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A General High-Performance Liquid Chromatography-Based Assay for the Hydrolysis of *N*-Acyl Glutamates

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A rapid and simple HPLC assay has been developed to separate and quantify *N*-acyl glutamates and the corresponding carboxylic acids of the acyl moiety. This method was specifically developed to assay hydrolytic activity for glutamate carboxypeptidases. Although established assays for specific substrates of such enzymes exist, they may not be amenable for examining the hydrolytic activity of new substrate probes. This assay was developed to accommodate such probes.

Glutamate carboxypeptidases represent a relatively diverse class of enzymes with respect to their distribution across living systems. A few such examples include the glutamate carboxypeptidases II, NAALADase[2] (1), and PSMA (2) which are noted to hydrolyze the neuropeptide *N*-acetylaspartylglutamate while two forms of pteroylpoly-γ-glutamate carboxypeptidase have been identified in human jejunum capable of hydrolytically cleaving C-terminal γ-glutamates from pteroylpoly-γ-glutamates.(3) Various forms of glutamate carboxypeptidase have been isolated from bacteria (4–7) which were noted to liberate glutamic acid from folates or folate derivatives, a representative of which (carboxypeptidase G_2 from *Psudomonas* sp. strain RS-16) has achieved considerable interest recently owing to its successful utilization in ADEPT (8) in addition to its use in therapeutic rescue techniques following severe chemotherapeutic dosages of antifolates such as methotrexate (9). While new prodrugs are developed for the use in ADEPT and novel substrate probes are developed to further characterize the structure and role of these enzymes noted above, methods for assaying enzymatic activity must also be established. Thus, the development of a convenient and general method should be invaluable for such pursuits.

Our primary interest in developing a general HPLC-based assay for glutamate carboxypeptidase arose from our interest in preparing putative prodrugs for ADEPT. Traditional assays for glutamate carboxypeptidase generally involve monitoring the change in absorbance at a selected wavelength coincident with the hydrolysis of the amide linkage (4). Although this method is appropriate for some substrates, shortcomings involving limits of detection may arise for substrates with low turnover rates. Of those assays which are HPLC-based, such as that employed for the hydrolysis of methotrexate, most suffer from long retention times and potentially high column pressures due to the makeup of the mobile phase (10). Thus, to establish a general HPLC-based assay, we sought to explore conditions to monitor the hydrolysis of several *N*-acyl glutamates (**I**–**III**) and methotrexate (Fig. 1).

MATERIALS AND METHODS

Chemicals. Glutamate carboxypeptidase (carboxypeptidase G from *Pseudomonas* sp. strain ATCC 25301, C 4053) and methotrexate were obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals and reagents used in the synthesis of substrates were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of the highest purity and were purchased from commercial sources.

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[2] Abbreviations used: NAALADase, rat brain N-acetylated-α-linked-acidic dipeptidase; PSMA prostate-specific membrane antigen; PPH, pteroylpolyglutamate hydrolase; ADEPT, antibody-directed enzyme prodrug therapy; I, *N*-[4-(4-nitrophenyl)]butyryl-L-glutamic acid; II, *N*-(2-naphthalenylacetyl)-L-glutamic acid; III, N-(1-naphthalenylacetyl)-L-glutamic acid; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenxotriazole hydrate.

