implanted subcutaneously on the back 12 h before injection of valproic acid. Blood was collected 30 min after the injection of valproic acid. A folic acid-deficient diet was fed from day 4 to day 9 of gestation.

Sample preparation

Stability tests of folates. In preliminary clean-up experiments, 5-formyl-H_4PteGlu was dissolved in water or plasma and stabilized with 4% sodium ascorbate or 1% 2-mercaptoethanol. The samples were deproteinated by heating for 2—10 min in a boiling water bath by an ordinally used method [16, 17, 20, 22, 24, 25]. Deproteinization with trichloroacetic acid was performed according to Siegmund et al. [29]. After precipitation, the proteins were removed by centrifugation for 3 min in an Eppendorf centrifuge 5124 and the supernatant was applied onto the analytical column.

Analysis of folates in blood plasma and animal diets. Blood was collected from the tail vein in heparinized capillaries (60 μl). The samples were centrifuged at 2°C at 3000 *g* for 5 min. The plasma was injected directly onto the precolumns of the HPLC system. All diet samples were dissolved in 50% methanol and 25 m*M* phosphate buffer (pH 7.0) with sonification for 5 min at room temperature and then injected directly onto the precolumn system.

High-performance liquid chromatography

Separation of folate monoglutamates was performed on an HPLC system using a Perkin-Elmer 2-2 system with two independent pumps, an autosampler ISS 100, a laboratory computing integrator LCI 100, a spectrophotometer

LC-75 (all from Perkin-Elmer; Überlingen, F.R.G.) and a column-switching module from Gynkothek (Munich, F.R.G.) with two pneumatic valves (Fig. 2).

The method was fully automated. The injection by the autosampler triggered a time relay in the precolumn-switching module; the time relay limited the washing period, and at the end of the clean-up period the integrator was started. The termination of the chromatographic run by the integrator started a new injection by the autosampler.

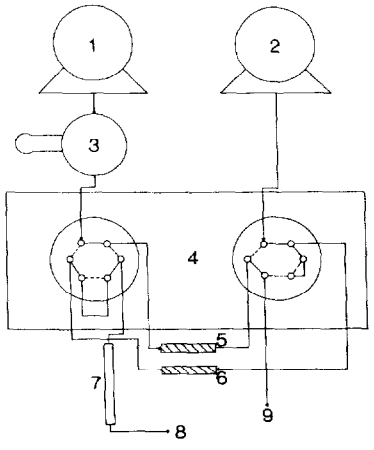

Fig. 2. Scheme of the precolumn-switching system. 1 = HPLC pump with solvent B; 2 = HPLC pump with solvent A; 3 = autosampler; 4 = column-switching module; 5 = precolumn 1; 6 = precolumn 2; 7 = analytical column; 8 = detector; 9 = waste.

The analytical column (250 × 4.6 mm I.D.) was packed with Spherisorb ODS-2 (5 μm) and the two precolumns (40 × 4.6 mm I.D.) were packed with Nucleosil C_{18} (5 μm). Solvent A (the analytical solvent) was 25 m*M* phosphate buffer (pH 7.0) and 5% methanol. Solvent B was 50 m*M* phosphate buffer (pH 7.0). Both solvents were used at a flow-rate of 1 ml/min. The analytical column was permanently flushed by solvent A, whereas both precolumns were alternately in solvent A or B stream.

Plasma samples were injected by the autosampler in volumes of 10—50 μl on precolumn 1, which was flushed by solvent B. The injection by the autosampler started a time relay in the precolumn-switching module, which stopped the washing period after 50 s. Plasma constituents such as proteins and salts were eluted. The folates were retained on the precolumn. During this time, precolumn 2 and the analytical column were flushed by solvent A, which was permanently monitored by UV detection at 280 nm.

At the end of the clean-up period, the time relay triggered the two pneumatically driven valves, and this directed solvent A to precolumn 1, which was now in-line with the analytical column. The retained folates were backflushed onto the analytical column. Precolumn 2 was now flushed with solvent B [30, 31]. Thus, both precolumns were used alternately for the sample clean-up procedure.

The folates were separated within 18 min with solvent A. This solvent separated all metabolites with good resolution.

RESULTS

Separation of folate monoglutamates

Resolution. Isocratic separation of folates and related compounds with solvent A (5% methanol—25 m*M* phosphate buffer) on a Spherisorb ODS-2 5-μm column resulted in good baseline resolution of all analysed metabolites; a typical chromatogram is presented in Fig. 3. With 5% methanol, the compounds were eluted within 18 min. This solvent was employed for the analysis of all metabolites.

