Journal of Chromatography, 417 (1987) 47–56
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3605

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PTEROYLPOLYGLUTAMATE HYDROLASE

JERAPAN KRUNGKRAI*,*

Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, and Department of Immunology and Biochemistry, Armed Forces Research Institute of Medical Sciences, Rajavithi Road, Bangkok 10400 (Thailand)

YONGYUTH YUTHAVONG

Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400 (Thailand)

and

H. KYLE WEBSTER

Department of Immunology and Biochemistry, Armed Forces Research Institute of Medical Sciences, Rajavithi Road, Bangkok 10400 (Thailand)

(First received November 4th, 1986; revised manuscript received January 12th, 1987)

SUMMARY

A highly sensitive assay for pteroylpolyglutamate hydrolase is described employing high-performance liquid chromatography (HPLC) with ultraviolet detection at 280 nm. The method is based on the separation of pteroylpolyglutamates containing various glutamyl residues on a C_{18} μBondapak reversed-phase column. Individual pteroylpolyglutamates are eluted by a gradient of 2.5–8.5% acetonitrile in 0.1 *M* potassium phosphate buffer (pH 6.0) within 20 min. The polyglutamates with higher glutamyl residues were less well retained in the reversed-phase column. The relationship between the peak area and the amount of pteroylpolyglutamate was observed to be linear over the range 10 pmol to 2.5 nmol. Human serum pteroylpolyglutamate hydrolase was studied using pteroylpentaglutamate as substrate in 0.1 *M* sodium acetate buffer (pH 4.5). The enzyme appeared to function as an exopeptidase based on the detection of intermediates, pteroyltetra-, -tri-, and -diglutamate, and the product, pteroylmonoglutamate. Using the HPLC assay, extracts of *Plasmodium falciparum* were found not to contain detectable enzyme activity.

*Address for correspondence: Department of Immunology and Biochemistry, U.S. Army Medical Component, AFRIMS, APO, San Francisco, CA 96346, U.S.A.

0378-4347/87/$03.50

INTRODUCTION

Enzymes that hydrolyze the polyglutamate side-chain of the widely distributed pteroylpolyglutamates ($PteGlu_n$), called variously pteroylpolyglutamate hydrolases (PPGH), γ-glutamyl hydrolases or conjugases (EC 3.4.22.12), play an important role in the bioavailability of dietary pteroylpolyglutamates and in the metabolism of pteroylpolyglutamates [1, 2]. There are several procedures available for the assay of PPGH. The first assay for PPGH was based on the microbiological measurement of folate activity released by enzyme action from a variety of crude or partially purified preparations of natural pteroylpolyglutamate, e.g. yeast extract [3], or synthetic pteroylpolyglutamates [2, 4]. A later radiometric assay was based upon the release and quantitation of labeled glutamic acid from a synthetic pteroylpolyglutamate used as substrate [5, 6]. Other methods were paper electrophoresis [7], acrylamide gel electrophoresis [8] and paper [1] and thin-layer chromatography [9]. Recently, methods using high-performance liquid chromatography (HPLC) were introduced for the separation of pteroylpolyglutamates [10–12].

We describe here a rapid, sensitive and simple PPGH assay based on reversed-phase HPLC. The basic principle involves the separation of pteroylpentaglutamate ($PteGlu_5$, substrate) and pteroylmonoglutamate (PteGlu, product) with the simultaneous detection of pteroyltetra-, -tri-, and -diglutamate ($PteGlu_4$, $PteGlu_3$, $PteGlu_2$, respectively), as intermediates. This method was used to study the kinetics of the PPGH activity in human serum (hydrolysis of $PteGlu_5$ to release PteGlu). The method was also used to examine the human malaria parasite, *Plasmodium falciparum*, for the presence of PPGH.