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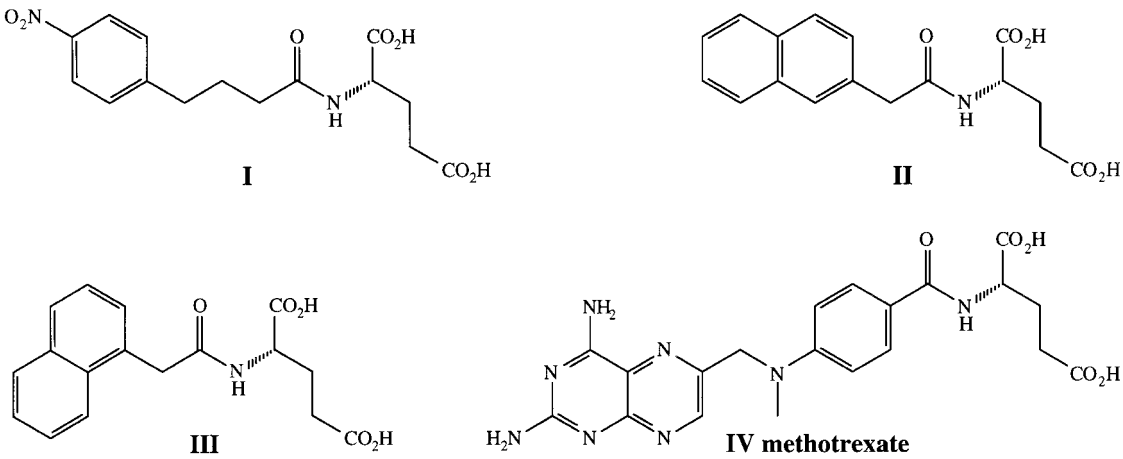
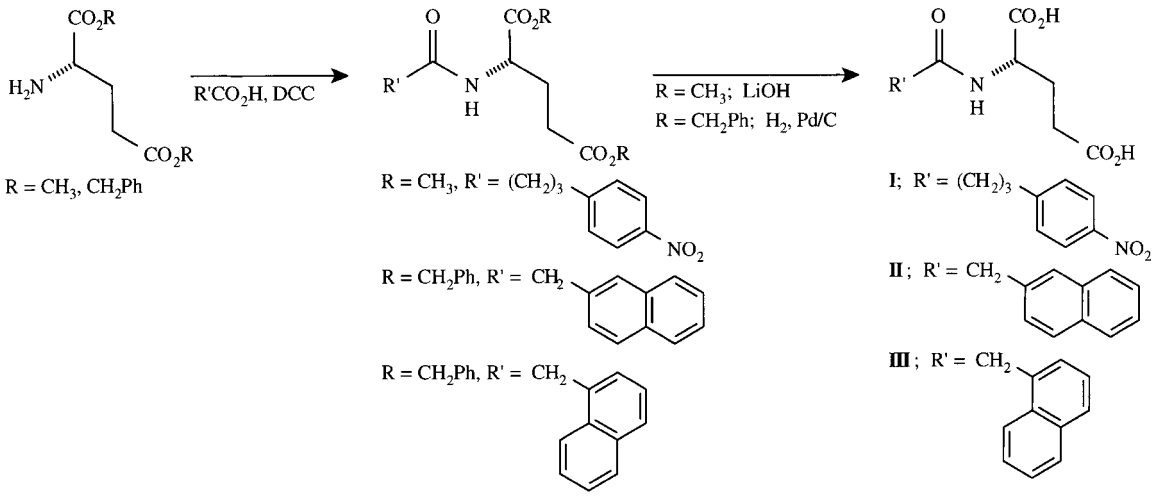


FIG. 1. *N*-Acylglutamate substrates for glutamate carboxypeptidase.

Incubation conditions and analytical methods. A typical incubation mixture (final volume 0.25 mL) was prepared by the addition of 200 μL Tris buffer (50 mM, pH 7.3) to 25 μL of an enzyme solution (1.3 μg protein/mL buffer). The enzymatic reaction was initiated by the addition of a 25-μL aqueous substrate solution. The reaction was allowed to proceed for 1 min with constant shaking at 30°C and was terminated by the addition of 100 μL ice-cold methanolic TFA (1% trifluoroacetic acid by volume in methanol) followed by vortexing and centrifugation (7000*g*). A 100-μL aliquot of the resulting supernatant was then quantified by HPLC. Substrates and their hydrolytic products were separated and quantified by analytical reversed-phase HPLC using a Microsorb MV-C18 column (5 μ, 100 Å, 4.6 × 250 mm, Varian, Walnut Creek, CA). For the determination of K_m and V/K, careful time-course studies (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 min) were performed to define the linear portion of the initial rate (0–2 min) for the enzymatic hydrolysis of **I** and **IV**. Under the assay conditions described above, it was noted that the initial substrate concentration was not substantially depleted during the time course of the incubation (e.g., approximately 5% conversion to product was observed for incubations with the lowest substrate concentration, 3 μM).