Calibration curves and precision. Each vitamer was dissolved in water, stabilized with 0.1% 2-mercaptoethanol and injected separately with and without the precolumn-switching module. The samples contained 0–200 pmol (10-pmol intervals) and 200—2000 pmol (100-pmol intervals). The standard curves were obtained by plotting peak areas versus the amounts of compound injected for each vitamer. The standard curve parameters for the compounds tested are shown in Table I. Standard curves are shown in Fig. 4. The precision ranged from 6.0 to 10.0% (35 pmol injected) and from 1.6 to 6.1% (1.5 nmol injected). The precision of the method was assessed by injection of the same sample ten times.

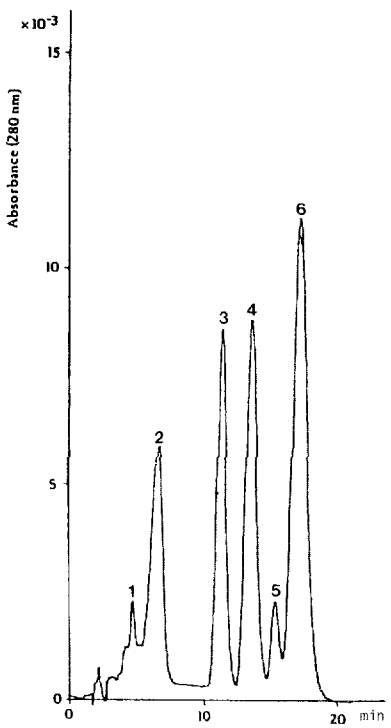

Fig. 3. Separation of standard folate monoglutamates. The eluent was 25 m*M* phosphate buffer (pH 7.0) and 5% methanol. Peaks: 1 = H_4PteGlu (42 pmol); 2 = 5-formyl-H_4PteGlu (475 pmol); 3 = 10-formyl-H_4PteGlu (1.7 nmol); 4 = 5-methyl-H_4PteGlu (900 pmol); 5 = H_2PteGlu (150 pmol); 6 = PteGlu (1.5 nmol).

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR EACH FOLATE ANALYSED

Compound	Retention time (min)	Detection limit*		Precision**		Standard curve parameters***			Recovery (%)	
		pmol	ng	35 pmol	1500 pmol	k	d	r	From water	From plasma
PteGlu	11.95	5	2.2	8.8	2.8	995	7376	0.994	98 ± 3	90 ± 3
H_2PteGlu	12.05	10	4.4	10.0	1.6	600	1830	0.997	96 ± 4	93 ± 5
H_4PteGlu	4.07	5	2.2	7.0	3.8	1102	5603	0.999	95 ± 2	94 ± 3
5-Formyl-H_4PteGlu	5.75	1	0.5	6.0	2.8	1101	4176	1.0	97 ± 2	95 ± 3
5-Methyl-H_4PteGlu	9.81	5	2.4	7.8	2.3	441	1801	1.0	96 ± 4	95 ± 6
10-Formyl-H_4PteGlu	16.33	10	4.8	6.8	7.1	262	2122	0.992	95 ± 3	93 ± 4

*Detection limit is defined as the amount of substance yielding a signal-to-noise ratio of 3.
**The precision in percentage deviation of the mean was assessed by repeated analysis of the same sample ten times. The precision was determined in water at concentrations found in mouse plasma.
***Standard curve parameters were determined by the equation $y = kx + d$, with y = area determined by the LCI 100 integrator and x = pmol of each folate compound injected.

Stability tests of folates

The reduced underivatized metabolites of PteGlu (H_4PteGlu, H_2PteGlu) are very susceptible to oxidation. The half-life of H_4PteGlu was ca. 10 min at room temperature without the addition of antioxidants, but was extended to 10 h at 4°C with the addition of 2% 2-mercaptoethanol. After one day, H_4PteGlu was converted to 55% 5-methyl-H_4PteGlu, 29% PteGlu and 16% H_4PteGlu (Fig. 6). In contrast, the recovery of 5-formyl-H_4PteGlu after 24 h at 37°C was 99.3%.

