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Editor:
Gert Lubec, Vienna

Non-Invasive Diagnosis of Kidney Disease

142 figures, 1 colour plate, and 44 tables, 1983

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Preface

What does non-invasive mean? Non-invasive means that diagnosis should be made without pain or harm to the patient, or, at least, with a minimum of pain or harm.

Why take this attitude to pain? Whether pain is to be looked upon as an instrument of divine punishment or not, I do not think that suffering is 'a sign of grace, not to be evaded but sought' (*Sigerist*, Medicine). A leading theologian permits alcoholic intoxication '...to cure typhus, snake bites, and *to relieve great pain*' (*Genicot*, Theologiae moralis institutiones), which should also be the major task of modern medical treatment.

This book, which reports non-invasive methods to the nephrologist for the benefit of his patients, presents a small contribution to humanity in medicine.

Vienna, January 1983 *Gert Lubec*

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1 γ-Glutamyltransferase in Kidney Disease

Peter R. Beck

Introduction

Distribution

γ-Glutamyltransferase (GGT) (EC 2.3.2.2) is a ubiquitous enzyme in nature which has its chief role in clinical medicine in the measurement of serum GGT for the diagnosis of hepatic disease [28, 68, 80]. However, the highest concentration of GGT occurs in the kidney [27], where it is found in the proximal tubular epithelium. Histochemical studies have shown that the GGT is localized to the brush border surface of the epithelial cells [2], and this has been confirmed by ultrastructural histochemistry [73].

Recent studies using isolated membranes have confirmed the close relationship which occurs between brush border membranes and the structure and possible functions of GGT [34, 36].

Structure and Orientation of Kidney GGT

GGT is a glycoprotein having an apparent molecular size of approximately 90×10^6 daltons [92]. Kidney GGT purified from a variety of species has a subunit structure. The subunits, which differ in size, are designated 'large' and 'small' forms [34]. The estimated size of these fractions vary between species [84, 92] but in the human appear to be of between 51 and 54×10^6 and 27×10^6 daltons, respectively [37, 92]. The active site of the enzyme resides on the smaller subunit [33]. Experimental studies suggest that this smaller subunit is oriented on the surface of the brush border membrane in contact with the tubular fluid, whilst the heavy subunit acts as an anchor piece to spatially arrange the GGT in relation to the lipid membrane [34, 36].

Function

The true physiological function of GGT is unknown. The action of GGT is to transfer the glutamyl radical in γ linkage from one peptide

or amino acid to another peptide or amino acid (transferase activity), or to water (hydrolytic activity) (fig. 1).

The most widespread γ-glutamyl containing peptide is glutathione (GSH), γ-glutamyl-cysteinyl-glycine. A multienzyme cycle has been described, the γ-glutamyl cycle [56], which appears to play an important part in the synthesis and degradation of GSH. GGT is an important constituent enzyme of this cycle, and it has been proposed that the function of GGT is in the mediation of amino acid or peptide transport across the proximal tubular epithelium through the activity of the γ-glutamyl cycle [82]. However, it should be noted that patients with abnormalities of the γ-glutamyl cycle [11, 89], or with tissue deficiencies of GGT do not suffer impairment of amino acid transport [29, 71].

Recently more emphasis has been placed on the possible hydrolytic actions of GGT. Evidence has been provided that in the presence of substrate concentrations which may be found intracellularly or in proximal tubular fluid then a hydrolytic action is much more likely to occur than a transferase action [23, 86]. On this basis it is suggested that the main function of GGT is to control the extracellular breakdown of GSH [19]. These suggestions await confirmation, and the ultimate significance of such a hydrolytic action is unknown.

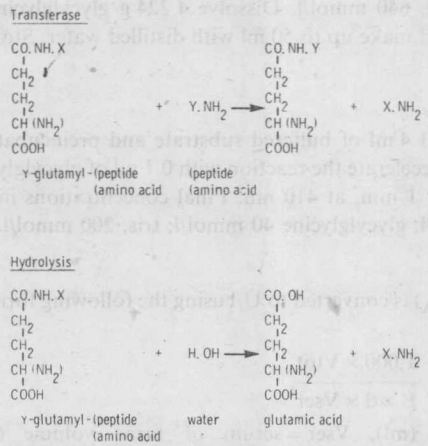

Fig. 1. Possible transferase and hydrolytic actions of GGT.