Synthesis. *N*-[4-(4-Nitrophenyl)]butyryl-L-glutamic acid: 4-(4-Nitrophenyl)butyric acid (0.741 g, 3.54 mmol), dimethyl glutamate hydrochloride (0.750 g, 3.54 mmol), and HOBT (0.487 3.54 mmol) were dissolved in CH_2Cl_2 (20 mL). Triethylamine (500 μL, 3.54 mmol) was added via syringe followed by a solution of DCC (0.803 g, 3.89 mmol) in CH_2Cl_2 (6 mL) and was stirred for 15 h. The reaction mixture was filtered and solvent removed *in vacuo.* The residue was purified by silica gel chromatography (hexane:ethyl acetate 1:3, v/v) to give a white solid (0.84 g, 65% yield). To a solution of this product in MeOH (2.3 mL) was added 1 N LiOH (2.3 mL) and the reaction mixture was stirred 15 h. The solvent was evaporated *in vacuo,* and the product was extracted twice from 10% HCl with CH_2Cl_2. The combined organic layers were dried over sodium sulfate, and the solvent was evaporated *in vacuo* to yield a pale yellow solid (0.739 g, 95% yield). ^{1}H NMR: (300 MHz, $CDCl_3$) δ 1.77–1.98 (m, 3H), 2.03–2.18 (m, 1H), 2.22 (t, J = 7.4 Hz, 2H), 2.33 (t, J = 7.5 Hz, 2H), 2.70 (t, J = 7.6 Hz, 2H), 4.36 (dd, J = 5.0, 9.1 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 8.06 (d, J = 8.4 Hz, 2H). mp 101–103°C. λ_{max} 274 nm, ε 19,800 M^{-1} cm^{-1}.

Representative procedure for ***II*** *and* ***III****.* *N*-(1-Naphthalenylacetyl)-L-glutamic acid: 1-Naphthalenylacetic acid (0.279 g, 1.5 mmol), dibenzyl glutamate *p*-toluenesulfonic acid salt (0.750 g, 1.5 mmol), and HOBT (0.203 g, 1.5 mmol) were dissolved in CH_2Cl_2 (20 mL). Triethylamine (209 μL, 1.5 mmol) was added via syringe followed by a solution of DCC (0.342 g, 1.65 mmol) in CH_2Cl_2 (6 mL) and was stirred for 15 h. The reaction mixture was filtered and the solvent was removed *in vacuo.* The residue was purified by silica gel chromatography (hexane:ethyl acetate 1:1, v/v) to give a white solid (0.330 g, 44% yield). To a stirred solution of this product in MeOH (15 mL) was added 10% Pd/C (0.060 g). The flask was then charged with $H_{2(g)}$ (balloon pressure) and stirred for 1.5 h. The solution was vacuum filtered through a bed of celite and the solvent was removed *in vacuo* to give **III** as a light-brown solid (0.184 g, 88% yield). **II**: ^{1}H NMR: (300 MHz, $CDCl_3$) δ 1.82–1.95 (m, 1H), 2.06–2.18 (m, 1H), 2.30 (t, J = 7.2 Hz, 2H), 3.66 (s, 2H), 4.38 (dd, J = 4.9, 8.5 Hz, 1H), 7.05–7.40 (m, 3H), 7.67–7.76 (m, 4H). mp 169–171°C, lit. 168–170°C (11). λ_{max} 275 nm, ε 10,800 M^{-1} cm^{-1}. **III**: ^{1}H NMR: (300 MHz, $CDCl_3$) δ 1.75–1.90 (m, 1H), 1.99–2.14 (m, 1H), 2.21 (t, J = 7.5 Hz, 2H), 3.91 (s, 2H), 4.33 (dd, J = 4.2, 8.2 Hz, 1H), 7.24–7.45 (m, 4H), 7.65



SCHEME 1. Synthesis of *N*-Acylglutamates.

