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Kinetic measurement by LC/MS of γ-glutamylcysteine ligase activity

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Abstract

γ-Glutamylcysteine ligase (GCL) combines cysteine and glutamate through its gamma carboxyl moiety as the first step for glutathione (GSH) synthesis and is considered to be the rate-limiting enzyme in this pathway. The enzyme is a heterodimer, with a heavy catalytic and a light regulatory subunit, which plays a critical role in the anti-oxidant response. Besides the original method of Seelig designed for the measurement of a purified enzyme, few endpoint methods, often unrefined, are available for measuring it in complex biological samples. We describe a new, fast and reliable kinetic LC/MS method which enabled us to optimize its detection. L-2-Aminobutyrate is used instead of cysteine (to avoid glutathione synthetase interference) as triggering substrate with saturating concentrations of glutamate and ATP; the γ glutamylaminobutyrate formed is measured at $m/z = 233$ at regular time intervals. Reaction rate is maximum because ATP is held constant by enzymatic recycling of ADP by pyruvate kinase and phosphoenolpyruvate. The repeatability of the method is good, with CV% of 6.5 and 4% for catalytic activities at, respectively 0.9 and 34 U/l. The affinities of rat and human enzymes for glutamate and aminobutyrate are in good agreement with previous published data. However, unlike the rat enzyme, human GCL is not sensitive to reduced glutathione and displays a more basic optimum pH.

Keywords: γ-Glutamylcysteine ligase; Glutathione metabolism; Oxidative stress; Catalytic activity; LC/MS

1. Introduction

Glutathione (GSH), the most abundant antioxidant in cells, plays an important role in the defense against oxidative stress [1–2]. GSH is synthesized from its constituent aminoacids in two sequential enzymatic reactions catalyzed by γ-glutamylcysteine ligase (GCL, EC 6.3.2.2) and GSH synthetase (EC 6.3.2.2). GCL, also known as γ-glutamylcysteine synthetase, is a heterodimer consisting of heavy (GCL-hs, 73 kD) and light (GCL-ls, 27 kD) subunits. The heavy subunit, which is up-regulated during oxidative stress, exhibits the catalytic activity of the enzyme whereas the light subunit plays a regulatory role by modulating the affinity of the heavy subunit for substrates and inhibitors. Its catalytic activity is redox dependent and this enzyme is considered to be the rate-limiting enzyme in de novo GSH synthesis [3–8]. In view of its main role in GSH homeostasis, GCL could be a target for new drugs in different fields such as cancer or parasitosis. Many results report on the transcriptional regulation of the protein, but the catalytic activity is rarely checked.

In fact, few assays of GCL activity have been described. The original method by Seelig and Meister [9] described for purified enzyme, relied on a spectrophotometric detection of NADH formed in enzymatic coupled reactions with pyruvate kinase and lactate dehydrogenase. Recently, HPLC methods with fluorometric or electrochemical detection have been described [10–13]. However, these methods are hampered by a tedious and time-consuming sample preparation (gel or membrane filtration) which is necessary to decrease the numerous metabolic side reactions due to low molecular weight compounds such as amino acids and their relatives (as recently recalled by Wu et al. [14]). Furthermore, these HPLC meth-

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ods, except for that of Nardi and Cipollaro [10], estimated catalytic activity on a single measurement of the reaction product formed by the enzyme. Another method involved a fluorescence-based microtiter plate assay with a single measurement point after naphtalenedicarboxaldehyde derivatisation but in fact it needed a blank measurement, at least for GSH and cysteine correction [15].

As GCL is a key enzyme in glutathione homeostasis and is regulated at numerous levels, the need for a precise measurement of its activity is important and explains why we describe a new method for its kinetic measurement, based on an HPLC–ESI-MS technique: L-2-aminobutyrate is used instead of cysteine as triggering substrate with saturating concentrations of glutamate and ATP, and the γ glutamylaminobutyrate formed is measured at $m/z = 233$ at regular time intervals. The reaction rate is maximum because ATP is held constant by enzymatic recycling of ADP by pyruvate kinase and phosphoenolpyruvate.

