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Note

Determination of folylpoly-γ-glutamate carboxypeptidase (folacin conjugase) activity using reversed-phase high-performance liquid chromatography*

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The intestinal absorption of dietary folacin requires the hydrolysis of folylpolyglutamate forms to monoglutamate forms. In mammals the hydrolytic enzyme folylpoly-γ-glutamate carboxypeptidase (E.C. 3.4.12.10), commonly referred to as folacin conjugase, cleaves the γ-glutamyl peptide bonds of folylpolyglutamates to ultimately yield folylmonoglutamates. Research concerning folacin conjugases from intestinal as well as other sources (foods and other plant and animal tissues) is dependent upon a reliable assay of folacin conjugase activity. In addition, the clinical evaluation of folacin malabsorption would be facilitated by a convenient assay procedure for determination of conjugase activity in intestinal biopsy specimens.

Folacin conjugase activities have been determined by a variety of assays. Early research during this period was based on the use of folacin-requiring microorganisms of varying nutritional requirements. These microbial growth assays are very sensitive, but are lengthy, imprecise and subject to interferences. Krumdieck and Baugh developed a rapid and sensitive radiometric folacin conjugase assay[1,2] using radiolabeled folyltriglutamate as a substrate[3,4]. The current exorbitant cost of synthesizing the substrate limits the use of the radiometric method. In addition, the identification of products is difficult with this procedure. Elsenhaus *et al.*[5] have described a folacin conjugase assay using radiolabeled folypolyglutamates and short-term bacterial uptake for product determination. Column chromatographic separations of folylpolyglutamates using ion exchange on DEAE-cellulose, TEAE-cellulose, DEAE-Sephadex A-25 or QAE-Sephadex A-25, or gel filtration on Sephadex G-15 or G-25 have been used in studies of folacin conjugase reactions. Separations also have been achieved using high-voltage paper[6,7] and polyacrylamide gel[8,9] electrophoresis. Folylpolyglutamate separation techniques have been developed involving prior $KMnO_4$ oxidative and Zn/HCl reductive cleavage of the folate C^9–N^{10} bond. These procedures, which could be applied to conjugase assays, have been criticized since certain folacin derivatives are resistant to these cleavage procedures or are converted to N-substituted *p*-aminobenzoylpolyglutamate derivatives[10–12]. However, recent cleavage techniques[13,14] permit quantitative conversion of all naturally occurring folacin derivatives to a single homologous series of unsubstituted *p*-aminobenzoylpolygluta-

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mate derivatives, which have been separated by high-performance liquid chromatography (HPLC)[15,16]. Suitable microparticulate columns having high efficiency have made possible rapid separation of folylpolyglutamatess by anion-exchange[17–19] or reversed-phase[18–20] HPLC. These techniques offer excellent resolution, high sensitivity and quantitative recovery of components.

The objective of this study was to develop a rapid chromatographic assay of folacin conjugase activity, which would permit simultaneous quantitation of substrate and products, as an alternative to radiometric methods. This communication describes a modification and application of the reversed-phase HPLC procedure of Cashmore *et al.*[18]. A recently developed 3-cm long HPLC column packed with 3-μm octadecylsilica was used in conjunction with isocratic mobile phase flow programming which allowed rapid and efficient separation and quantitation of folylpolyglutamates. A preliminary report of this procedure and its application in studies of intestinal conjugases has been published previously[21].

EXPERIMENTAL

Folylpentaglutamate (pteroylpentaglutamate; PG-5) and triglutamate (PG-3) were synthesized by the solid-phase procedure of Krumdieck and Baugh[3,4] with minor modifications. The PG-5 and PG-3 standards contained traces of folyltetraglutamate (PG-4) and folyldiglutamate (PG-2), respectively. Folic acid (FA) was obtained from Sigma (St. Louis, MO, U.S.A.) and N^{10}-trifluoroacetylpteroic acid (TFAPA) was prepared from pteroic acid by incubation in trifluoroacetic anhydride[4,22]. Standard solutions of PG-5, PG-3, FA and TFAPA were prepared by dissolution in several millilitres of 5% (w/v) K_2HPO_4, pH 9.1, before immediately diluting to volume (25 ml) with 0.01 *M* KH_2PO_4, pH 7.0. Aliquots (100 μl) of these solutions were taken for dilution and spectrophotometric determination of concentration[4,22]. These solutions were used immediately or flushed with nitrogen and stored at −20°C. Minimal degradation was found after 3 months of storage at −20°C.

