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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PHYSIOLOGICAL FOLATE MONOGLUTAMATE COMPOUNDS

INVESTIGATION OF ABSORPTION AND CONVERSION OF PTEROYL-GLUTAMIC ACID IN THE SMALL INTESTINE OF THE RAT *IN SITU*

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SUMMARY

A new high-performance liquid chromatographic method to investigate many folate monoglutamate compounds was developed. This method employed a Cosmosil 5-Ph column eluted with 10 mM potassium phosphate buffer (pH 7.0) containing 1 % methanol at a flow-rate of 1.0 ml/min. Under these conditions, almost all of the physiological folate monoglutamate compounds were effectively separated and determined within 22 min. By applying this method to investigate the absorption and conversion of pteroylglutamic acid in the rat small intestine, it was demonstrated that pteroylglutamic acid was converted into 5-methyltetrahydrofolic acid during absorption, and that the conversion was a saturable process, whereas unchanged pteroylglutamic acid was absorbed almost linearly in proportion to the initial amount administered.

INTRODUCTION

Folate compounds are essential coenzymes in the biosynthesis of amino acids, proteins and nucleic acid bases, and folate deficiency and genetic defects of folate metabolism cause serious diseases in humans. Folate compounds consist of many derivatives which differ in the reduced states of the pteridine ring, in the substitution at N^5 and N^{10} positions and in the number of glutamic acid residues.

Analysis of various folate compounds has been performed by ion-exchange columns with DEAE-cellulose[1] or QAE-Sephadex A-25[2], and by gel filtration with Sephadex G-10, G-15 and G-25[3]. However, all these methods are not practical for precise investigations because they have low sensitivity, are time-consuming and separate only a minor number of folate compounds.

Recently, several groups have reported high-performance liquid chromatographic (HPLC) methods for the separation of folate compounds[4–13]. In this paper, we describe a useful HPLC method to perform more sensitive and rapid analysis of

many physiological folate monoglutamate compounds. The method was applied to an investigation of the pteroylglutamic acid (PteGlu) absorption in the rat small intestine *in situ*.

EXPERIMENTAL

Chemicals

PteGlu, 7,8-dihydrofolic acid ($H_2PteGlu$), 5,6,7,8-tetrahydrofolic acid ($H_4PteGlu$), 5-methyltetrahydrofolic acid (5-CH_3-$H_4PteGlu$) sodium salt, 5-formyl-tetrahydrofolic acid (5-CHO-$H_4PteGlu$) calcium salt, pterin, pteroic acid, 6-hydroxy-methylpterin (6-CH_2OH-pterin), 6-carboxypterin (6-COOH-pterin) and *p*-amino-benzoylglutamic acid (*p*-ABG) were purchased from Sigma (St. Louis, MO, U.S.A.). 10-Formyltetrahydrofolic acid (10-CHO-$H_4PteGlu$) and 5,10-methylidy-netetrahydrofolic acid (5,10-CH = $H_4PteGlu$) were synthesized by the method of Scott[14]. [³H]PteGlu potassium salt (5 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). The solutions of standard folate compounds were stored with 1% 2-mercaptoethanol at $-20°C$ under nitrogen.

Liquid chromatography

HPLC was performed on a Shimadzu HPLC system using a liquid chromatograph LC-3A, spectrophotometric detector SPD-2A and Chromatopac-E1A. A column (150 × 3.6 mm I.D.) packed with Cosmosil 5-Ph (5 μm; Nakarai Chemicals, Kyoto, Japan) and a guard column (40 × 3.6 mm I.D.) packed with LiChrosorb RP-2 (30 μm; E. Merck, Darmstadt, G.F.R.) were used. As the mobile phase, a degassed solution consisting of 10 mM potassium phosphate buffer (KPB, pH 7.0) and 1% methanol was used at a flow-rate of 1.0 ml/min.