Methods

Introduction

There have been a large number of substrates used for the assay of GGT in serum, urine and tissues varying in complexity, stability, and convenience of use [16, 26, 27, 61, 64, 83]. Although experimental studies, especially recently, have concentrated on 'physiological' substrates for GGT [19, 86], most clinical studies have utilized one of a variety of synthetic chromogenic or fluorimetric substrates. Any of the generally available assay methods for serum GGT are equally suitable with little or no modification for the assay of GGT in urine. Differences relate only to preparation of the sample before assay, and this is discussed more fully below after a description of the assay used in the author's own laboratory.

Principle

γ-Glutamyl-*p*-nitroanilide + glycylglycine $\rightarrow$ *p*-nitroaniline + γ-glutamyl-glycylglycine

$\downarrow$

yellow colour
measure at 405,410 nm

Automated Kinetic Assay

Reagents

(1) Tris buffer, 0.23 mol/l. Dissolve 27.83 g tris (tris (hydroxymethyl) aminomethane) in approximately 800 ml distilled water. Correct the pH to 8.1 using concentrated hydrochloric acid and make up to 1 litre with distilled water. Store at 4 °C and discard if turbidity develops.

(2) Buffered substrate, 4.57 mmol/l. Weigh 130.4 mg of γ-l-glutamyl-*p*-nitroanilide monohydrate (GLUPA) into a conical flask and add approximately 95 ml of tris buffer. Warm to 60 °C on a magnetic stirrer-hot plate, which should dissolve the substrate, then allow to cool to room temperature. Transfer to a volumetric flask and make up to 100 ml with tris buffer. Store at room temperature, and use within 48 h.

(3) Starting reagent, glycylglycine, 640 mmol/l. Dissolve 4.224 g glycylglycine in approximately 30 ml distilled water, and make up to 50 ml with distilled water. Store at 4 °C. It is stable for 2–3 weeks.

Method

Add 0.1 ml of serum or urine to 1.4 ml of buffered substrate and preincubate to 37 °C for the minimum time possible. Accelerate the reaction with 0.1 ml of glycylglycine and monitor increasing absorbance for 1 min. at 410 nm. Final concentrations in the reaction mixture are GLUPA, 4 mmol/l; glycylglycine 40 mmol/l; tris, 200 mmol/l.

Calculation

Absorbance change per minute (Δ_A) is converted to U/l using the following formula [38]:

$$\text{GGT activity (U/l)} = \Delta_A \times \frac{1{,}000 \times \text{Vtot}}{\text{E} \times \text{d} \times \text{Vser}},$$

where Vtot = total reaction volume (ml), Vser = serum or urine volume (ml), E = millimolar absorptivity of *p*-nitroaniline/cm path length = 8.76 at 410 nm, d = path length (cm).

The assay [9] was originally used on an LKB 8600 Reaction Rate Analyser, measuring at 37 °C, but by appropriate adjustment of stock solution concentrations, and the factor for conversion to U/l, the assay may be used on any reaction rate instrument. It has been shown [5, 10] that the activities obtained using this assay are not significantly different from those kits using GLUPA-carboxylate as substrate as marketed for example by BCL (Lewes, Sussex, England).

Manual Assay

The method given below [8] is suitable for use in the absence of automated equipment. This method and the kinetic method described above give results which are not significantly different [5].

Reagents

(1) Buffered substrate, GLUPA, 114 mg; glycylglycine 660 mg and 2-amino-2-methyl-propan-1,3-diol (Ammediol), 1.8375 g is weighed into a 100-ml volumetric flask and made up to volume with distilled water. Transfer to a beaker and warm to 60 °C with constant stirring until dissolved. Allow to cool, adjust the pH to 8.25 with concentrated hydrochloric acid and make the volume up to 100 ml with distilled water. Make up daily.