Human intestinal mucosal brush border and hog kidney folacin conjugases used in this study were partially purified by minor modifications of the procedures of Menard and Cousins[23] and Brody *et al.*[24], respectively. Hog kidney folacin conjugase assays were performed in separate Beckman (Palo Alto, CA, U.S.A.) Airfuge tubes (tube volume 250 μl) which were incubated for various times at 37°C. After incubation of 70 μl folacin conjugase preparation, 90 μl 0.1 *M* potassium acetate (pH 4.5) and 20 μl 100 nmol ml^{-1} PG-5, the enzyme was inactivated by addition of 20 μl 50% (w/v) trichloroacetic acid. Centrifugation at 150,000 *g* for 3 min was sufficient to sediment the protein before HPLC analysis. Human intestinal mucosal brush border folacin conjugase assays were similarly performed in separate Beckman Airfuge tubes incubated for various times at 37°C. After incubation of 70 μl folacin conjugase preparation, 70 μl 0.033 M Tris · HCl (pH 7.0) and 20 μl 100 nmol ml^{-1} PG-3, the folacin conjugase was inactivated by addition of 20 μl 50% (w/v) trichloroacetic acid, and 20 μl 500 nmol ml^{-1} TFAPA was added as an internal standard. After centrifugation at 150,000 *g* for 3 min, supernatant aliquots were analyzed by HPLC. Recoveries of the added PG-5 and PG-3 standards exceeded 90% and precision using the TFAPA internal standard was within 5%.

HPLC analyses were performed using a commercial system (Altex, Berkeley, CA, U.S.A.; Model 312) which consisted of a solvent metering pump (Model 110A), solvent programmer (Model 410), ultraviolet absorption detector (280 nm, 8-μl flow cell, Model 153) and loop-type injection valve (20 μl, Model 905-42). A precolumn packed with 37–53-μm silica (Whatman, Clifton, NJ, U.S.A.) was installed between the pump and the injection valve to provide saturation of the mobile phase with silica, which prolonged the life of the Perkin-Elmer "3 × 3" analytical column (3 μm octadecylsilica, 30 × 4.6 mm I.D., Perkin-Elmer, Oak Brook, IL, U.S.A.). The elution was monitored by a Model 153 ultraviolet absorption detector (280 nm). Quantitation was based on peak height measurement. Separations were performed at ambient temperature with an isocratic mobile phase (2.5% (v/v) acetonitrile in 0.1 *M* potassium acetate pH 5.0) and non-linear flow program gradient (flow exponent 5) from 0.3 to 3.0 ml min^{-1}. The gradient time was adjusted between 5 and 25 min as needed to allow adequate resolution between folylpolyglutamates of varying glutamate chain length and other sample components in different folacin conjugase assay systems.

RESULTS AND DISCUSSION

In reversed-phase chromatography the stationary phase is non-polar while the mobile phase is polar. Enhancement of electrostatic interactions between the solute

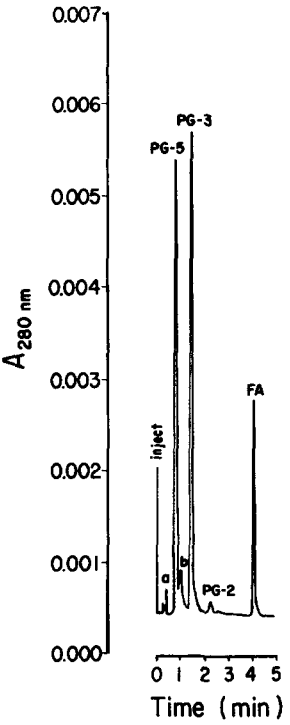

Fig. 1. HPLC elution profiles of folacin standards (0.2 nmol, each). The PG-5 and PG-3 standards contained traces of PG-4 and PG-2 respectively. a = Solvent front; b = PG-4.