EXPERIMENTAL

Materials

Pteroylhepta-, -penta-, and -triglutamate ($PteGlu_7$, $PteGlu_5$, $PteGlu_3$, respectively) were kindly synthesized by Dr. Carlos L. Krumdieck (Department of Nutrition Sciences, University of Alabama, Birmingham, AL, U.S.A.). PteGlu or folic acid was obtained from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Sephadex G-25 (medium size) was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). All other chemicals were purchased from Sigma.

HPLC apparatus and chromatographic conditions

The HPLC system was a Spectra-Physics (Santa Clara, CA, U.S.A.) Model SP 8100 liquid chromatograph equipped with a Valco Model C6U injector (50-μl loop), a Model SP 8440 variable-wavelength detector and a Model SP 4270 computing integrator.

Pteroylpolyglutamates were separated using a C_{18} μBondapak reversed-phase column (300×3.9 mm I.D.; particle size 10 μm) (Waters Assoc., Milford, MA, U.S.A.) used in conjunction with a C_{18} Corasil μBondapak (27–40 μm) guard

column. The eluting solvent (solvent A) was 0.1 *M* potassium phosphate buffer, pH 6.0, at a flow-rate of 1.5 ml/min. A programmed gradient elution from 75% of the phosphate buffer (solvent A) and 25% of 10% acetonitrile in the phosphate buffer (solvent B) to 15% of solvent A and 85% of solvent B at 20 min was applied. The effluent was monitored at 280 nm and retention times and peak areas were computed by the SP 4270 integrator. All analyses were conducted at ambient temperature (approximately 25 °C). The concentrations of pteroylpoly- and -monoglutamate were estimated from E_{283} 23 400 M^{-1} cm^{-1} [13].

Enzyme preparations

Human serum (stored at −20 °C for five to seven days) and malaria parasites were used as a source of PPGH. The human parasite, *P. falciparum,* (K_1 and G_{112} isolates [14]), was maintained in culture by the method of Trager and Jensen[15]. The serum or parasite extract (freeze–thaw lysate) was applied to a Sephadex G-25 column (2 ml bed volume) to separate the enzyme from low-molecular-weight contaminants. The void volume eluate in 0.1 *M* sodium acetate buffer pH 4.5 (600 μl) was saved for the enzyme assay. Protein concentration was determined by the method of Bradford [16] using bovine serum albumin as standard.

Enzyme assay

The assay of PPGH activity was based on the rate of release of PteGlu from $PteGlu_5$. The standard reaction mixture (250 μl) contained the following components: 200 μl of enzyme eluate from the Sephadex G-25 column in 0.1 *M* sodium acetate buffer (pH 4.5), 25 μl of 0.1 *M* sodium acetate buffer and 25 μl of 1.0 *M* $PteGlu_5$ in the buffer. The reaction was preincubated at 37 °C for 5 min and initiated by adding 25 μl of the $PteGlu_5$ solution to give a final concentration of 0.1 *M*, and then allowed to proceed at 37 °C for 1 h in the dark. After incubation, 250 μl of ice-cold 2% ascorbic acid in 0.1 *M* phosphate buffer (pH 6.5) were added and the reaction mixture was then immediately placed in a boiling water-bath for 10 min. The mixture was cooled in an ice-bath and then centrifuged in an Eppendorf microfuge for 10 min. The supernatant was kept at −20 °C until HPLC analysis.

RESULTS

Using the C_{18} μBondapak column and HPLC procedure described, pteroylpolyglutamate derivatives were found to be well separated within 15 min (Fig. 1). The small peaks observed at retention times (t_R) of 6.53, 9.33 and 14.85 min were $PteGlu_4$, $PteGlu_2$ and pteroic acid (Pte), respectively. Highly reproducible retention times [5.44 ± 0.34 min, $n=10$, coefficient of variation (C.V.) = 6.3% for $PteGlu_5$] and peak areas (2 534 410 ± 128 281 integrator response, $n=5$, C.V. = 5.1% for 1.25 nmol $PteGlu_5$) for the pteroylpolyglutamates were observed. The relation between the number of glutamate residues (n) of the pteroylpolyglutamates, $PteGlu_n$, and the t_R of each pteroylpolyglutamate is shown in Fig. 2. The logarithm of t_R and square root of n of the pteroylpolyglutamate has a linear

