

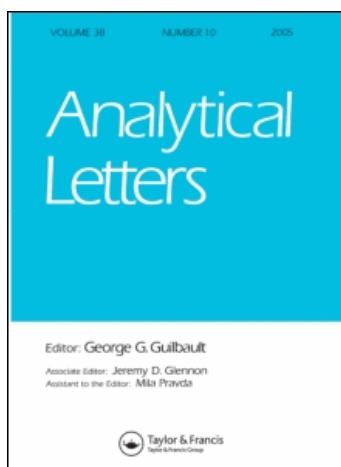
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FAST AMPEROMETRIC DETERMINATION OF ENZYMATIC ACTIVITY OF GLUTAMINASE

Key Words: glutaminase, enzyme activity, glutamate biosensor, amperometry, tissue.

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ABSTRACT

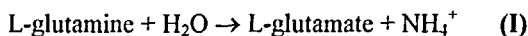
The activity of the enzyme glutaminase has been measured using a glutamate electrochemical biosensor based on H₂O₂ detection. Calibration curves for glutamate detection and for glutaminase activity using standard glutaminase from *Escherichia coli* demonstrated the high sensitivity and the rapid analysis time of

this novel amperometric procedure, which was 100 times more sensitive than that reported in literature.

Porcine liver and kidney tissue and human kidney tissue samples have been tested for glutaminase activity, demonstrating the possibility to perform measurements directly on whole tissues, with no need of sample extraction and purification.

INTRODUCTION

Glutaminase is an enzyme present in high concentration in the kidney of humans and of other mammals, with variable amounts in liver, brain, platelets and others tissues¹. This enzyme catalyses the catabolism of the aminoacid glutamine, giving stoichiometric amounts of glutamate and ammonia according with the following reaction:



Two isoforms of the enzyme are known: a liver-type glutaminase, which effectively couples the ammonia production with urea synthesis, and a kidney-type glutaminase, which releases ammonia without further metabolism. Hepatic glutaminase increases during starvation, diabetes, and in high protein diet, while kidney-type glutaminase increases during metabolic acidosis². Both the isoforms of glutaminase are localised within the mitochondria and also its activity appears to be strictly associated with the mitochondrial fraction of liver and kidney. Extraction and solubilisation of glutaminase have proven to be difficult, and the measurement of its activity needs long procedures because of mitochondrial sample preparation³. These measurements have been carried out in most of the cases by UV-spectrometry: glutamate was detected using a glutamate dehydrogenase procedure⁴ in kidney mitochondria³, in liver mitochondria⁵⁻⁷ and

in neutrophils⁸; or by measuring the formation of [¹⁴C] glutamate from [U-¹⁴C] glutamine, both in kidney mitochondria⁹ and platelets¹⁰.

In this paper we report a fast and sensitive method for the determination of the glutaminase activity by the measurement of the rate of the production of glutamate using an amperometric biosensor. In fact, glutamate comes from the glutaminase catalyzed reaction (I), and is then oxidised by glutamate oxidase immobilised on a membrane on the tip of a H₂O₂ sensor. The reaction is as follows:



The hydrogen peroxide produced in this second reaction is detected at the H₂O₂ electrode and related to the glutaminase enzyme activity.

This procedure has been also applied to measure the glutaminase activity directly on the kidney and liver whole tissues obtained from human and animal samples, without any extraction and purification.

EXPERIMENTAL

Reagents and materials

L-Glutamate Oxidase (GLOD) (EC 1.4.3.11) from *Streptomyces sp.* was purchased from Toyobo Co. (Choshi, Japan); Glutaminase (GMN) (E.C. 3.5.1.2) from *Escherichia Coli*, L-Glutamine, L-Glutamate (sodium salt) and all other reagents were of analytical grade and purchased from Sigma Chemical (ST. Louis, MO, USA).

Immobilon AV Affinity Membrane 0.65 μm pore size, 125 μm thick, and Polycarbonate membrane, 0.2 μm pore size, 6 μm thick, were obtained from Millipore Corporation, (Bedford, MA, USA).