2. Materials and methods

2.1. Chemicals and reagents

L-Glutamic acid, L-2-aminobutyrate and benzylamine hydrochloride were purchased from Aldrich (St Louis, MO, USA). Pyruvate kinase, phosphoenolpyruvate, ATP, Tris were from Roche.

γ Glutamylaminobutyrate was from Bachem (Bubendorf, Switzerland), L-2-aminobutyric 3,3-d2 acid from CDN Isotopes (Canada) and other common reagents from Sigma (St Louis, MO, USA).

2.2. Enzymatic assay

All the concentrations were final concentrations in the kinetic reaction (before the addition of sulfosalicylic acid).

The general mixture (without or with glutamate) was Tris (25 mM), KCl (150 mM), $MgCl_2$ (20 mM), EDTA Na_2 (2 mM), glutamate (40 mM), adjusted at pH 8.6 with NH_4OH. This mixture was aliquoted (9 ml) and frozen at −20 °C.

2.3. Reagents for the enzymatic reaction

The incubation reagent (R1) was obtained daily by adding ATP (5.6 mM), PEP (2.1 mM) benzylamine hydrochloride (5 μM) and pyruvate kinase (30 000 U/l) to the general mixture with glutamate, the pH being checked at 8.6 after addition.

The starting reagent (R2) was L-2-aminobutyrate (20 mM) in the general mixture without glutamate.

2.4. γ glutamylaminobutyrate calibration

A stock solution of γ glutamylaminobutyrate (24 mM) was prepared in water, and aliquots (100 μl) were frozen at −20 °C. Standards (24, 12, 6, 3 μM) were prepared daily by dilution of the stock solution in water. Then each calibration point was treated as a sample, i.e. 10 μl with 70 μl of R1, 20 μl R2 and 40 μl of sulfosalicylic acid (SSA, 6% in water).

2.5. Samples: human fibroblasts and rat liver extracts or microsomes

Human fibroblasts were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum containing penicillin (98 U/ml), streptomycin (98 μg/ml) and fungizone (2.5 μg/ml) at 37 °C in a 5% CO_2 humidified atmosphere. Cells were harvested by trypsinization, washed twice in phosphate buffered saline (PBS) solution, then lysed by two cycles of freezing and thawing associated with ultrasounds for 15 s and kept frozen at −20 °C until analysis.

Rat liver microsomes were prepared by tissue homogeinisation with Tris–HCl buffer 100 mM, pH 7.4, containing 100 mM KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4000 × *g* for 15 min at 4 °C. For tissue assays, aliquots of the supernatant were frozen at −80 °C until use. To complete the microsome extraction, the supernatant was centrifuged at 100,000 × *g* for 90 min at 4 °C. The resulting microsomes were washed in a volume (equal to that of the original homogenate) of 100 mM sodium pyrophosphate buffer, pH 7.4. The washed microsomes were re-suspended at a protein concentration of 40–50 mg/ml, in 50 mM KH_2PO_4 buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) and 20% glycerol and frozen at −80 °C until use.

2.6. GCL kinetic measurement

For the assay of the enzymatic activity, 40 μl of sample were mixed with 280 μl of R1, distributed in three Eppendorf tubes (80 μl each) and incubated for 2 min at 37 °C. The reaction was started simultaneously with 20 μl of R2 in each tube and stopped individually with 40 μl of SSA at 2, 4 or 6 min. After centrifugation at 13,000 × *g* for 2 min, the γ glutamylaminobutyrate was measured by LC/MS.

2.7. LC–MS analysis of the reaction product γ glutamylaminobutyrate

LC–MS separations were performed on a Thermo liquid chromatograph with a pump P 4000, a thermostated autoinjector AS 3000 and a Navigator Aqua mass spectrometer detector equipped with a diverting valve (between the column and the detector). The separation was carried out on a 150 mm × 2 mm UP3 HDO cartridge (octadecylsilane silica with 3 μm particle size, Interchim, Montluçon, France), kept at 45 °C in an oven, with a flow rate of 0.2 ml/min.