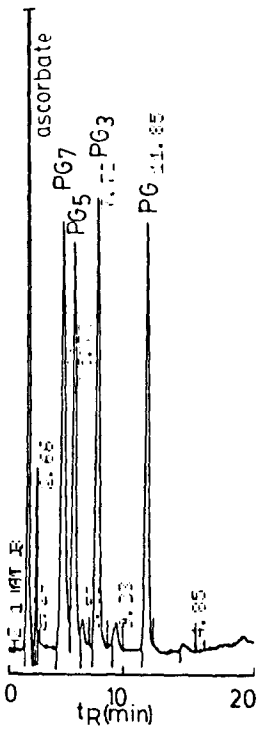

Fig. 1. HPLC profile of pteroylpoly- and -monoglutamates. Column, μBondapak C_{18} (10 μm) with Corasil μBondapak C_{18} (27–40 μm) as guard column. A programmed gradient elution from 75% of 0.1 *M* phosphate buffer, pH 6.0 (solvent A) and 25% of 10% acetonitrile in solvent A (solvent B) to 15% of solvent A and 85% of solvent B at 20 min with a flow-rate of 1.5 ml/min was used. The effluent was monitored at 280 nm. The concentration of each compound injected was 1 nmol/ml in a sample volume of 50 μl. Peaks: PG = pteroylmonoglutamate; PG_3 = pteroyltriglutamate; PG_5 = pteroylpentaglutamate; PG_7 = pteroylheptaglutamate.

relationship. The *n* of pteroylpolyglutamates can therefore be determined from the linear relationship, such as $PteGlu_6$, $PteGlu_4$ and $PteGlu_2$. From the development of the HPLC separation on $PteGlu_n$ ($n = 1$–7), it was possible to assay the PPGH activity by following disappearance of substrate and appearance of enzymatic products in the reaction assay. As shown in Fig. 3, the chromatogram corresponds to the reaction mixture in the presence of human serum PPGH (eluate from Sephadex G-25 column) compared with the assay containing no enzyme for 30 min incubation using $PteGlu_5$ as substrate (Fig. 3A). In the complete mixture assay at 0-min incubation, the only peak found was the substrate $PteGlu_5$ (Fig. 3B). At 30-min incubation the final product (PteGlu) and the reaction intermediates ($PteGlu_4$, $PteGlu_3$ and $PteGlu_2$) were demonstrated (Fig. 3C). The UV spectra of peaks collected after HPLC separation of $PteGlu_5$, $PteGlu_4$, $PteGlu_3$, $PteGlu_2$ and PteGlu were the same. By UV measurement at 280 nm and use of an SP 4270 integrator, it was possible to detect the presence of 10 pmol PteGlu, and the integrator response was linear over the concentration range 10 pmol to 2.5 nmol PteGlu. The addition of PteGlu to the enzyme reaction