and mobile phase leads to a decrease in retention time. Therefore, the elution order of folylpolyglutamates in reversed-phase chromatography, when the pH of the eluent is sufficiently high for the carboxylic acid groups to be predominantly ionized, should be the opposite of that observed in anion-exchange chromatography. Fig. 1 shows a typical separation of PG-5, PG-4, PG-3, PG-2 and FA within 5 min. The elution order is the same as found by Bush *et al.*[20] and Cashmore *et al.*[18], *i.e.* the retention time decreases with increasing number of glutamyl residues at pH 5.0. The use of isocratic mobile phase flow programming was chosen in preference to solvent programming to eliminate the need for reequilibration between injections. The folylpolyglutamate standards had different apparent molar responses since the residence time in the analytical flow cell varied with elution time.

Fig. 2 illustrates a typical time course experiment utilizing hog kidney conjugase with PG-5 as substrate. Fig. 3 illustrates a typical time course experiment using human intestinal mucosal brush border folacin conjugase, PG-3 as substrate and TFAPA as an internal standard. Human intestinal mucosal brush folacin conjugase has been reported to have an apparent Michaelis constant (K_M) for PG-3 of 1.6 μM (ref. 25). Fig. 4 shows that the PG-3 substrate concentration of 12.5 μM ensured zero-order kinetics with respect to incubation time up to 25% hydrolysis of PG-3 after which the reaction rate slowed slightly. Analysis of these curves indicates predominantly *exo*-γ-glutamyl carboxypeptidase activity for the conjugases of hog kidney and human intestinal mucosal brush border.

This folacin conjugase assay is currently being used in studies of the characteristics of intestinal mucosal folacin conjugases from various mammalian species[21]. Studies of folacin conjugases in other research and clinical applications would be facilitated using this assay. Distinct advantages compared to the radiometric assay methods are that substrate and products are individually quantitated by a direct instrumental technique, and that the less expensive non-radioactive substrate is used in very small amounts (2 nmol per assay). The radiometric and microbiological assays

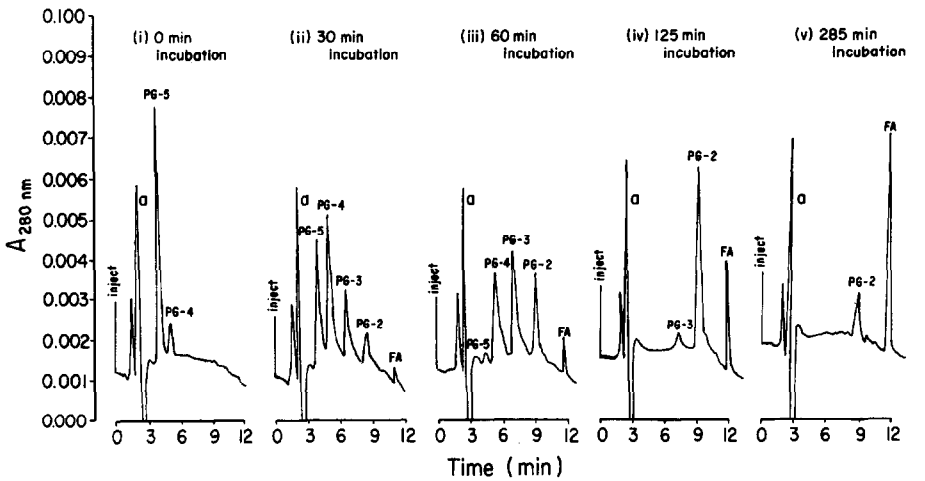

Fig. 2. Time course hydrolysis of PG-5 (12.5 μM) by hog kidney folacin conjugase at 37°C. a = Solvent front.