The mobile phase was ammonium acetate 5 mM (final concentration) adjusted to pH 2.5 with formic acid and 5% (v/v) methanol. The standards and samples were kept at 15 °C in the autosampler and the injection volume was 5 μl.

Detection was carried out with a single quadrupole mass spectrometer in ESI+. The sample probe was set at 400 °C and 4 kV with an entrance cone voltage at 10 V. Chromatograms were recorded in single ion monitoring mode $[M+H]^+$ for 6 min and integrated with XCalibur software (Version 2.51) at $m/z = 233$ for γ glutamylaminobutyrate and $m/z = 108$ for the internal standard benzylamine.

3. Results

3.1. LC/MS measurement of γ glutamylaminobutyrate

Optimisation of the three main parameters of the ionization source showed that a 10-fold increase in sensitivity, at least, could be obtained in three steps: the effects of the probe voltage (between 3 and 4.5 kV), of the probe temperature (100–400 °C) and cone voltage are shown in Fig. 1. The best sensitivity, together with high reproducibility (CV% < 1.5% for each parameter, $n = 3$ in 3 different experiments), is obtained at 4 kV, 400 °C and 10 V for the probe voltage, probe temperature and cone voltage.

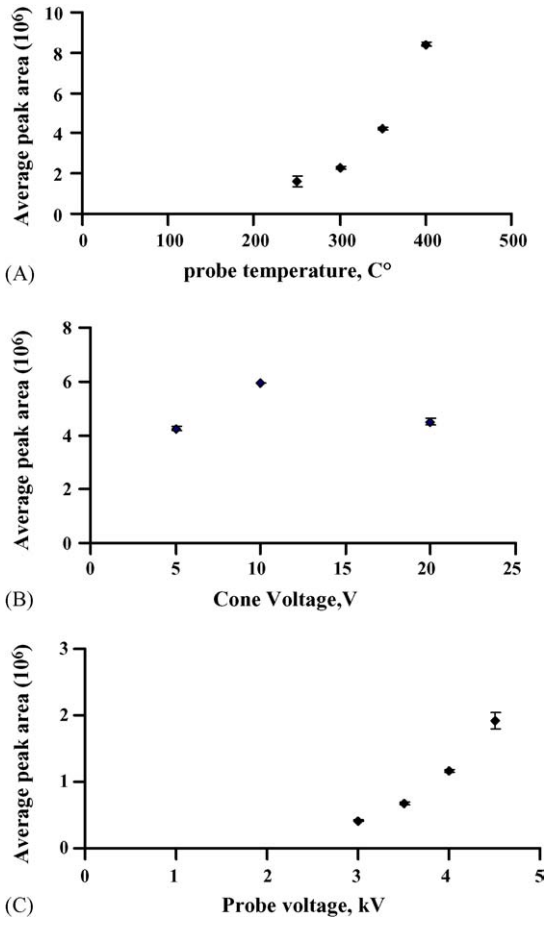

Fig. 1. Optimization of the three main parameters of the ionization source in the MS detector.

A typical chromatogram, acquired in full scan mode, for a liver sample with a relatively high catalytic activity incubated for 6 min, is shown in Fig. 2: γ glutamylaminobutyrate is eluted at 4.5 min whereas all the main components of the reaction mixture are detected at or near the void volume of the column.

3.2. Enzymatic reaction measurement

By measuring the amount of γ glutamylaminobutyrate in each of the three tubes incubated, respectively, for 2, 4 and 6 min, it was possible to calculate the rate of production of γ glutamylaminobutyrate, that is, in fact, the catalytic activity of the sample (μM/min at 37 °C). This catalytic activity is represented by the slope of the regression line calculated with three points, and any kinetic analysis with a coefficient of correlation below 0.99 was checked (Fig. 3).