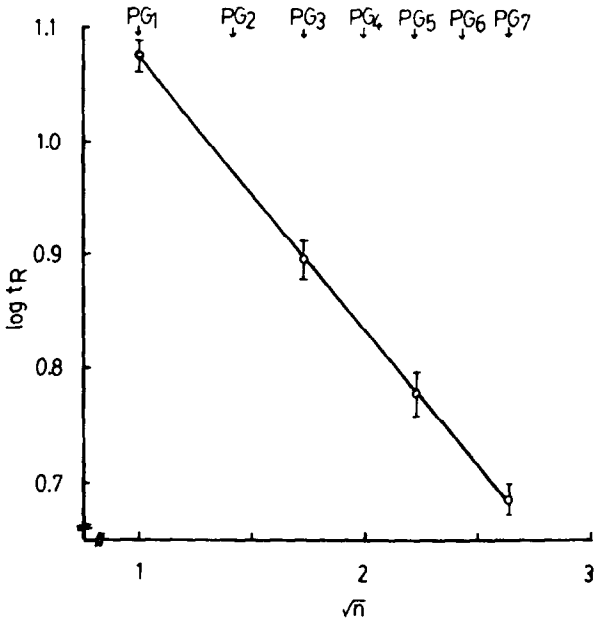

Fig. 2. Relationship between retention time (t_R) and number of glutamate residues (n) in pteroylpolyglutamates (PG). Logarithm of retention time ($\log t_R$) was plotted against the square root of the number of glutamate residue in pteroylpolyglutamates ($\sqrt{n}$). Each point was from five to seven determinations. The subscript number of each PG indicates the number of glutamate residues in pteroylpolyglutamates.

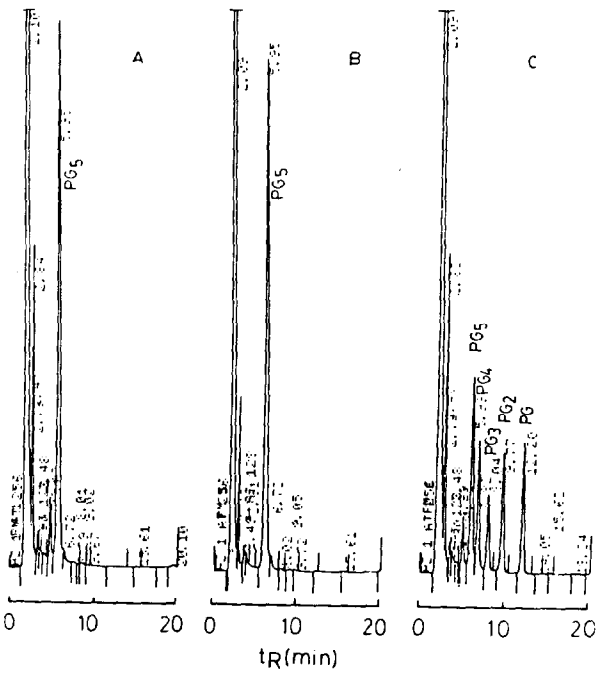

Fig. 3. Conditions for the human serum conjugase assay by HPLC. The HPLC analysis of human serum PPGH was done as described in Fig. 1, using 0.1 m*M* $PteGlu_5$ as substrate with the enzyme (3.0 mg in 200μl of 0.1 *M* acetate buffer, pH 4.5) and incubation for 30 min at 37°C in dark. (A) 30-min incubation without added enzyme, (B) 0-min incubation in the presence of enzyme and substrate, (C) 30-min incubation with the addition of enzyme and substrate. For peak identification see Fig. 1.

TABLE I

RECOVERY OF PteGlu IN THE HUMAN SERUM PPGH REACTION MIXTURE

Amount of PteGlu added (nmol)	Peak area (mean ± S.D., $n=5$)		Amount of PteGlu recovered (nmol)	Recovery (%)
	PteGlu added	PteGlu recovered		
1.25	2 523 410 ± 128 281	2 427 407 ± 124 434	1.202 ± 0.062	96.2
2.50	4 957 177 ± 58 670	4 822 072 ± 60 016	2.432 ± 0.030	97.3

mixture containing the human serum PPGH at 0-min incubation was found to give a high recovery during the folate extraction process and HPLC analysis (Table I).

The enzymatic hydrolysis of substrate $PteGlu_5$ and the formation of $PteGlu_4$, $PteGlu_3$, $PteGlu_2$ and PteGlu was linear with incubation time at 37°C for up to 60 min (Fig. 4). A plot of the amount of human serum PPGH after the Sephadex G-25 column against nmol of the product formation is shown in Fig. 5. It was demonstrated that at least 5 μl of human serum PPGH eluate (approximately 220 μg protein) were required in order to show a detectable enzyme activity.