(d, J = 7.2 Hz, 1H), 7.72 (d, J = 7.8 Hz, 1H), 7.91 (d, J = 8.1 Hz, 1H). mp 189–190°C. λ_{max} 280 nm, ε 14,700 M^{-1} cm^{-1}.

RESULTS AND DISCUSSION

The preparation of substrate **II** had been previously described via direct acylation of dibenzyl glutamate (11). However, in coordination with an ongoing research effort in our laboratory, we systematically prepared this compound, along with **I** and **III**, via DCC coupling of glutamate esters with the respective carboxylic acids (Scheme 1) followed by deprotection; via hydrolysis for **I** and hydrogenolysis for **II** and **III**.

Once in hand, several HPLC methods were investigated to allow for the separation and quantification of the substrates (**I**–**IV**) from their respective hydrolytic products, 4-(4-nitrophenyl)butyric acid, 2-naphthylacetic acid, 1-naphthylacetic acid, and 4-[*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino]benzoic acid. Of the traditional reversed-phase mobile phases explored, most consisted of buffer/organic mixtures such as phosphate or acetate and acetonitrile or methanol varying in pH from 3.2 to 5.5. Brief investigations into ion pairing with tetrabutylammonium phosphate in these mobile phases also failed to provide acceptable resolution. Although some effort was made to examine gradients for the above mobile phases, these often required long retention times or steep gradients requiring significant equilibration time.

As a result of inadequate resolution from methods derived from common approaches, we chose to examine more simple mobile phases consisting of methanol/water mixtures containing 0.1% TFA under isocratic conditions. Indeed, under such conditions, convenient retention times along with superior resolution were observed as delineated in Table 1. It is worthwhile to note that analogous *N*-acyl derivatives of glutamine and γ-aminobutyric acid could also be conveniently separated from their corresponding carboxylic acids using similar conditions.

TABLE 1

Isocratic HPLC Separation of Substrates and Hydrolytic Products

	Retention times (min)		k'^{a}			
Substrate	Substrate	Product	Substrate	Product	α^{b}	Mobile phase[c]: MeOH:H_2O:TFA (v/v)
I	4.8	8.3	1.0	2.5	2.5	58:42:0.1
II	4.6	8.4	0.93	2.5	2.7	65:35:0.1
III	4.4	7.6	0.85	2.2	2.6	65:35:0.1
IV	6.1	11.2	1.6	3.7	2.3	36:64:0.1

[a] Capacity factor.
[b] Selectivity factor.
[c] Separations were monitored at 274 nm except **III** (280 nm) and **IV** (304 nm).

It was important to verify that the established HPLC conditions for substrates **I–IV** were suitable for the analysis of enzymatic incubations. Specifically, we were concerned with interfering peaks arising from the assay mixture. However, chromatograms from aliquots of quenched assay mixtures revealed clean separation of substrates from products with no superimposing or interposing peaks (Fig. 2). Due to the purified nature of the glutamate carboxypeptidase and simple assay conditions employed in this study, repeated and daily use of the column for enzymatic analysis indicated that no substantial accumulation of hydrophobic impurities occurred as evidenced by a lack of baseline drift, extraneous peaks, or a rise in column back pressure.

