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Optimized Determination of γ-Glutamyltransferase by Reaction-Rate Analysis

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We describe a method for measuring γ-glutamyltransferase (EC 2.3.2.2) activity in serum, which can be used with automated enzyme analyzers (such as the LKB 8600 Reaction Rate Analyzer) that require enzyme reactions to be initiated with substrate. The method also permits optimal determination conditions to be obtained at 37 °C. The enzymatic reaction is commenced by adding γ-glutamyl-*p*-nitroanilide dissolved in dilute hydrochloric acid to samples pre-incubated with tris(hydroxymethyl)aminomethane–glycylglycine buffer. The *p*-nitroaniline liberated is continously monitored at 37 °C at 405 nm. The pH of the pre-incubation buffer is such that the optimal pH for the enzyme reaction results from addition of the acid substrate solution.

Additional Keyphrases: *LKB Analyzer • liver disease • kinetic enzyme assay*

The enzyme γ-glutamyltransferase (EC 2.3.2.2) catalyzes the transfer of the γ-glutamyl group from γ-glutamyl-peptides to suitable acceptors, and is widely determined in clinical laboratories in the investigation of liver disease. In modern determination procedures γ-L-glutamyl-*p*-nitroanilide is almost invariably used as substrate for the transferase and glycylglycine as the glutamyl group acceptor. The enzyme liberates *p*-nitroaniline from the substrate, and this compound has a pronounced yellow color, whereas the substrate is practically colorless. The rate of increase in absorbance at a suitable wavelength (such as 405 nm) thus provides a measure of enzyme activity.

Because γ-glutamyl-*p*-nitroanilide is poorly soluble in many buffers and solvents, the enzymatic reaction cannot readily be begun by adding concentrated substrate to sample. This has caused difficulty in attempts to adapt the enzyme reaction to automated or semi-automated enzyme analyzers (such as the LKB 8600 Reaction Rate Analyzer) in which enzyme reactions must be started by a small volume of concentrated substrate solution (*1–3*). Such difficulties are even more pronounced at 37 °C, for at this temperature the substrate Michaelis constant (K_m) is almost double that at 25 °C (*4,5*), and its optimal concentration significantly greater.

We describe here a method for γ-glutamyltransferase determination by reaction-rate (kinetic) analysis in which the enzymatic reaction is started by adding a concentrated substrate solution and that permits optimal reaction conditions to be obtained at 37 °C. The enzymatic reaction is started by adding γ-glutamyl-*p*-nitroanilide, dissolved in dilute hydrochloric acid, to samples pre-incubated with tris(hydroxymethyl)aminomethane–glycylglycine buffer at 37 °C. The pH of the pre-incubation buffer is such that the pH for the enzymatic reaction is optimal when the acid substrate solution is added.

Materials and Methods

Reagents

Tris(hydroxymethyl)aminomethane (115 mmol/liter)–glycylglycine (138 mmol/liter)buffer. Dissolve 6.96 g of tris(hydroxymethyl)aminomethane (British Drug Houses Ltd., Poole, England), and 9.11 g of glycylglycine (British Drug Houses Ltd.) in about 400 ml of distilled water. Adjust the pH to 8.5 at 37 °C with sodium hydroxide solution (0.5 mmol/liter), and dilute to 500 ml with water. This buffer is stable for at least three months at 4 °C.

γ-Glutamyl-p-nitroanilide substrate (104 mmol/liter). Dissolve 29.5 mg of γ-glutamyl-*p*-nitroanilide monohydrate (DADE Division American Hospital Supply Corp., Miami, Fla. 33152; Sigma, London; or Boehringer Mannheim Corp.) per milliliter of dilute hydrochloric acid (0.5 mol/liter). (Prepare a sufficient amount for the day's determinations; 0.1 ml is required per test. Use as soon as possible, preferably within 2 h.)

Procedure

Pre-warm 50 μl of sample and 1.0 ml of buffer to 37 °C.

Start the reaction by adding 0.1 ml of substrate, and continuously monitor the rate of absorbance increase at 405 nm at 37 °C.

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Apparatus

Initial reaction rates at 37 °C were continuously monitored in cells of 10-mm lightpath, with the LKB 8600 Reaction Rate Analyser (LKB Instruments Ltd., Surrey, England) equipped with a 405-nm interference filter. Instrument background absorbance compensation was set to an absorbance value of 0.7, and absorbance increases were monitored for 1 min.