In situ experiment

Male Wistar rats (230–260 g) were anaesthetized with 55 mg/kg of sodium pentobarbital, and given an intravenous infusion of heparinized saline via the thigh vein. The jejunal loop (10 cm) was identified through a midline abdominal incision. The mesenteric vein from the loop was cannulated with a needle and all venous blood was collected every 2.5 min into centrifuge tubes on ice. The [³H]PteGlu solution was immediately injected into the loop. The venous blood collected was centrifuged at 700 *g* for 10 min, and aliquots of the supernatant were counted in a Packard automatic Tri-carb liquid scintillation spectrometer, Model 3255. A mixture of 0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene and 30% Triton X-100 was used as the scintillation cocktail.

Preparation of blood sample

All operations were carried out below 4°C unless otherwise noted, and shielded from light. To the sample solution, HCl and 2-mercaptoethanol were added to final concentrations each of 50 mM. The solution was placed in boiling water for 1 min and the precipitate was removed by centrifugation at 2000 *g* for 5 min. The supernatant was filtered with a diaflo ultrafiltration membrane YM-2 (Amicon, Lexington, MA, U.S.A.), and the filtrate was lyophilized. The sample was dissolved in 50 μl of 1% 2-mercaptoethanol, and aliquots (5–10 μl) were used for HPLC analysis.
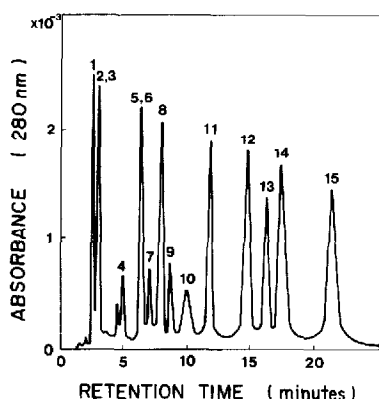
Fig. 1. Chromatographic separation of various folate compounds and their related compounds. Conditions: column, Cosmosil 5-Ph (5 μm, 150 × 3.6 mm); guard column, LiChrosorb RP-2 (30 μm, 40 × 3.6 mm); mobile phase, 10 mM KPB (pH 7.0) containing 1 % methanol; flow-rate, 1.0 ml/min; detector, UV absorbance at 280 nm, 0.01 a.u.f.s. Standard samples: 1 = p-ABG; 2 = p-aminobenzoic acid; 3 = 6-COOH-pterin; 4 = 2-mercaptoethanol; 5 = 10-CHO-H_4PteGlu; 6 = 6-CH_2OH-pterin; 7 = pterin; 8 = H_4PteGlu; 9 = 10-CHO-PteGlu; 10 = 5-CHO-H_4PteGlu; 11 = PteGlu; 12 = H_2PteGlu; 13 = 5,10-CH = H_4PteGlu; 14 = pteroic acid and 15 = 5-CH_3-H_4PteGlu; 10–30 pmol of each compound were injected.

RESULTS

Separation of folate monoglutamate compounds by HPLC on Cosmosil 5-Ph

Fig. 1 shows the elution profile obtained when eight folate monoglutamate compounds and their related compounds were applied on a Cosmosil 5-Ph column and eluted with a mobile phase consisting of 10 mM KPB (pH 7.0) and 1 % methanol at a flow-rate of 1.0 ml/min. It was found that almost all of the physiological folate monoglutamate compounds were effectively baseline-separated within 22 min. Under

TABLE I

DETERMINATION OF SIX REPRESENTATIVE FOLATE COMPOUNDS

The quantitative chromatographic parameters were obtained by least squares regression analysis of the peak area, determined at 280 nm with a detector setting of 0.01 a.u.f.s., *versus* the amount of each compound injected on the column. Mobile phase: 10 mM KPB (pH 7.0) containing 1 % methanol; flow-rate, 1.0 ml/min.

Folate compound	Range of linearity (pmol)	Linear regression equation*	Regression, r
PteGlu	1.0–200	$y = 273x + 644$	0.999
H_2PteGlu	2.0–240	$y = 235x - 403$	0.993
H_4PteGlu	3.0–300	$y = 183x + 208$	0.985
5-CH_3-H_4PteGlu	2.5–500	$y = 191x - 314$	0.998
5-CHO-H_4PteGlu	5.5–300	$y = 298x + 572$	0.921
5,10-CH=H_4PteGlu	2.5–300	$y = 187x + 181$	0.997