High temperatures, also after the addition of sodium ascorbate, as commonly used for deproteinization, caused a rapid decline and interconversion of folates. The treatment of 5-formyl-H_4PteGlu in boiling water (1% sodium ascorbate, 25 m*M* phosphate buffer, pH 7.0) resulted in a decrease to 35% of the initial concentration after 2 min. The same results were obtained with 5-formyl-H_4PteGlu-spiked plasma (Table II). Using trichloroacetic acid for deproteiniza-

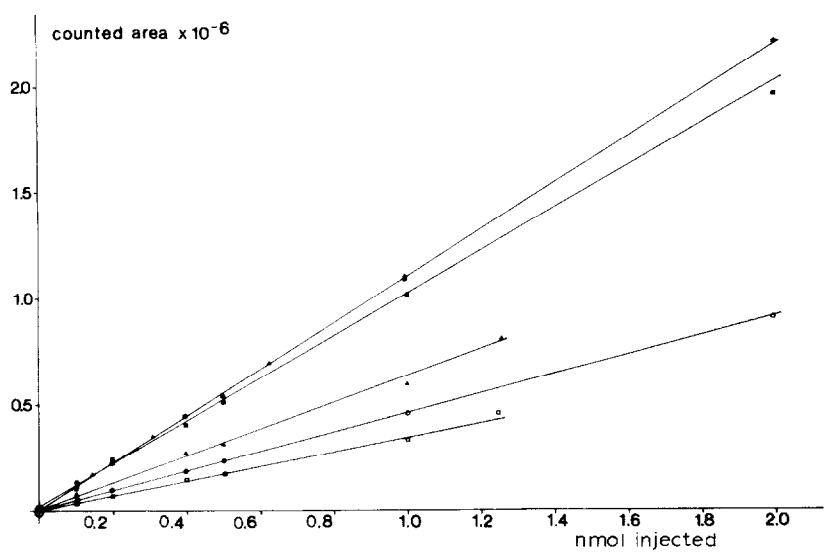

Fig. 4. Standard curves of the tested folate monoglutamates: H_4PteGlu (△), H_2PteGlu (▲), 5-methyl-H_4PteGlu (○), 5-formyl-H_4PteGlu (●), 10-formyl-H_4PteGlu (□) and PteGlu (■).

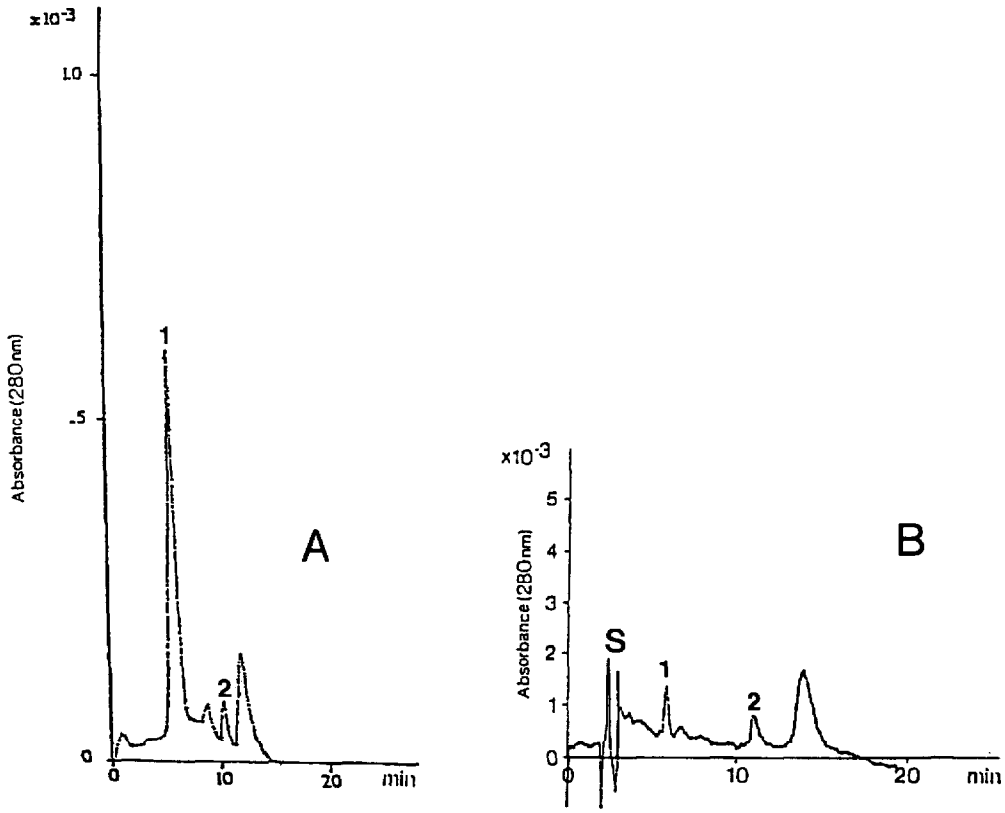

Fig. 5. Separation of folate monoglutamates in mouse plasma. Chromatogram A: 20 μl of plasma of an animal supplemented with 12 mg 5-formyl-H_4PteGlu per 24 h via osmotic minipump. Blood was collected 12 h after implantation of the minipump. Peaks: 1 = 5-Formyl-H_4PteGlu (4.1 μg/ml); 2 = 5-methyl-H_4PteGlu (0.45 μg/ml). Chromatogram B: 20 μl of plasma of an untreated animal. Peaks: S = solvent front; 1 = 5-formyl-H_4PteGlu (0.29 μg/ml); 2 = 5-methyl-H_4PteGlu (0.43 μg/ml).

