New Substrate for Fluorometric Determination of γ-Glutamyltransferase Activity in Serum

Julie D. MacQueen, Richard C. Driscoll, and Robert J. Gargiulo

We describe the preparation of a new substrate, \( N^\gamma L \)-glutamyl-5-aminoisophthalic acid dimethyl ester hydrochloride, for the fluorometric determination of \( \gamma \)-glutamyltransferase activity in serum by the "front-surface" technique. Details of the resulting method are provided. The final reaction mixture contains 4 mmol of the substrate per liter of tris(hydroxymethyl)aminomethane (100 mmol/liter) and glycylglycine (75 mmol/liter) at pH 8.2 (25 °C).

We report a fluorometric method for measuring \( \gamma \)-glutamyltransferase (EC 2.3.2.2) activity for use on a prototype front-surface fluorometer with fixed wavelengths. Two commercially available fluorogenic substrates for this enzyme are the \( \gamma \)-glutamyl(\( \alpha \) or \( \beta \))napththalamides, which yield the hydrolysis products \( \alpha \) or \( \beta \)-napththalamine. These substrates are poorly water-soluble, and the fluorophores are toxic and lack sensitivity at the wavelengths of the prototype instrument.

We think that \( N^\gamma L \)-glutamyl-5-aminoisophthalic acid dimethyl ester hydrochloride overcomes these disadvantages. It is water soluble and, although the toxicities of it and its major hydrolysis product are not known, analogous compounds are usually noncarcinogenic.

In conventional fluorescence techniques, the fluorescence of a solution contained in a 10-mm pathlength cuvet is measured. This technique has some limitations, particularly if high concentrations of fluorescent substrates or cofactors are used. We examined the front-surface fluorescence technique, in which the fluorescence of a solution is measured at the surface of a cuvet of 1-mm pathlength. This method can mean improved sensitivity for fluorescence methods because reagent-induced quenching is reduced.

Materials and Methods

All chemicals are reagent grade.

Substrate Synthesis

The substrate was prepared by a modification of Orłowski's (1) procedure. Add phthaloyl glutamyl anhydride (13.2 g, 51 mmol) and 5-aminoisophthalic acid dimethyl ester (10.4 g, 50 mmol; Pfaltz and Bauer, Inc., Stamford, Conn. 06902) to 60 ml of dioxane. Stir the mixture for 1.5 h at 55–60 °C in a water bath. Evaporate the solvent at ambient temperature (22–28 °C) and redissolve the residue in 200 ml of methanol containing hydrazine hydrate (7.5 g, 0.15 mol). Filter this mixture and allow it to stand at ambient temperature for two days. Collect the resulting white precipitate, wash it with 100 ml of distilled water and then 25 ml of ethanol, agitate for 20 min

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in 100 ml of 0.5 mol/liter HCl, and filter. Treat the filtrate with sodium bicarbonate to adjust the pH to 6.5-7.0, collect the precipitate, and dry it under reduced pressure. The yield is 45%, the mp 194-197 °C.

Prepare the hydrochloride salt by dissolving 1.0 g of the precipitate in a mixture of 6.0 ml of methanol and 0.3 ml of concentrated HCl. Evaporate the solvent and dry the solid under reduced pressure overnight. The melting point of the final product is 153-155 °C; [α] D = +15 ° (5% in 0.5 mol/liter HCl). Calculated for C18H18N4O2·Cl·C: C, 45.80; H, 5.34; N, 7.12; H2O, 4.6%. Found: C, 45.96; H, 5.18; N, 7.18; H2O, 4.4%. Preliminary data indicate that the hydrochloride salt is stable for a year at 2-8 °C.

**Instrumentation:** We explored the feasibility of using a simple front-surface fluorometer with fixed wavelengths. The wavelengths—excitation (ex) = 365 nm and emission (em) = 465—were selected to enable measurement of a variety of fluorophores.

The prototype instrument we used for these studies was built to our specifications. The light source was a fluorescent tube (General Electric, F4-T4-BL). The reaction cell was a 1-mm flow-through cuvet (Coleman #30-304, Spectrasil), thermally regulated at 37 ± 0.2 °C. The cuvet was filled by an AutoAnalyzer (Technicon Corp., Tarrytown, N. Y. 10591) two-speed Proportioning Pump II. Changes in fluorescence were followed on a linear chart recorder (Heath-Schumberger, Benton Harbor, Mich. 49002). Stability of the light source was monitored with solutions of quinine sulfate. A Perkin-Elmer spectrophotofluorometer, Model MPF-2A, was used for wavelength scans and conventional fluorescence studies.

**Reagents**

**Stock buffer:** Dissolve 6.05 g of tris(hydroxymethyl)aminomethane and 4.95 g of glycyglycine in sufficient distilled de-ionized water to yield 400 ml. This stock buffer is stable for several months at 2-8 °C.