3.3. pH optimum for the enzyme activity

The effect of the pH on the catalytic rate of the enzyme was checked for rat liver and human fibroblasts between 6 and 10 with two different buffers, then optimised by 0.2 pH unit steps between 7.8 and 8.8 with Tris buffer. The pH effect was different for the human and rat enzymes, but a common optimum pH was found at around 8.6 (Fig. 4).

3.4. Measurement of K_m for glutamate and L-2-aminobutyrate

The catalytic activity was measured with increasing concentrations of substrates, between 0.2 and 30 mM for glutamate and 0.05 and 20 mM for L-2-aminobutyrate. The K_m values were extrapolated from Lineweaver–Burk plots ($1/V$ as a function of $1/S$) and checked according to Hofstee (V as a function of V/S) and were reported as the mean value. The affinities of the human and rat enzymes are 2.4 and 2.3 mM for glutamate and 1.4 and 0.8 mM for L-2-aminobutyrate.

3.5. Effect of GSH on the reaction rate

The catalytic activity of human and rat samples was measured in the presence of increasing concentrations of reduced glutathione (0, 1 and 2 mM, final concentrations). As shown in Fig. 5, unlike the rat enzyme, the human GCL is not sensitive to GSH.

3.6. Within-run imprecision

Samples of human fibroblasts and rat liver (diluted 1/10 in NaCl 9 g/l)) were measured six times in the same series. The within-run CV was 6.5% and 4% with catalytic activities at 0.9 and 34 U/l.