TABLE II

STABILITY OF 5-FORMYL-H_4PteGlu UNDER VARIOUS PHYSICAL AND CHEMICAL TREATMENT PROCEDURES AND COMPARISON OF THESE DATA WITH THE NEW PRECOLUMN-SWITCHING SYSTEM

Sample	Recovery of 5-formyl-H_4PteGlu (% of initial concentration)		
	2 min heating	10% Trichloro-acetic acid	Precolumn system
100 pmol 5-Formyl-H_4PteGlu per ml water containing 0.1% sodium ascorbate	35	Not detectable	97 ± 2
Plasma sample spiked with 5-formyl-H_4PteGlu and containing 0.4% sodium ascorbate	35	Not detectable	95 ± 3

tion, there was a complete decomposition of 5-formyl-H_4PteGlu in plasma containing 0.4% sodium ascorbate. These results emphasize that heating the samples or using chemical procedures to extract folates from biological fluids are unsuitable (Table II).

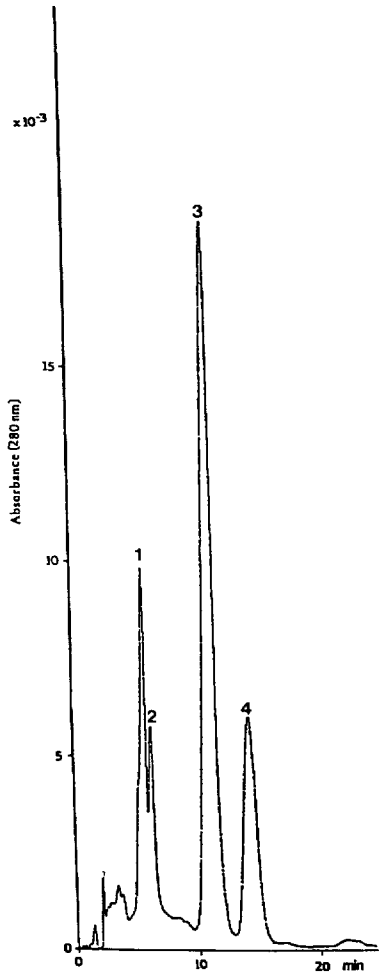

Fig. 6. Interconversion products of H_4PteGlu: 20 µl of a 0.5 m*M* standard solution of H_4PteGlu, stabilized with 2% 2-mercaptoethanol and stored for 24 h at 4°C. Peaks: 1 = Pteridin; 2 = H_4PteGlu; 3 = 5-methyl-H_4PteGlu; 4 = PteGlu.

Precolumn-switching system

All metabolites were retained on the precolumn with solvent B and liberated during the backflush with solvent A. A recovery of 95–98% was obtained for each vitamer within the concentration range studied (30 pmol and 1 nmol). Recovery rates between 93 and 95% were obtained for plasma samples spiked with 100 pmol/ml of each vitamer, except in the case of PteGlu with a recovery of 88–93% (Table I). Interconversions of folates were not detectable.

Analysis of folates in mouse plasma and diets

The mouse plasma contained 260 ± 90 ng/ml 5-formyl-H_4PteGlu and 390 ± 100 ng/ml 5-methyl-H_4PteGlu (Fig. 5). There was no significant difference between pregnant (day 8 of gestation) and non-pregnant mice (Table III). The Altromin 1324 diet showed 83 ± 2.5 mg/kg 5-formyl-H_4PteGlu and 6.5 ± 0.3 mg/kg PteGlu (analysis of three parallel samples). Ssniff 1/0 normal diet con-

TABLE III

FOLATE CONCENTRATIONS DETERMINED BY HPLC WITH A PRECOLUMN-SWITCHING SYSTEM

Sample	*n*	5-Formyl-H_4PteGlu	PteGlu	5-Methyl-H_4PteGlu
Mouse plasma (μg/ml)				
Pregnant untreated*	23	0.26 ± 0.1	N.D.**	0.39 ± 0.2
Non-pregnant untreated	5	0.25 ± 0.1	N.D.	0.40 ± 0.1
Pregnant with minipumps treatment***	29	4.2 ± 0.9	N.D.	0.20 ± 0.1
Diets (mg/kg)				
Altromin 1324	3	83 ± 2.5	6.5 ± 0.3	N.D.
Ssniff 1/0 normal	3	3.5 ± 0.3	8.4 ± 0.2	N.D.
Ssniff 1/0 folic acid deficient diet	3	3.8 ± 0.8	N.D.	N.D.
Ssniff 1/0 folic acid supplementation	3	5.5 ± 0.8	690 ± 40	N.D.