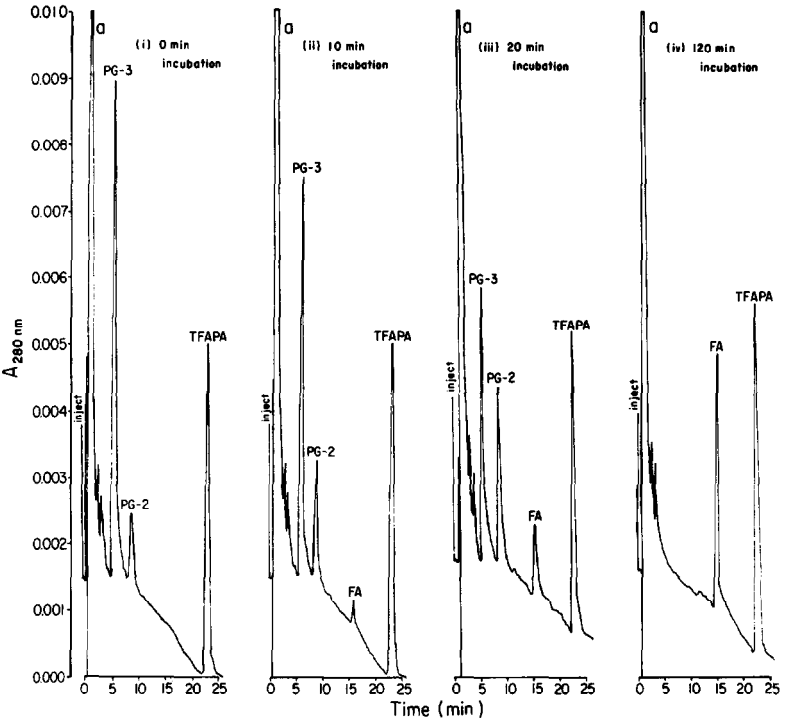

Fig. 3. Time course hydrolysis of PG-3 (12.5 μM) by human intestinal mucosal brush border folacin conjugase at 37°C. a = Solvent front.

may be more suitable for studies requiring analyses of greater than 30 samples a day where information concerning product identity is not required. Preliminary work with different folacin conjugases indicates that the speed of the HPLC analysis may be increased when using more purified enzyme preparations since the width of the

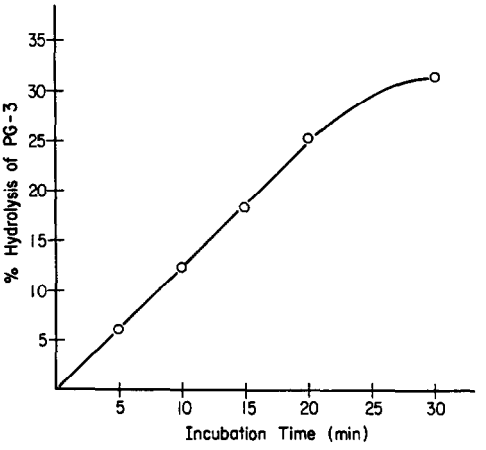

Fig. 4. Percent hydrolysis of PG-3 with respect to incubation time at 37°C. Human intestinal mucosal brush border protein concentration is 2.8 mg per ml of reaction mixture.

solvent front peak is shortened dramatically. Selection of an alternate internal standard that elutes between PG-2 and FA would reduce analysis time. Work recently completed indicates that an internal standard may not be necessary since repetitive injections of folylpolyglutamate standards showed injection loop precision to be within 5%, as previously indicated by 5% precision obtained using TFAPA. Chromatography of samples in which TFAPA is omitted minimizes analysis time. The use of PG-3 as a routine substrate also facilitates rapid analysis.

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