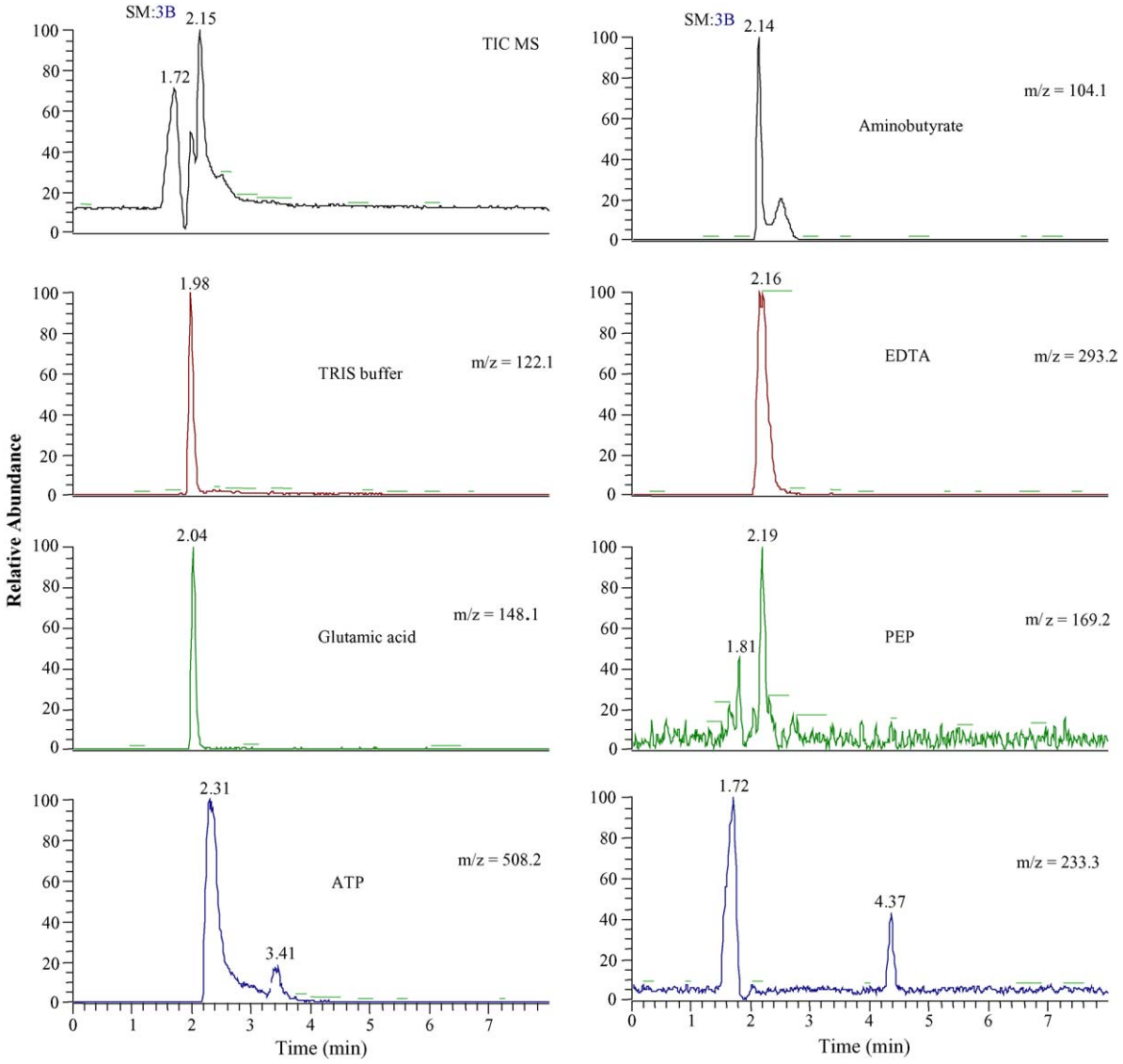

Fig. 2. Chromatogram of a liver extract sample (after 6 min), acquired in full scan mode: top left corner. The other chromatograms are extracted at specific *m*/*z* ratios corresponding to the different compounds present in the enzymatic reagent. The last chromatogram (bottom right corner) corresponds to γ glutamylaminobutyrate produced by GCL.

3.7. Between-run precision and transferability

Aliquots of the same liver extract were prepared and measured in duplicate, one at a time, on three different LC/MS (with a specific optimization of the source parameters for each mass spectrometer) for three weeks: the mean value was 29.9 with a SD = 1.3 (CV% = 4.35, $n = 6$).

4. Discussion

The original method of Seelig was devised for purified enzyme and, whatever the detection chosen, the adaptation of this method to tissue or cell extracts needs a prerequisite: time-consuming sample preparation to decrease the effects of interfering reactions, particularly that of GSH synthetase, present in the sample, which uses directly the product of the GCL reaction as a substrate.

Except for the method with an electrochemical detection [13], recent HPLC techniques with flurometric detection present another drawback: the necessary derivatisation of the thiol function of the γ glutamyl-cysteine product by orthophthalaldehyde (OPA), monobromobimane (MB) or *N*-(1-pyrenyl) maleimide (NPM). Furthermore, most of them measure the catalytic activity as a single endpoint.

That is why we decided to explore the potential contribution of LC/MS detection to set up a meticulous but simple, fast and robust kinetic method for GCL measurement. This new method combined the advantages of a highly selective detection and the use of L-2-aminobutyrate instead of cysteine (to avoid GSH synthetase interference):