  * y = Area count on Shimadzu Chromatopac-E1A integrator recorder; x = pmol of each folate compound injected.
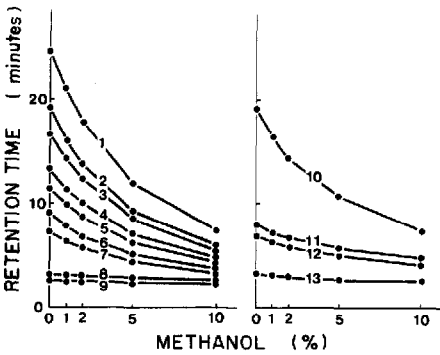
Fig. 2. Effect of methanol concentration in the mobile phase on the retention time of each compound. The HPLC conditions were as in Fig. 1 except for the methanol concentration in the mobile phase. Standard samples: 1 = 5-CH_3-H_4PteGlu; 2 = 5,10-CH=H_4PteGlu; 3 = H_2PteGlu; 4 = PteGlu; 5 = 5-CHO-H_4PteGlu; 6 = H_4PteGlu; 7 = 10-CHO-H_4PteGlu; 8 = p-aminobenzoic acid; 9 = p-ABG; 10 = pteroic acid; 11 = pterin; 12 = 6-CH_2OH-pterin and 13 = 6-COOH-pterin.

these conditions, various amounts of six folate compounds (PteGlu, H_2PteGlu, H_4PteGlu, 5-CH_3-H_4PteGlu, 5-CHO-H_4PteGlu and 5,10-CH = H_4PteGlu) were separately injected and determined at 280 nm with a detector setting of 0.01 absorbance units full scale (a.u.f.s.). The standard curves were obtained for each folate compound by plotting peak area *versus* the amount of compound injected, and the quantitative chromatographic parameter was calculated by least squares regression analysis. As shown in Table I, the curves for all the compounds tested were linear with excellent correlation coefficients. The minimum levels detectable under these conditions were 1.0 pmol (0.44 ng) as PteGlu and 2.5 pmol as 5-CH_3-H_4PteGlu.

The retention times of the folate compounds tested were dependent on the methanol concentration in the mobile phase, as shown in Fig. 2. Thus, it is possible to choose an appropriate methanol concentration for the particular analysis and the
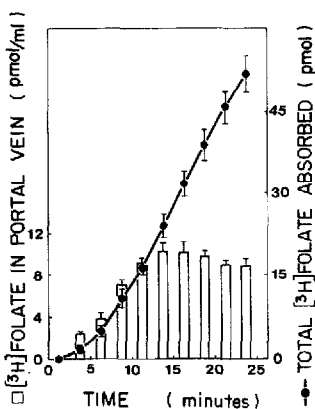

Fig. 3. Amounts of [³H]folate compounds absorbed into portal venous blood *in situ*. Anaesthetized rat was given an intravenous infusion of heparinized saline. The mesenteric vein from the jejunal loop (10 cm) was cannulated and venous blood was collected every 2.5 min (about 1 ml per tube). Immediately, 1 ml of the [³H]PteGlu solution (0.2 nmol/ml) was injected into the loop.
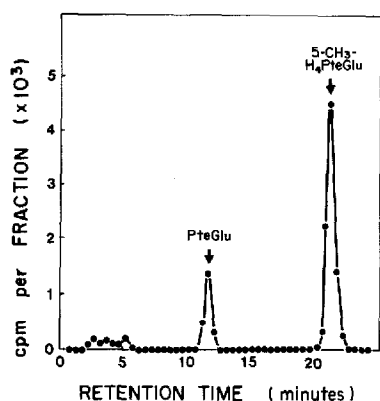
Fig. 4. Chromatographic separation of [³H]folate compounds in the portal venous blood. The [³H]PteGlu solution (0.2 nmol/ml) was injected into the rat jejunal loop *in situ*. The portal venous blood from 10 to 20 min after the administration was prepared as described under Experimental. HPLC conditions as in Fig. 1.

number of folate compounds to be separated. Usually, we employed a mobile phase containing 1 % methanol for routine work.