Calculation

One International Unit (U) of γ-glutamyltransferase is that enzyme activity that will liberate 1 μmol of *p*-nitroaniline per minute under the test conditions. The absorbance at 405 nm of a 1 mol/liter solution of *p*-nitroaniline is 9900 in cells of 10-mm lightpath, so that a solution containing 1 μmol of *p*-nitroaniline in 1.0 ml would have an absorbance of 9.9, and the formation of 1 μmol of *p*-nitroaniline in the 1.15 ml final test volume would produce an absorbance increase (ΔA) of 9.9/1.15 = 8.609. The number of micromoles of *p*-nitroaniline formed per minute in the test cuvet is therefore equal to the observed ΔA/min divided by 8.609. Under the conditions of assay, this many micromoles of *p*-nitroaniline are liberated by 0.05 ml of serum or other enzyme-containing sample. The equivalent number of micromoles of *p*-nitroaniline liberated per minute from 1 liter of sample is therefore equal to observed ΔA/min × (20 000/8.609). Sample γ-glutamyltransferase activity in International Units (U) per liter at the chosen temperature is therefore equal to observed ΔA/min × 2323.

Notes on the Method

1. A substrate blank, in which water is substituted for sample, should be included in each daily run, to correct for spontaneous decomposition of substrate; any increase in blank absorbance being subtracted from that observed with the test samples. In practice, substrate blank activity did not exceed the equivalent of 3 U/liter at 37 °C. Nevertheless, slight substrate decomposition on standing increases the initial substrate absorbance, so that substrate should be used within 2 h of preparation.

2. Absorbance increases should be linear and were invariably so in practice. Sera with activities >2000 U/liter at 37 °C should be appropriately diluted with distilled water and re-determined, because their rate of absorbance increase is inconveniently high. The method was shown to be linear without dilution up to 2000 U/liter at 37 °C.

3. The pH upon addition of 0.1 ml of substrate to 1.0 ml of buffer should be 8.0 at 37 °C. If it is not, adjust the pH of the buffer with 0.5 mol/liter sodium hydroxide or hydrochloric acid as appropriate. Several sera were pre-incubated with pH 8.5 to pH 9.0 buffers for 2 h, and their activities compared with these obtained with 5-min pre-warming. Near-identical activities were obtained, confirming the pre-incubation step to be noninhibitory.

4. The final reaction conditions are: substrate, 9.0 mmol/liter; tris(hydroxymethyl)aminomethane, 100 mmol/liter; glycylglycine, 120 mmol/liter; pH 8.0 at 37 °C.

Results

The procedure was compared with the manual end-point method of Rosalki et al. (*4*) (which is also done at 37 °C) carried out at its optimal pH of 8.3. The two methods were run within 24 h of each other, on sera stored at 4 °C meanwhile. Comparisons were made on sera from patients with liver disease. Forty-two sera were examined, and gave a correlation coefficient (r) of 0.982 with a regression equation of $y = 1.29x - 9.44$ (where y = activity with the optimized reaction-rate method, x = activity with the end-point method) (Figure 1). Precision studies with the reaction-rate procedure gave a within-day precision (coefficient of variation, CV) of 2.5% (n = 20) at a mean activity of 35 U/liter at 37 °C, and 1.4% (n = 20) at a mean activity of 273 U/liter at 37 °C on replicate analyses of single sera. Between-day precision (CV) was 6.0% (n = 51) at a mean activity of 83 U/liter at 37 °C and 3.5% (n = 30) at a mean activity of 120 U/liter at 37 °C. Between-day precision was studied during five days, the sera being re-examined 24 h after their initial determination and stored at 4 °C meanwhile.

Fresh sera from 193 apparently healthy volunteer blood donors (120 men, 73 women) were examined. The distribution of enzyme values in both sexes was unimodel but skewed to the right. Enzyme values for each sex were therefore plotted as a cumulative frequency distribution on logarithmic probability paper (*6*). This gave linear plots, extrapolation of which indicated an upper limit of normal (98% probability) of 50 U/liter at 37 °C for men and 30 U/liter at 37 °C for women.

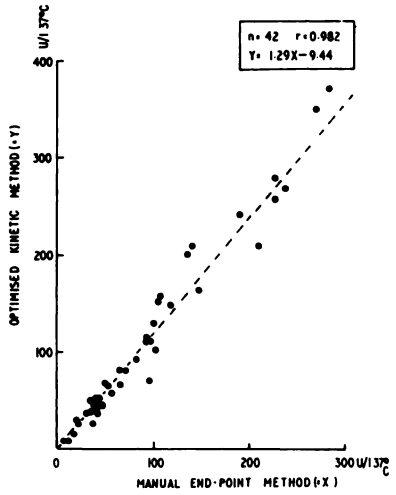

Fig. 1. Comparison of optimized reaction-rate (kinetic) γ-glutamyltransferase method described in the text, with the manual end-point method of Rosalki et al. (*4*), on sera from patients with liver disease

Derivation of Optimal Reaction Conditions

Optimal conditions for the enzyme reaction at 37 °C were established by variation of pH, substrate, acceptor, and buffer concentration, by using sera from patients with alcoholic liver disease.