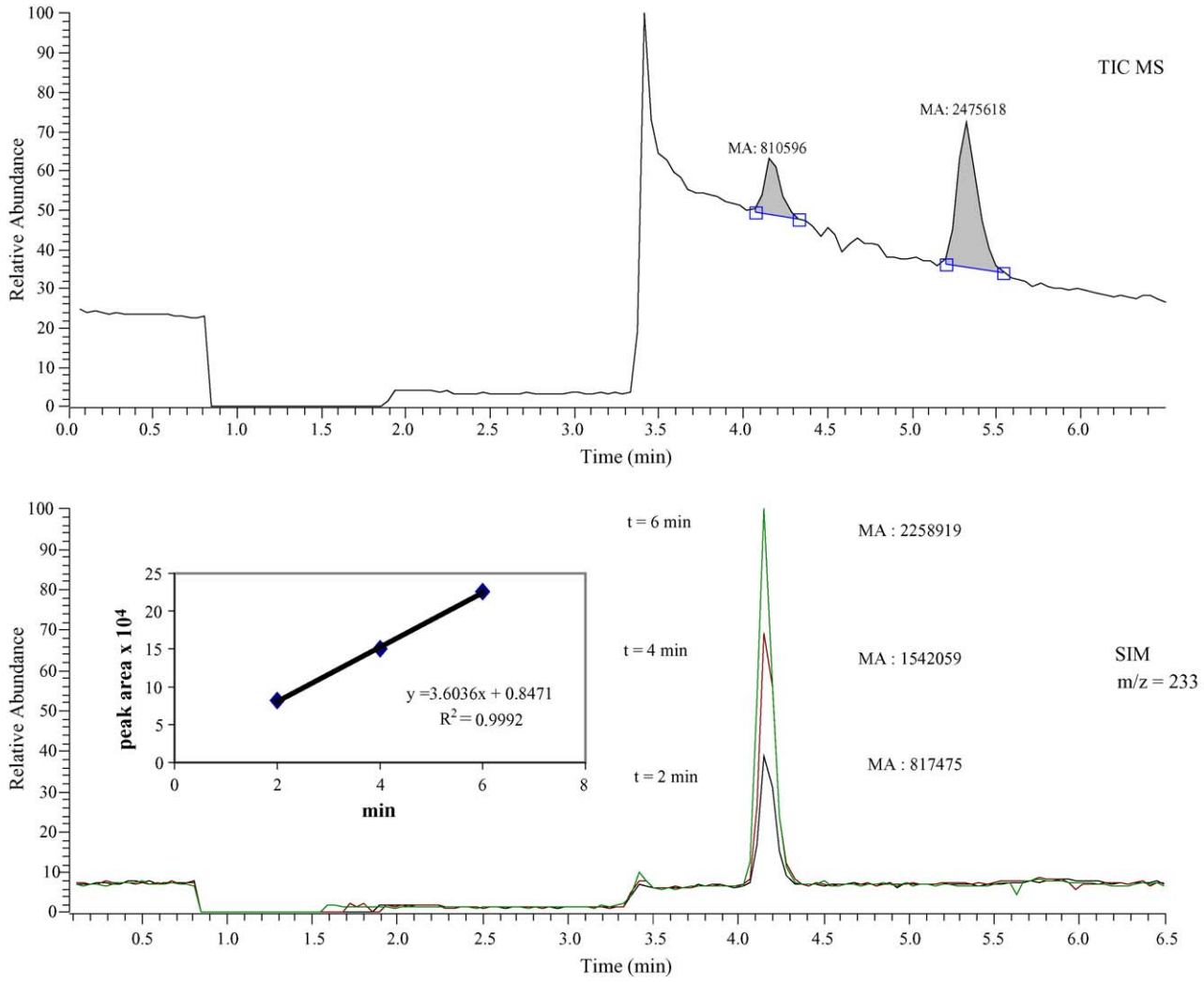

Fig. 3. The upper chromatogram is acquired, in full scan mode, 2 min after the triggering of the enzymatic reaction: γ glutamylaminobutyrate is eluted at 4.20 min and benzylamine (the internal standard) at 5.40 min. The lower figure is an overlay of the three chromatograms (one enzymatic analysis) integrated in single ion monitoring mode at $m/z = 233$ and at 2, 4 and 6 min. In the inset is the linear plot of the three areas. The slope corresponds to the enzymatic reaction rate.

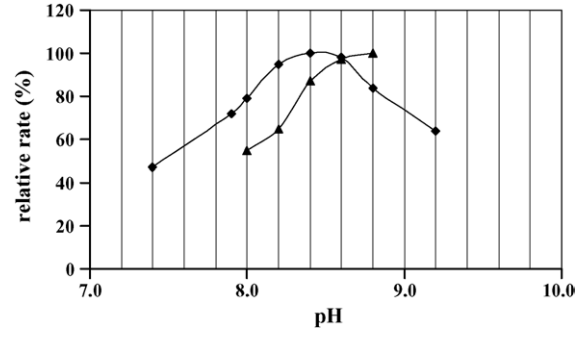

Fig. 4. Effect of various pH on the catalytic activity of γ-glutamylcysteine ligase in human fibroblasts (triangles) and rat liver (diamonds). Each point was carried out in triplicate. A common optimum pH was found at around 8.6.