Absorption of PteGlu in the rat small intestine in situ

When 1 ml of the [³H]PteGlu solution (0.2 nmol/ml) was injected into rat jejunal loop *in situ*, the concentration of [³H]folate compounds in the portal venous blood increased during the first 10 min after administration, and subsequently remained constant, as shown in Fig. 3. The [³H]folate compounds, absorbed into the portal blood from 0 to 20 min after administration, were investigated by HPLC analysis. As shown in Fig. 4, approximately 82 % of PteGlu had been converted into 5-CH₃-H₄PteGlu during absorption.

When test solutions containing various amounts of [³H]PteGlu were injected into rat jejunal loops, the ratio of conversion of PteGlu into 5-CH₃-H₄PteGlu was dependent on the initial amount of [³H]PteGlu administered into the loop, as shown in Table II.

TABLE II

CONVERSION RATIO OF PteGlu INTO 5-CH₃-H₄PteGlu

The sample solutions, containing various amounts of [³H]PteGlu, were injected into rat jejunal loops *in situ*. The [³H]folate derivatives in the portal venous blood were determined by HPLC as described under Experimental. Values are mean ± S.D. of the data from four rats.

[³H]PteGlu administered (nmol)	Conversion ratio (%)
0.1	88.0 ± 2.1
0.2	82.3 ± 5.4
0.4	64.5 ± 3.7
1.0	52.5 ± 2.8
2.0	41.7 ± 5.6
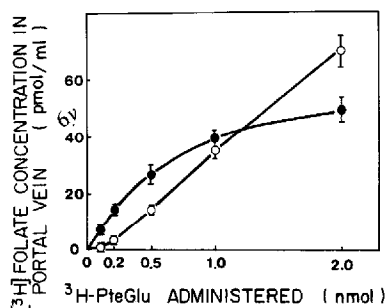
Fig. 5. The concentration of [³H]PteGlu (○) and [³H]5-CH₃-H₄PteGlu (●) in the portal venous blood. The sample solutions, containing various amounts of [³H]PteGlu, were injected into rat jejunal loops *in situ*. The portal venous blood was collected and prepared as described under Experimental, and analyzed by HPLC. Values are the mean ± S.D. of the data from four rats.

Fig. 5 shows the concentration of each [³H]folate compound in the portal venous blood, plotted against the initial amount of [³H]PteGlu injected. The concentration of unchanged [³H]PteGlu increased in the blood almost linearly with the initial amount of [³H]PteGlu administered. However, the conversion of PteGlu into 5-CH₃-H₄PteGlu was a saturable process, which obeyed Michaelis–Menten kinetics ($r = 0.99$).

DISCUSSION

The analytical technique of HPLC has made remarkable progress over the past decade. Recently, several methods for the HPLC separation of folate compounds were reported[4–13]. Some investigators[10,11] employed ion-pair chromatography with tetrabutylammonium phosphate (PIC A) on a C_{18} column eluted with a non-linear gradient of ethanol or methanol. Another group[12,13] reported the use of ion-pair chromatography with two C_{18} phenyl columns. However, these methods required a solvent program and re-equilibration prior to injection of the next sample. Moreover, the detectable levels of folate compounds under these conditions were 20–50 times higher than that in our HPLC system which is almost the same level as that of a microbiological assay[10]. Microbiological assay has been employed for the determination of folate compounds. However, it cannot distinguish between the individual folate compounds, which differ in physiological functions and bioavailability for both microorganisms and animals. Our HPLC system is a much more useful method to separate many physiological folate monoglutamate compounds, more rapidly and effectively than previous methods.

We applied this HPLC system to investigate the absorption and conversion of PteGlu in the rat small intestine. Smith *et al.*[15] previously reported that PteGlu transported into the scrosal side of jejunal everted sac was largely in an unaltered form. On the contrary, other groups reported that reduction, methylation[16–18] and formylation[19] occurred during intestinal transport of PteGlu *in vitro*. However, in these cases, unchanged PteGlu was also detected in the serosal side as well as $H_4PteGlu$, $5\text{-CHO-}H_4PteGlu$ and $5\text{-CH}_3\text{-}H_4PteGlu$. The correct ratio of conversion of PteGlu into the reduced forms of folate compounds was not reported in the