Initially, substrate concentration was varied at a constant buffer concentration of 100 mmol/liter, constant acceptor concentration of 120 mmol/liter [a value 10 times the acceptor K_m value previously observed by the end-point method (*4*)], a buffer pH of 8.5 at 37 °C, and substrate dissolved in 0.5 mol/liter hydrochloric acid such that the final pH was 8.0 at 37 °C. Final substrate concentrations up to 20 mmol/liter were obtainable for short periods without substrate precipitation. The optimal substrate concentration was found to be between 8 and 10 mmol/liter, and above this value inhibition was observed (Figure 2). Lineweaver–Burk plots (*7*) showed an average K_m value for γ-glutamyl *p*-nitroanilide of 1.9 mmol/liter (Figure 3)—a value identical with that previously observed with the end-point method (*4*).

At a 9 mmol/liter substrate concentration, varying the glycylglycine acceptor concentration up to 200 mmol/liter indicated an optimal glycylglycine concentration to be approached at about 50 mmol/liter, but no inhibition was found at higher values (Figure 4). The average K_m value found for glycylglycine was 12.0 mmol/liter (Figure 5), a value also nearly identical with that previously observed with the end-point method (*4*). An acceptor concentration of 120 mmol/liter, i.e., some 10-fold the K_m value, was therefore adopted. Wide variation in tris(hydroxymethyl)aminomethane concentration (25 to 200 mmol/liter) was without effect, and for this reason 100 mmol/liter was chosen as a convenient final concentration. Under these conditions of substrate, acceptor, and buffer concentration, a final pH of 8.0 at 37 °C was optimal (Figure 6).

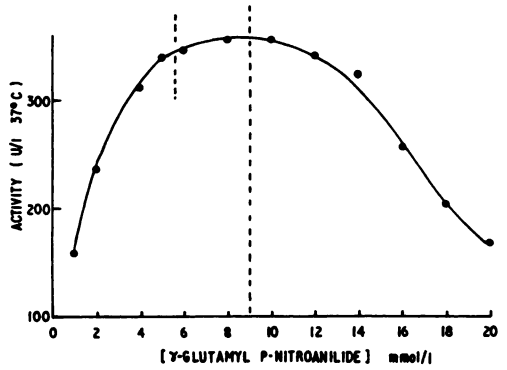

Fig. 2. Effect of variation of γ-glutamyl-*p*-nitroanilide substrate concentration on serum γ-glutamyltransferase activity at 37 °C, in tris(hydroxymethyl)aminomethane (100 mmol/liter)–glycylglycine (120 mmol liter)buffer and at a final pH of 8.0

The long vertical dotted line indicates the substrate concentration chosen for the text-method, the short dotted line indicates the substrate concentration of the comparison end-point method

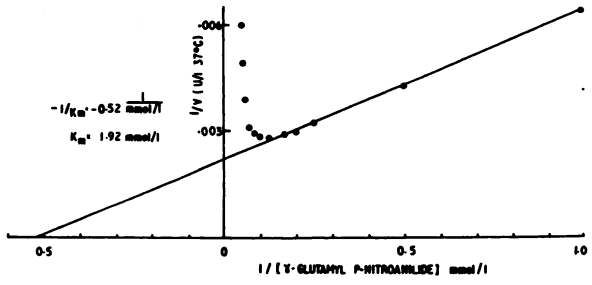

Fig. 3. Lineweaver–Burk plot of the data illustrated in Figure 2

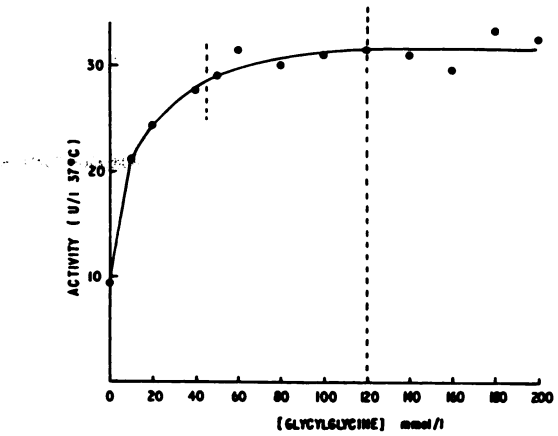

Fig. 4. Effect of variation of glycylglycine concentration on serum γ-glutamyltransferase activity at 37 °C in tris(hydroxymethyl)aminomethane (100 mmol/liter) buffer, a γ-glutamyl-*p*-nitroanilide concentration of 9 mmol/liter, and a final pH of 8.0