(2) Hydrochloric acid, 0.04 mol/l.

Method

Pipette 1 ml of buffered substrate into a test tube (test), and 1 ml of water into a second test tube (serum/urine blank). Allow to stabilize at 37 °C in a water-bath and initiate the reaction with 0.1 ml of serum or urine added to both test and blank tubes. Include a reagent blank (1 ml of buffered substrate + 0.1 ml of water) with each batch of assays. Incubate at 37 °C for 45 min. and stop the reaction with 5 ml of 0.04 mol/l hydrochloric acid. Read the absorbances in a spectrophotometer at 405 nm. Correct the measured absorbances both for the serum/urine blank absorbance and for the reagent blank absorbance.

Calculation

$$\text{GGT activity (U/l)} = (\text{absorbance (test)} - \text{absorbance (serum/urine blank)} - \text{absorbance (substrate blank)}) \times \frac{1000 \times \text{Vtot}}{\text{E} \times \text{d} \times \text{Vser} \times \text{incubation time}},$$

where Vtot = total reaction volume = 6.1 ml, Vser = serum/urine volume = 0.1 ml, E = millimolar absorptivity of *p*-nitroaniline at 405 nm = 8.76, d = path-length of spectrophotometer cell (usually 1 cm), min = incubation time = 45 min.

Sample Handling

Stability

Serum GGT is stable for 24 h at room temperature, at least 1 week at 4 °C and for more than 1 year at −20 °C [5]. By contrast GGT in urine is not stable when frozen [15, 69, 78] except in some patients with renal failure [8], but should be stored at 4 °C prior to assay which should be within 1 week. It appears that urea is a major factor responsible

for the instability of GGT in urine which has been frozen [8]. Stability may be improved either by prior dialysis [8, 78] or by addition of dimethyl-sulphoxide [78] to the urine, but this is unnecessary unless long-term storage is required.

Inhibitors

Szasz [79] originally described the presence of dialyzable inhibitors of GGT in urine. Although this has been confirmed [66], other authors, including the present one [8, 20, 26, 78], have been unable to detect an increase in urinary GGT following dialysis. It is likely that any inhibitors which are present are of no significance in an assay system using less than 10% sample volume [18]. *Maruhn* et al. [52, 53] have used microcolumn gel permeation chromatography to remove inhibitors of urinary GGT and other enzymes prior to assay.

Centrifugation

Following centrifugation of urine the GGT activity of the supernatant is less than that of well-mixed urine prior to centrifugation [15, 59, 63]. This may be related to the GGT activity of the tubular cells and leucocytes contained in the deposit [79].

Bacterial Contamination

GGT is both produced [4, 57, 81] and destroyed [4] by a variety of bacterial species. Although defined acute bacterial infection of the urinary tract has not been described as a cause of increased urinary GGT activity, bacterial contamination must be considered as a cause of anomalous results. Sodium azide and thiomersalate do not affect GGT activity and may be used as preservative [4].

Temporal Variation

Diurnal Variation

40 normal individuals collected urine over a 24-hour period divided into the following time periods: 0800–1300, 1300–1800, 1800–2300, 2300–0800. The GGT activity was measured for each time period and the results expressed as U/5-hour period. The results are indicated in fig. 2. There was a significantly greater excretion rate during the period 0800–1300. There was no difference between sexes. A similar variation was found by *Cho* et al. [17].

Day-to-Day Variation

Studies on small numbers of individuals [4] suggests that the day-to-day variation in GGT excretion (U/24 h) is normally no more than 5–10 U.

Sex Variation

Whilst some authors have found no differences between males and females [44, 84], others have found males to excrete more GGT than females [50, 51, 79].

Expression of GGT Excretion

A variety of methods have been used for expressing the results of urinary GGT excretion, including as a concentration (U/l) [35, 61]; as an excretion rate, e.g. U/3 h [51], U/8 h [50], U/24 h [85, 91]; as a ratio to creatinine excretion [51, 59, 67]; or as a ratio to creatinine clearance [6, 7, 84]. The basis for relating GGT excretion to creatinine excre-