With an HPLC method established to monitor glutamate carboxypeptidase-mediated hydrolysis of substrates **I–IV**, preliminary enzyme activity for the substrates **I–III** was determined relative to methotrexate **IV** as shown in Fig. 3. Because both substrate **I** and methotrexate **IV** exhibited similar relative activities, Eadie–Hoffstee analysis was performed to determine the kinetic parameters K_m and V/K (12) for each (substrate concentrations were varied from 3 to 6 μM). It was shown that substrate **I** exhibited a kinetic profile to similar that of the traditional substrate methotrexate **IV** as evidenced by K_m values of 3.3 and 3.9 μM, respectively. The respective V/K values for **I** and **IV** were determined to be 7.0 and 4.2 L min^{-1} mg protein^{-1}. These results not only verified the utility of this HPLC method but also indicated that substrate **I** may now serve as a suitable substitute substrate for monitoring glutamate carboxypeptidase activity.

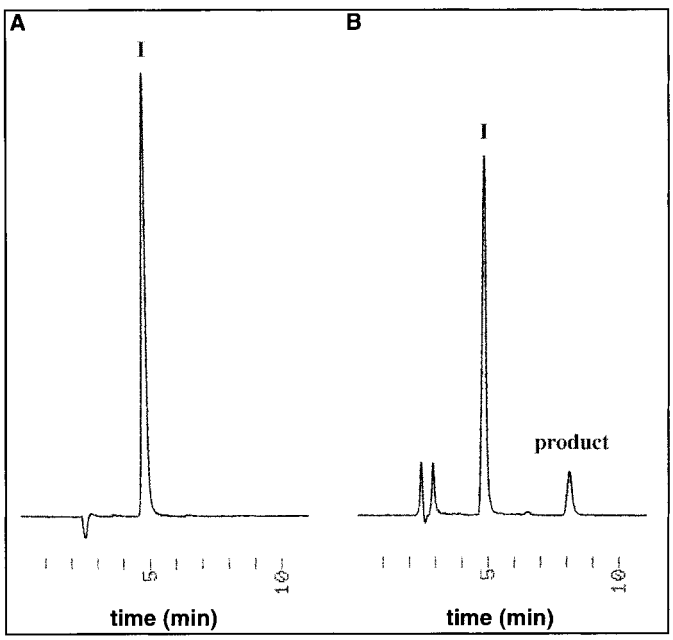


FIG. 2. Chromatogram of **I** alone (A) and after 5 min incubation with 0.032 μg glutamate carboxypeptidase (B) under established HPLC conditions (Table 1).

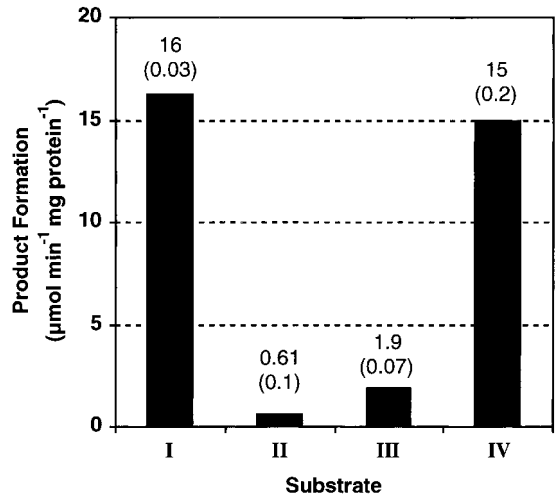


FIG. 3. Hydrolytic activity of substrates **I–IV**. Data presented represent the incubation of each substate (100 μmM) incubated with 0.032 μmg protein for 2 min. Each value represents the average of triplicate determinations with standard error in parentheses.

In conclusion, a simple and versatile isocratic reversed-phase HPLC method for the rapid separation of *N*-acyl glutamates from their corresponding carboxylic acids of the acyl moiety has been identified. This development now allows for the rapid screening of both novel substrate probes for glutamate carboxypeptidases and putative prodrugs for glutamate carboxypeptidase-based ADEPT. Although the series of substrates examined was limited, the results reported herein suggest that the HPLC conditions identified will be amenable to a variety of *N*-acyl glutamates.

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