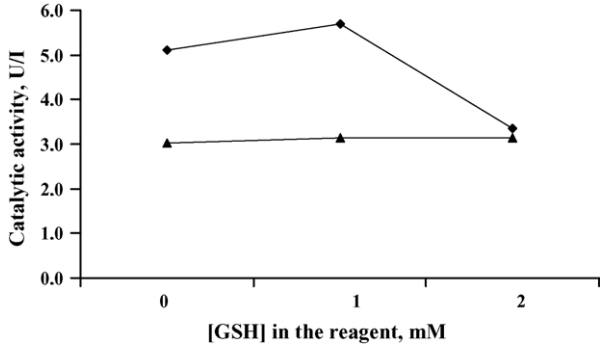

Fig. 5. Effect of increasing concentrations of reduced glutathione on the catalytic activity of human (triangles) and rat (diamonds) GCL enzyme. Each point is the mean of two measurements.

- the first advantage of L-2-aminobutyrate was to avoid any cumbersome sample preparation and it was checked by a simple trial. Thanks to the high activity of the rat liver extract, γ-glutamyl-d2-aminobutyrate was enzymatically synthetised and used as an internal standard instead of benzylamine for a short series of ten measurements: the results were comparable to those obtained with benzylamine.
- the second benefit was that the final product γ glutamylaminobutyrate, unlike γ-glutamyl-cysteine, has sufficient retentivity to be chromatographed easily on a standard C18 column in order to make precise quantification by MS detection.

From a practical point of view, another advantage of this method is that the time points for the kinetic analysis may be