The long vertical dotted line indicates the glycylglycine concentration chosen for the text method, the short dotted line indicates the glycylglycine concentration of the comparison end-point method

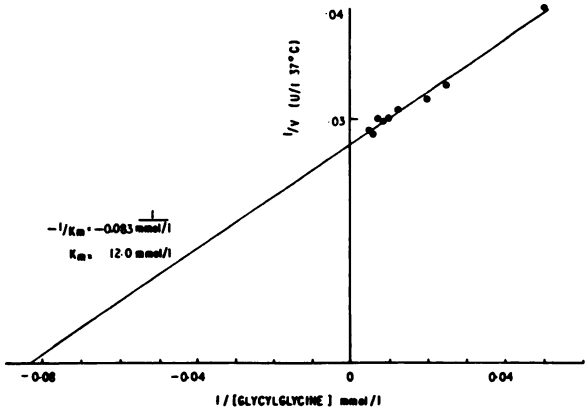

Fig. 5. Lineweaver–Burk plot of the data illustrated in Figure 4

Discussion

Previous attempts to automate γ-glutamyltransferase determination by using discrete enzyme analyzers and reaction-rate analysis have not been entirely satisfactory. Thus, with the Unicam AC 1800 apparatus at 30 °C, Moss and Whitfield (*1*) were

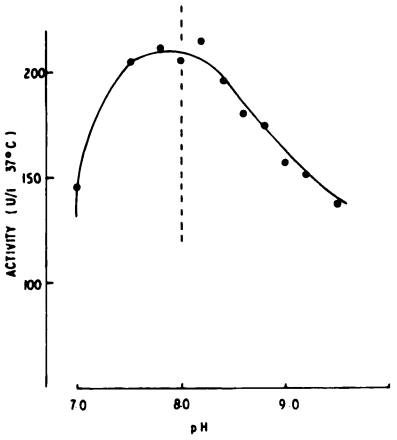

Fig. 6. Effect of pH variation on serum γ-glutamyltransferase activity at 37 °C, in tris(hydroxymethyl)aminomethane (100 mmol/liter))–glycylglycine (120 mmol/liter) buffer and at a substrate concentration of 9 mmol/liter

The vertical dotted line indicates the final pH chosen for the text method

forced to modify the instrument to permit initiation with sample rather than substrate. Yap et al. (*2*), using the Gilford 2000 system, were able to initiate the kinetic reaction with substrate; they used a concentrated substrate solution dissolved in diethylene glycol. However, this solvent caused absorbance decreases with hemoglobin and hemolyzed samples, making it necessary to add solvent to the sample diluent. In addition, it produced approximately 10% inhibition of enzyme activity. Using the LKB 8600, Delarue (*8*) described a procedure (run at 35 °C), in which the reaction was initiated with substrate dissolved in buffer, but at such a low final concentration (1.03 mmol of γ-glutamyl *p*-nitroanilide and 10.3 mmol of glycylglycine per liter) that the method cannot be recommended. Indeed, both substrate and acceptor concentrations are below their K_m values, so that enzyme activities cannot represent even 50% of their theoretical maximal velocity. Another undesirable practice is that adopted by some commercial reagent manufacturers, of initiating the enzyme reaction with the acceptor glycylglycine after pre-incubation of enzyme with substrate. Substrate hydrolysis and transpeptidation to substrate ("internal" transpeptidation) may take place during such pre-incubation, resulting in substrate concentration variation from sample to sample by the time the reaction is initiated with glycylglycine. A solution to this difficulty has been proposed by Seiffart and Chaves (*3*) for use at 25 °C. They pre-incubate sample and substrate at pH 6.0, returning the pH to optimal (8.2) with initiation of the reaction by glycylglycine addition. At pH 6.0 they saw no substrate hydrolysis by sample during pre-incubation and no spontaneous substrate breakdown or sample inactivation.

A more satisfactory procedure for γ-glutamyltransferase determination and automation is proposed here, utilizing the solubility of γ-glutamyl *p*-nitroanilide in hydrochloric acid (*9*). The procedure is simple and can be highly precise. It allows reaction initiation with substrate, and enables a far higher substrate concentration to be achieved than in any of the preceding methods, permitting the use of optimal substrate concentration and reaction conditions at 37 °C. As a result of this optimization, it yields values some 20% higher than the manual end-point method with which it was compared.

We gratefully acknowledge the loan of the LKB 8600 Reaction-Rate Analyser from LKB Instruments, Surrey, England: the gift of substrate from DADE Division, American Hospital Supply Corporation, Miami, Fla. 33152; and the assistance of Dr. T. E. Cleghorn in the provision of sera from blood donors.

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