previous papers. The *in situ* technique used here was demonstrated to be much more useful for studying intestinal functions[20], because more physiological conditions were maintained compared to other techniques *in vitro*. It was previously reported[21-26] that $5\text{-}CH_3\text{-}H_4PteGlu$ differed from PteGlu in the plasma clearance and in its uptake into many tissues and cells. Our results showed that the ratio of conversion of PteGlu into $5\text{-}CH_3\text{-}H_4PteGlu$ in the rat small intestine *in situ* was higher than any value *in vitro* previously[16-18]. Moreover, this conversion *in situ* was a saturable process, whereas unchanged PteGlu was absorbed almost linearly in proportion to the initial amount injected into the jejunal loop. These results indicate that in the investigation on the intestinal absorption of PteGlu, not only the measurement of the total amount of folate compounds absorbed but also the analysis of each folate compound is important. We are now applying this HPLC technique to other physiological samples to investigate the metabolism of folate compounds *in vivo*.

ACKNOWLEDGEMENT

REFERENCES

1 B. T. Kaufman, K. O. Donaldson and J. C. Keresztesy, *J. Biol. Chem.*, 283 (1963) 1498.
2 K. U. Buehling, T. Tamura and E. L. R. Stockstad, *J. Biol. Chem.*, 249 (1974) 1081.
3 Y. S. Shin, K. U. Buehling and E. L. R. Stockstad, *J. Biol. Chem.*, 247 (1972) 7266.
4 L. S. Reed and M. C. Archer, *J. Chromatogr.*, 121 (1976) 100.
5 R. W. Stout, A. R. Cashmore, J. K. Coward, C. G. Horvath and J. R. Bertino, *Anal. Biochem.*, 71 (1976) 119.
6 S. K. Chapman, B. C. Greene and R. R. Streiff, *J. Chromatogr.*, 145 (1978) 302.
7 J. Lankelma and E. van der Kleijn, *J. Chromatogr.*, 182 (1980) 35.
8 M. C. Archer and L. S. Reed, *Methods Enzymol.*, 66 (1980) 452.
9 K. E. McMartin, V. Virayotha and T. R. Tephly, *Arch. Biochem. Biophys.*, 209 (1981) 127.
10 B. A. Allen and R. A. Newman, *J. Chromatogr.*, 190 (1980) 241.
11 D. W. Horne, W. T. Biggs and C. Wagner, *Anal. Biochem.*, 116 (1981) 393.
12 R. N. Reingold, M. F. Picciano and E. G. Perkins, *J. Chromatogr.*, 190 (1980) 237.
13 R. N. Reingold and M. F. Picciano, *J. Chromatogr.*, 234 (1982) 171.
14 J. M. Scott, *Methods Enzymol.*, 66 (1980) 437.
15 M. E. Smith, A. J. Matty and J. A. Blaiw, *Biochim. Biophys. Acta*, 219 (1970) 37.
16 J. H. Selhub, H. Brin and N. Grossowicz, *Eur. J. Biochem.*, 33 (1973) 433.
17 E. J. Olinger, J. R. Bertino and H. J. Binder, *J. Clin. Invest.*, 52 (1973) 2138.
18 W. B. Strum, *Biochim. Biophys. Acta*, 554 (1979) 249.
19 I. Perry and I. Chanarin, *Brit. Med. J.*, 1 (1973) 558.
20 S. Kimura, T. Totani and T. Murachi, *Jap. Exp. Med.*, 43 (1973) 163.
21 H. Sotobayashi, *Nippon Ketsuekigaku Kaishi*, 36 (1973) 509.
22 P. F. Nixon and J. B. Bertino, *New Engl. J. Med.*, 286 (1972) 175.
23 I. D. Goldman, *Ann. N.Y. Acad. Sci.*, 186 (1971) 400.
24 A. Nahas, P. F. Nixon and J. R. Bertino, *Fed. Proc.*, 28 (1969) 289.
25 K. C. Das and A. V. Hoffbrand, *Brit. J. Haematol.*, 19 (1970) 203.
26 N. S. Lichtenstein, V. T. Oliverio and I. D. Goldman, *Biochim. Biophys. Acta*, 193 (1969) 456.