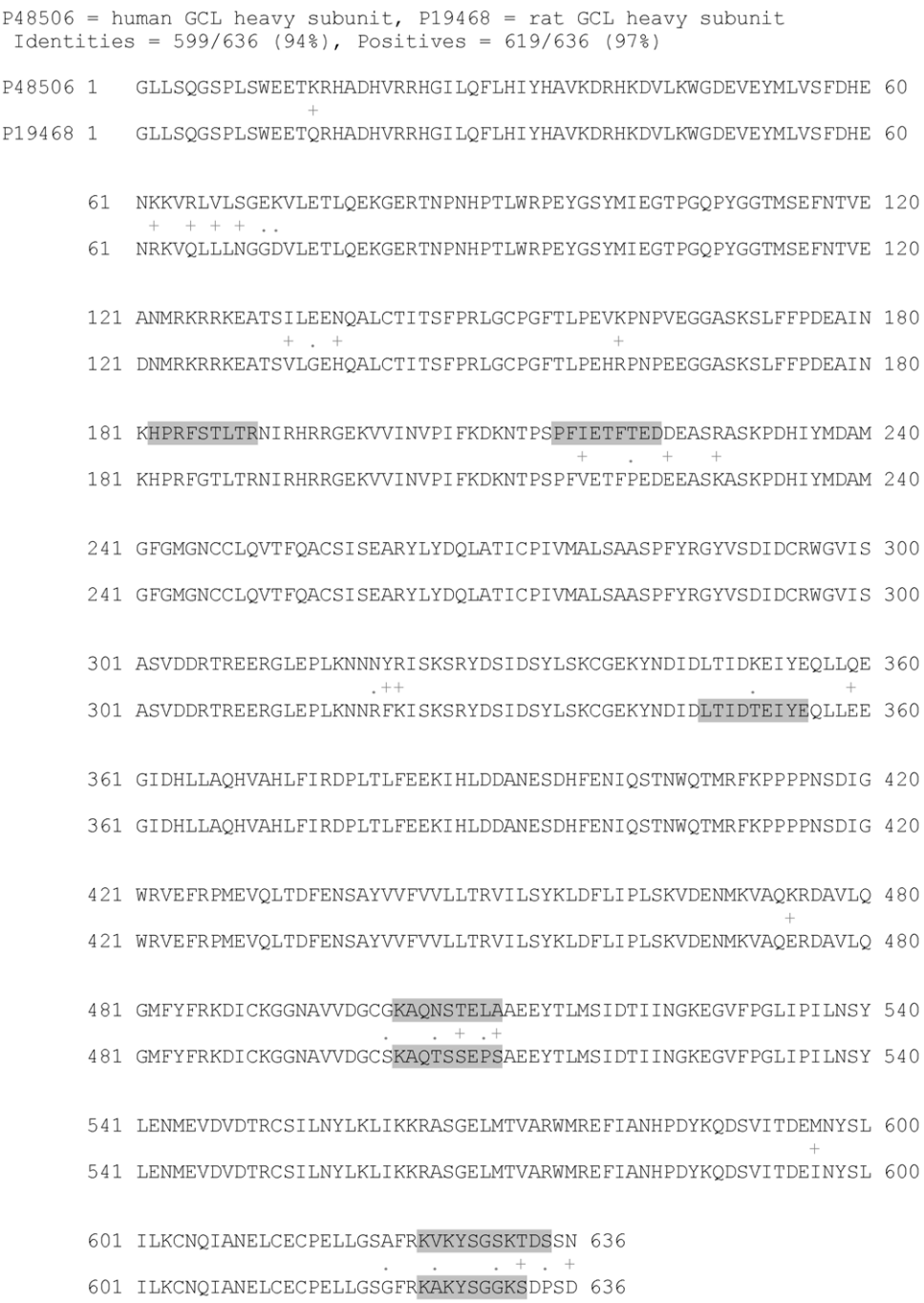

Fig. 6. Sequence comparison between human GCL heavy subunit (accession number: P48506) and rat GCL heavy subunit (accession number: P19468). The phosphorylation sites with a prediction score higher than 0.8 (except for the first one) appear in the shaded areas. The non similar amino-acids are marked with a point.

shortened at 1, 2 and 3 min for high activity or lengthened to 3, 6 and 9 min for very low activities. In our situation, the practicability of the method relies on the use of a multi-channel electronic pipettor to trigger and stop the enzymatic reaction at a precise time with, respectively, the second substrate and the sulfosalicylic acid.

The reaction pH used in previous studies was between 8 and 8.2. While this pH is close to the optimum for rat samples, the catalytic activity for human samples is only 55% of the maximum, which is reached at pH 8.8. For this reason, we retained a pH of 8.6 as the best compromise.

The K_m of human and rat enzymes for L-2-aminobutyrate and glutamate are in the same range as that previously published and recently reviewed by Griffith [8]. However, they are very different from the only human values measured by Sriman and Ali-Osman [16] from a malignant astrocytoma cell line.

As described previously, rat GCL is known to be inhibited by GSH. Unexpectedly, the human enzyme, at least in fibroblasts, is not sensitive to GSH; the catalytic activity, expressed in U/mg, is roughly of the same order of magnitude for both samples and, if we remember that the enzyme is diluted 10 times before measurement, the 2 mM GSH concentration used during the assay corresponds to an upper concentration of 20 mM within cells. Even if we remain cautious, it seems plausible to conclude that the human enzyme does not behave like rat enzyme. The comparison of both amino-acid sequences of heavy GCL subunits with Blastp [17] shows that these catalytic subunits share 94% of identity and 97% of similarity, confirming a highly conserved structure. Surprisingly most of the rare non-similar amino-acids (identified by a point in Fig. 6, with human GCL as the reference on the upper line) are serine (S), threonine (T) or tyrosine (Y), all located in potential phosphorylation sites as determined by NetPhos [[18], www.cbs.dtu.dk]. Furthermore, four of these five sites have a prediction score higher than 0.8. As the data on phosphorylation are available only for rat enzyme [19,20] and describe five phosphopeptides [20], further work will be necessary to know whether this difference, in the presence of GSH, could be related to differences in protein phosphorylation: the new method we propose seems to be the right tool for this task, as it is fast, precise and reliable.

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