The reaction products of human serum PPGH were identified by the described HPLC procedure at different incubation times in order to determine the number of glutamate residues. Fig. 6 shows different chromatograms of the reaction products at different incubation times from 5 to 180 min. It was found that the rate of formation of PteGlu was linear over the period of incubation (Fig. 7), whereas the first product of the reaction ($PteGlu_4$) accumulated rapidly within 45 min and soon decreased. $PteGlu_3$ accumulated during 60 min and then rapidly decreased. The accumulation of $PteGlu_2$ took place within 90 min, after which it slowly decreased.

Crude extracts of *P. falciparum* isolated from host red cells after saponin lysis of the infected red cells contained no detectable PPGH activity with $PteGlu_5$ as substrate under the same assay conditions as for human serum PPGH (in 0.1 *M* acetate buffer, pH 4.5) or with 0.1 *M* Tris–HCl, pH 7.8. To see whether enzyme from the intra-erythrocytic malaria parasites was secreted into the extracellular medium (ECM) during cultivation, the RPMI 1640 medium normally supplemented with 10% human serum was used for cultivation of both normal and infected red cells at 8% hematocrit for four days. The ECM of red cells infected with K_1 and G_{112} isolates and of normal red cells had PPGH activities of 2.83 ± 0.16, 2.75 ± 0.12 and 2.61 ± 0.15 ($n=3$) nmol/min/ml ECM, respectively. These enzyme activities represented the supplemented human serum in the medium. In addition, cultured normal red cells showed no PPGH activity.

DISCUSSION

PPGH activity was determined by simultaneous detection of the disappearance of substrate ($PteGlu_5$) and appearance of hydrolytic products ($PteGlu_4$, $PteGlu_3$, $PteGlu_2$ and PteGlu) using reversed-phase HPLC with UV detection at 280 nm.

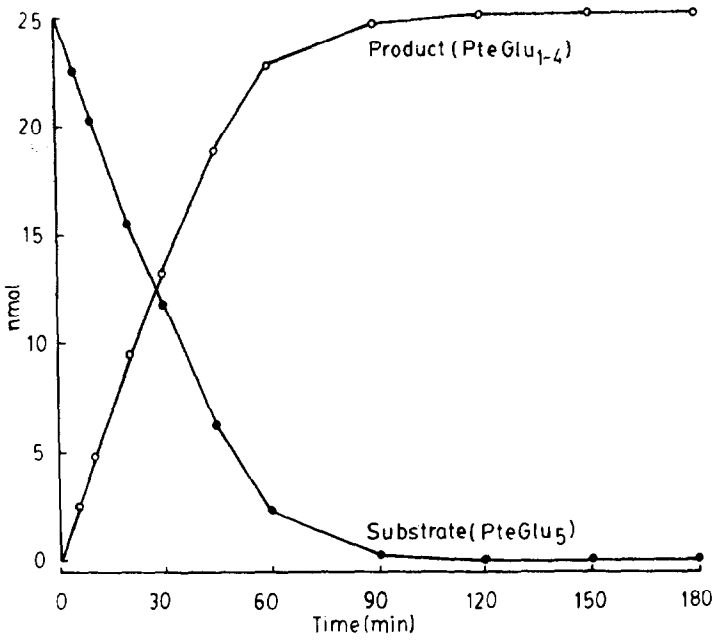

Fig. 4. Time course of human serum PPGH hydrolysis with respect to the formation of $PteGlu_{1-4}$. The rate of substrate ($PteGlu_5$) disappearance was equal to the rate of $PteGlu_{1-4}$ formation.