**Buffered substrate:** Dissolve 160 mg of N-γ-L-glutamyl-5-aminoisophthalic acid dimethyl ester hydrochloride in 80.0 ml stock buffer. Adjust the pH to 8.2 at 25 °C with 0.1 mol/liter HCl; bring the volume to 100 ml with distilled de-ionized water. The final reaction mixture contains 4 mmol of the substrate per liter of tris(hydroxymethyl)aminomethane (100 mmol/liter) and glycyglycine (75 mmol/liter). This reagent is stable at 2-8 °C for at least two days. For use, warm 3.0 ml of the reagent to 37 °C, add 0.1 ml of sample, mix, and aspirate into the instrument. Record the change in relative fluorescence (RF) during 4-5 min. Determine ΔRF/min for a linear segment of the recorded reaction.

**Quinine sulfate:** The diluent for all preparations is 0.1 mol/liter H2SO4. Prepare a stock solution of 1.00 g of quinine sulfate per liter of diluent; dilute the stock solution 100-fold in diluent. Prepare working solutions by further diluting this second preparation: dilute 9.0 ml to 100 ml with the diluent, which yields a solution containing 900 μg of quinine sulfate per liter. The solutions are stable for at least two months stored at 2-8 °C in amber-colored bottles.

**Calibration:** γ-Glutamyltransferase activity (in U/liter) in a control serum was measured by a colorimetric comparison procedure (GGTP reagent set, Dade). The control serum was used to determine the relative fluorescence (RF) in the fluorometric system and was also determined. A factor relating RF/min of the control serum in the fluorometric system to the U/liter value established by the reference method was derived and used to convert RF/min of unknown samples to U/liter.

**Colorimetric comparison procedure:** The DADE GGTP reagent set method is based on Rosalki’s (2) procedure, with γ-glutamyl-p-nitroanilide as substrate and measurement of the highly colored product, p-nitroanilide. Normal values with this system are less than 45 U/liter for men, less than 30 U/liter for women.

**Analytical Variables**

Absorption spectrum of product: The fluorescent hydrolysis product, aminoisophthalic acid dimethyl ester, was dissolved in dioxane and diluted in buffer. Excitation and emission spectra were determined on the Perkin-Elmer spectrophotofluorometer; peak wavelengths observed were: ex = 335 nm, em = 435 nm.

Buffer, pH, and acceptor: Tris(hydroxymethyl)aminomethane, 100 mmol/liter, is commonly used in assays for γ-glutamyltransferase. Figure 1 relates glycyglycine concentrations and γ-glutamyltransferase activity for nine of 15 samples tested. Activity was increased up to 90% by glycyglycine in concentrations ranging from 65 to 95 mmol/liter. Because no one concentration provided maximal activity for all samples, we elected to use 75 mmol/liter routinely. Four serum samples were used in verifying the optimum pH for the system. The range of pH studied was 7.8-8.8, in 0.2 pH increments; activity was optimal at pH 8.2 (25 °C).
Substrate concentration: Five sera with enzyme activities as great as three times the upper limit of normal were used to determine substrate requirements. The optimum substrate concentration was 4 mmol/liter (Figure 2).

Precision: Within-run reproducibility of the fluorometric system was evaluated with two sera with activities of 70 and 225 U/liter. Ten replicates of each were run, with these results: the CV at 70 U/liter was 4.9%, at 225 U/liter it was 2.8%.

Intermethod comparison: The clinical validity of the fluorometric assay was confirmed by comparing values for patients' sera by the fluorometric system to results derived by a clinically proven colorimetric method (GGTP Reagent Set, Dade), with the results shown in Figure 3. For 27 sera with activity as much as six times the upper limit of normal, the correlation is excellent (least squares equation, \( y = 1.01x + 0.9 \), where \( x = \) comparison method).

Comparison to another fluorometric assay system: \gamma\)-Glutamyl (\(\alpha\) or \(\beta\))naphthalamides have been used to measure \gamma\)-glutamyltransferase activity colorimetrically, the naphthylamine produced being coupled to a diazo dye (3). Both of these substrates have also been used in fluorometric techniques (4, 5).

We briefly compared \(N\)-\gamma-\(L\)-glutamyl-5-aminoisophthalic acid dimethyl ester hydrochloride and \(\gamma\)-\(L\)-glutamyl-\(\alpha\)-naphthylamide as fluorescent substrates for the enzyme. The latter system consisted of 4 mmol of the substrate per liter of tris(hydroxymethyl)aminomethane (100 mmol/liter) and glycyglycine (75 mmol/liter); owing to the limited solubility of this substrate, the pH of the system was 9.0 at 25 \(^\circ\)C. The single serum sample used had an activity of 220 U/liter by the comparison method. The \(N\)-\gamma-\(L\)-glutamyl-5-aminoisophthalic acid dimethyl ester hydrochloride yielded a 10-fold greater rate of change in RF than did the \(\gamma\)-naphthylamide on the prototype fluorometer. At equimolar concentrations, aminoisophhalate dimethyl ester yields two times more fluorescence than \(\alpha\)-naphthylamine does at the wavelengths of the prototype fluorometer.

Our fluorogenic substrate has distinct advantages over others now available. The substrate has recently been used to identify the isoenzymes of \gamma\)-glutamyltransferase separated on cellulose acetate and observed under ultraviolet light (6).

The elemental analysis was done by Galbraith Laboratories, Knoxville, Tenn.

References