*Untreated mice without application of any drug.
**N.D. = Not detectable.
***Osmotic minipumps filled with 12 mg 5-formyl-H_4PteGlu per 240 μl water with a content of 241.0 ± 3.8 μl and a delivery rate of 7.6 ± 0.2 μl/h were implanted 12 h before the injection of valproic acid. Blood for the determination of folates was collected 30 min after the treatment with valproic acid.

tained 3.2 ± 0.7 mg/kg 5-formyl-H_4PteGlu and 8.4 ± 0.2 mg/kg PteGlu. Ssniff folic acid supplementation diet contained 5.2 ± 0.7 mg 5-formyl-H_4PteGlu/kg diet and 690 ± 40 mg PteGlu/kg diet. Ssniff folic acid deficient diet showed 3.2 ± 0.3 mg 5-formyl-H_4PteGlu, but PteGlu was not detectable (Table III).

DISCUSSION

Our investigations, in accordance with Wilson and Horne [11], showed that all commonly used extraction methods of folates were performed either with trichloroacetic acid or with elevated temperatures and sodium ascorbate or 2-mercaptoethanol. Partial or total decomposition or interconversion of the vitamers due to their susceptibility to oxidation was shown by the following separation by HPLC. Thus, the isolation of folates from biological materials with the conventional methods previously described [16, 17, 20, 22, 24, 25] was unsuitable.

The tissue folates are polyglutamates, and conjugase treatment in order to obtain folate monoglutamates is necessary. Wilson and Horne [32] reported the isolation of folate polyglutamates from tissues at decomposition rates between 44% (H_2PteGlu) and 5.6% (5-methyl-H_4PteGlu). In contrast, plasma folates are monoglutamates and can be determined directly after deproteinization. Our newly developed method is the first chromatographic technique for the isolation and determination of folate monoglutamates in plasma by a single HPLC system. No time-consuming or difficult clean-up steps are needed for the deproteinization.

With this simple, fully automated precolumn-switching technique, inter-

conversion of folates during clean-up and chromatographic separation was not detectable.

Volumes of 10—50 μl could be injected directly onto the precolumns by the autosampler. Up to 300 samples were applied onto the precolumns before it was necessary to change the precolumns, which was performed in a few minutes.

The detection limit is as low as in most of the published methods, but not low enough to measure human plasma samples, which contain between 3 and 20 ng/ml (6—45 pmol/ml) folates [33—36]. The determination of folates with the presented HPLC method using UV detection is limited to samples with a relatively high level of folates. We have applied this new method to the analysis of diets and mouse plasma. The total content of folates in the Altromin 1324 diet was not described by the manufacturer. Altromin added 3 mg PteGlu/kg diet to the crude protein, crude fat and crude fibre. The analysis of the diet showed 90 ± 3 mg folate per kg diet. Ssniff offers diets with controlled folic acid contents, which were confirmed by our HPLC method.

Untreated NMRI mice showed high plasma concentrations of folates (0.6 ± 0.3 μg/ml). There was no significant difference between pregnant and non-pregnant mice. Previously described plasma levels of mice [37], rats [38- 40] and sows [41] showed plasma folate levels of 40—100 ng/ml. Only Baker et al. [42] reported 0.56 ± 0.12 μg/ml plasma folates in the rat. This can be partially due to the nutritional supply of folates by the diet. With Altromin 1324 diet, the daily uptake was 18—20 mg folate per kg body weight of the mouse (daily uptake was ca. 7 g diet). This daily supplementation with folates is very high in comparison to human recommended values. Adults need ca. 100 μg folate per day and during pregnancy 350 μg folate per day [1, 43].

After supplementation of 5-formyl-H_4PteGlu via osmotic minipumps, the applied compound was the main metabolite in the plasma. This indicated that the application of 5-formyl-H_4PteGlu did not result in an increase of other folate metabolites. The determination of the metabolic pattern is of interest, because a change in this pattern could be due to malnutrition or to therapy with drugs and could cause many diseases. To perform this presented fully automated method to the determination of folates in human plasma, it is necessary to develop a sensitive detection method.

In our animal model, however, the measurement of the pattern of the folate metabolites and the correlation to neural tube defects was possible and extended the experimental studies [10, 44].

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