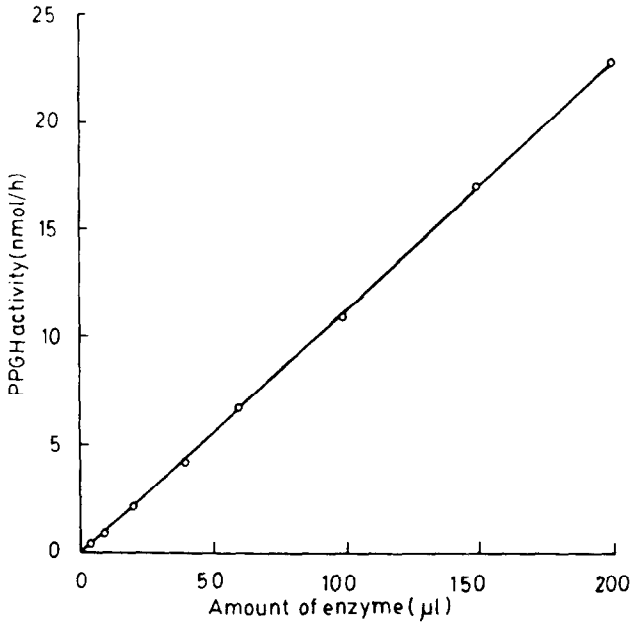

Fig. 5. Formation of product from human serum PPGH activity with respect to the amount of enzyme in the incubation mixture. Conditions of incubation were 60 min and 37°C in dark.

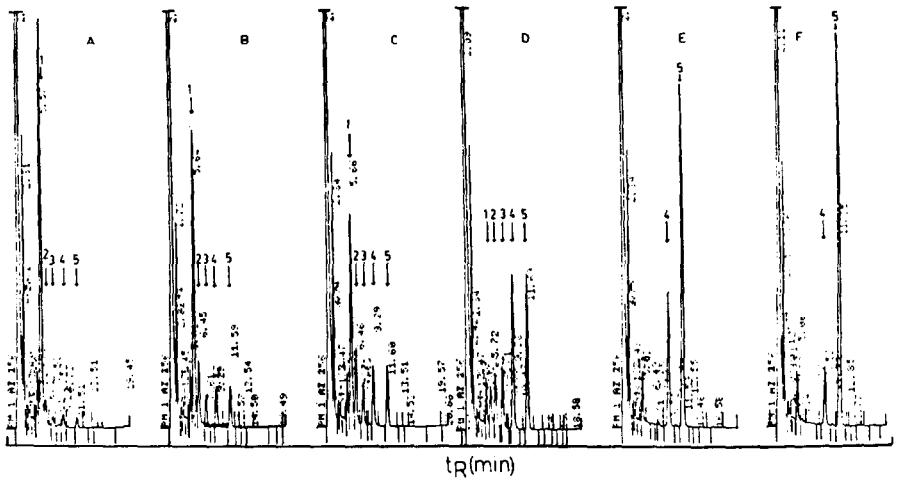

Fig. 6. HPLC profiles of human serum PPGH reaction intermediates and products at different incubation times. Conditions of the enzyme assay and HPLC analysis were as described in Fig. 1. The incubation times were (A) 5 min, (B) 20 min, (C) 30 min, (D) 60 min, (E) 120 min and (F) 180 min. Peaks 1–5 are $PteGlu_5$, $PteGlu_4$, $PteGlu_3$, $PteGlu_2$, and PteGlu, respectively.