Cellulose Acetate membranes of about 100 Dalton Molecular Weight Cut-Off were prepared in our laboratory as described in the literature¹¹.

Pig liver and kidney samples were purchased from local shops. Human samples of blood and renal tissues were collected from the Department of Urology of the "Umberto I" Hospital in Rome.

Instrumentation

Amperometric measurements were carried out with an ABD Amperometric Detector (Universal Sensor, Metairie, LA, USA), equipped with its H₂O₂ sensor. A LINSEIS L-250E Recorder (Selb, Germany) recorded all measurements.

Experiments at 37° C were performed in a double wall beaker by the use of an Haake F3 Thermostat (Berlin, Germany).

Procedures

Preparation of L-glutamate biosensor

The biosensor was assembled as reported in a previous paper¹². The biosensor was inserted in a thermostatted cell containing buffer and let to equilibrate for few minutes. The glutamate, added to the solution or coming from the glutaminase enzymatic reaction, is oxidised by GLOD immobilised on the membrane, producing hydrogen peroxide that diffuses across the acetate cellulose membrane and is oxidised at the electrode.

The GLOD membrane was prepared by covalently binding the enzyme onto the preactivated Immobilon membrane. A solution (10 µl, containing 0.3 U GLOD, 4 µl of BSA 10% (w/v) and 1 µL of glutaraldehyde 2.5% (v/v), all in phosphate buffer pH 7.4) was spread out uniformly on a disk of the membrane (1 cm²). It was allowed to dry at room temperature for 1-2 h, then the same operation was repeated on the opposite face. The dry membrane was washed for 30 minutes in a glycine solution 0.1 mol/L and then in phosphate buffer pH 7.4. The membrane was stored in the same buffer with sodium azide 0.01 mol/L at 4° C when not in use.

Enzyme activity measurement

The glutamate electrode was equilibrated in 3 ml of a stirred solution, kept at 37°C in a jacketed beaker connected to the thermostat in presence of glutamine 0.030 mol/L in acetate buffer, pH 5.4. Different concentrations of Glutaminase solutions were then injected, and the variation of the current caused by the glutamate, produced by the enzymatic reaction, was monitored for 6 minutes. The value of the first minute was discarded, while the current values during the subsequent 5 minutes were recorded and averaged.

Tissue measurement

Small pieces of fresh porcine kidney and liver were cut and accurately weighed, then washed in phosphate buffer with NaN_3 0.01 mol/L to eliminate cell fragments possibly generated during the initial cutting of the tissue from the bulk organ.

The glutaminase activity was then measured adding the weighed pieces of tissue to the thermostatted beaker containing glutamine 0.030 mol/L in phosphate buffer 0.01 mol/L pH 7.4, then the rate of production of glutamate was recorded through the glutamate biosensor as in the previous protocol.

RESULTS AND DISCUSSION

Glutamine and glutamate biosensors based on glutaminase and glutamate oxidase have been reported in a large number of papers¹²⁻²⁴; to our best knowledge, a glutamate biosensor has never been applied to the measurement of glutaminase activity.

In the past we assembled biosensors immobilising GLOD for the measurements of transaminases activity in serum¹⁴, or together with others enzymes for the detection of glutamate and aspartate in food and pharmaceutical products¹⁵, or alanine in serum¹⁷.

In this paper GLOD has been immobilised on a preactivated membrane, thus obtaining a biosensor with very stable and reproducible features. Fig. 1 reports the calibration curves of glutamate at different temperatures, showing a linear range from the detection limit of 1×10^{-7} mol/L up to 5×10^{-4} mol/L at 37 °C. The lifetime of the probe was more than four months, if stored in buffer with NaN_3 , 10^{-2} mol/L, at 4°C when not in use. The optimum pH range of the biosensor was between 7 and 8, but at pH 5.4, where some measurements have been carried out, the output of the biosensor was still about 60 % of the optimum values.