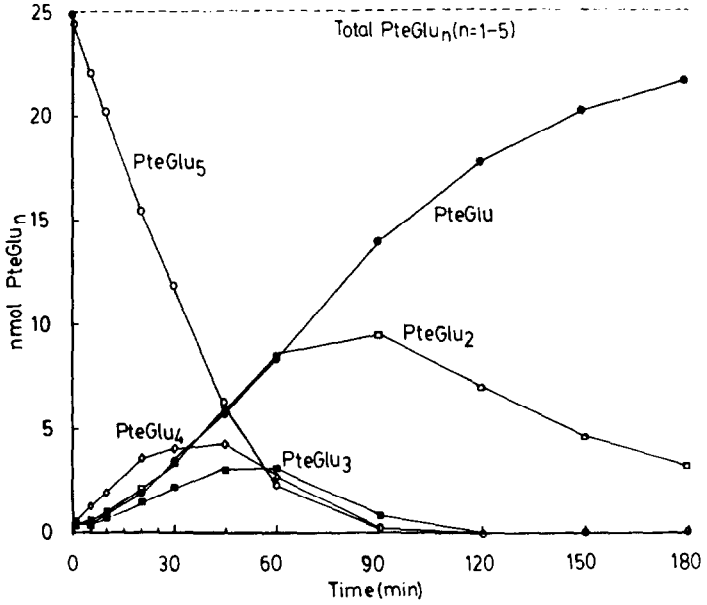

Fig. 7. Activity of human serum PPGH as an exopeptidase showing disappearance of substrate ($PteGlu_5$), accumulation of intermediates ($PteGlu_{4-2}$) and product formation (PteGlu). Each point represents duplicate experiments. The area under each peak from Fig. 6 was calculated into nmol per total reaction volume (250 μl). The amount of each pteroylpolyglutamate was plotted against incubation time. (○) $PteGlu_5$; (◇) $PteGlu_4$; (■) $PteGlu_3$; (□) $PteGlu_2$; (●) PteGlu.

The method derives from the fact that the pteroylpolyglutamates with higher number of glutamate residues are less well retained on the reversed-phase column when the pH of the eluent is sufficiently high to permit dissociation of carboxylic

acid groups [17]. Using human serum as a source of PPGH, modified enzyme incubation conditions, followed by folate extraction and HPLC analysis as described, PPGH activity was determined with high sensitivity and reproducibility. The enzyme assay was rapid and simple. The deproteinized reaction mixture was directly analyzed by HPLC. Furthermore, there was no need to isolate $PteGlu_{1-5}$ from the reaction mixture to detect radioactive glutamate as in the case of radiometric assay [5, 6].

The HPLC assay developed here is different in other ways from the two commonly used established methods: radiometric assay [5, 6] and selective short-term bacterial uptake of labeled products hydrolyzed from radiolabeled substrate [2, 4]. These two methods require substrates of pteroylpolyglutamates labeled at different portions of the molecule and do not allow direct identification of the mode of enzymatic hydrolysis as being an endo- or exopeptidase action. Cashmore et al. [12] reported the separation of pteroylpolyglutamates by anion-exchange and reversed-phase HPLC using different eluting systems in which a linear relationship was observed between t_R and number of glutamate residues.

The described HPLC method is suitable not only for quantitation of substrate and product simultaneously, but also allows detection of intermediates, permitting the study of the mode of hydrolytic action of the enzyme. The method can also be applied for detecting products of pteroylpolyglutamate synthetase activity.

Based on our observation it can be concluded that human serum PPGH functions as an exopeptidase (at pH 4.5) with release of glutamate residues from the substrate one at a time (viz., intermediates $PteGlu_4$, $PteGlu_3$ and $PteGlu_2$ from $PteGlu_5$, and with product PteGlu) (Figs. 6 and 7). The human serum PPGH appeared similar to the enzyme from human liver [18] and jejunal brush borders [2].

We used the PPGH assay to look for activity of this enzyme in several strains of *P. falciparum*. Enzyme activity was not detected either at pH 4.5 (acid enzyme) or at pH 7.8 (alkaline enzyme) by this method suggesting that the malarial parasite lacks PPGH activity. The activity was also not detected in host red cells. Since it appears that intracellular folate of the malaria-infected red cell is predominantly 5-methyltetrahydropteroylpentaglutamate (5-methyl-$H_4PteGlu_5$) [19], it is likely that the malaria parasite utilizes this folate form for one-carbon transferred reactions of folate-dependent enzymes without hydrolysis into the monoglutamates. Support for this supposition comes from the observation that pteroylpolyglutamates may be more effective than monoglutamates as substrates for the folate-dependent enzymes [20].

ACKNOWLEDGEMENTS

This work was supported in part by the Rockefeller Foundation Program on Great Neglected Diseases of Mankind and by a grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases as well as the Walter Reed Army Institute of Research, U.S. Army Research and Development Command.

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