From the previous mentioned work on alanine¹⁷ we know that glutamine is also a substrate of GLOD, giving about 7% of the response compared with that of glutamate tested at the same concentration. Because of the enzymatic activity measurement, a concentration of substrate higher than the K_m of the enzyme should be necessary. We used a glutamine concentration of about 30 mmol/L, which is higher than the K_m of the liver-type glutaminase (2-5 mmol/L), but of the same order of magnitude (20-30 mmol/L) of the kidney-type glutaminase². Nevertheless this concentration has been selected for two reasons: firstly, the values and the period of time of the enzyme activity measurements performed, resulting in negligible amounts of glutamine consumed; second, concentrations of glutamine higher than 30 mmol/L showed a small current drift that could interfere with the enzyme activity measurements. Calibration curves of glutamate have been repeated in the presence of such amounts of glutamine and the response of the biosensor was about 20 % higher than in the absence of it; the linearity range was only up to 10^{-4} mol/L, while the detection limit remained the same.

The human glutaminase is a quite complex enzyme, which is activated by phosphate and ammonia and inhibited by high concentration of glutamate. This enzyme shows an optimum activity at a pH between 7.8-8.2^{2,25}. However, using a high concentration of phosphate, (0.1 mol/L), the enzyme becomes insensitive to these compounds⁷. Therefore all measurements with animal and human

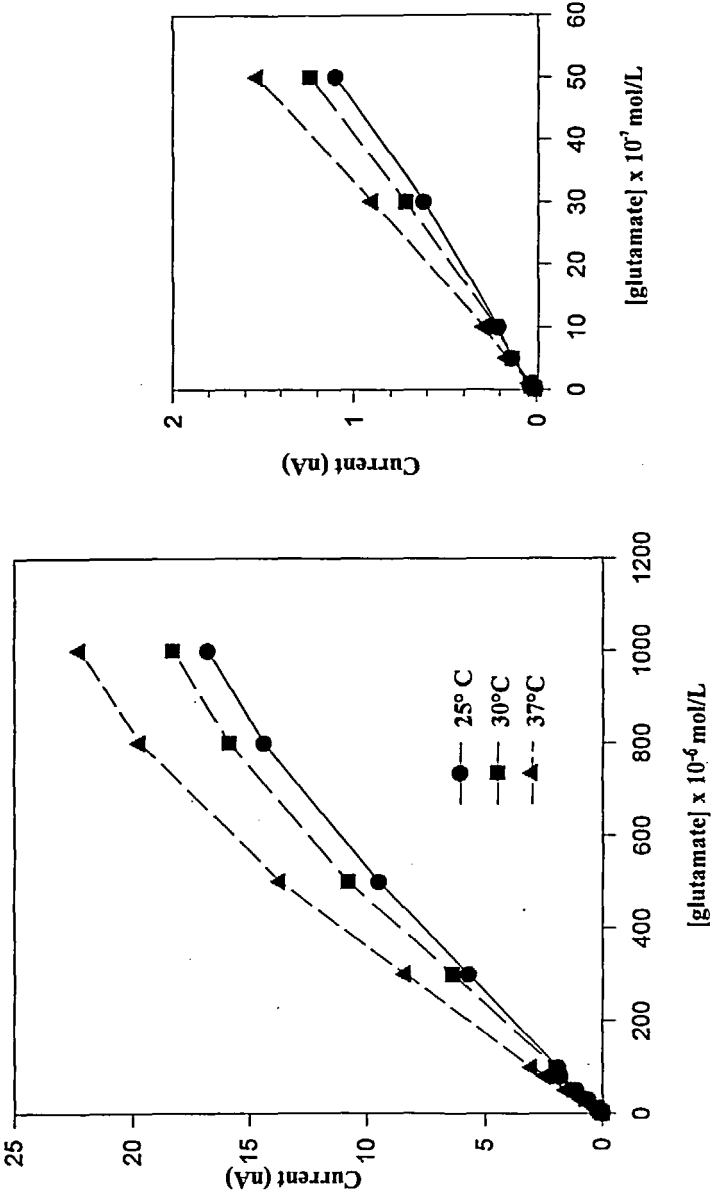


Fig. 1: Calibration curves of glutamate at different temperatures. Phosphate buffer 0.1 mol/L, pH 7.4.

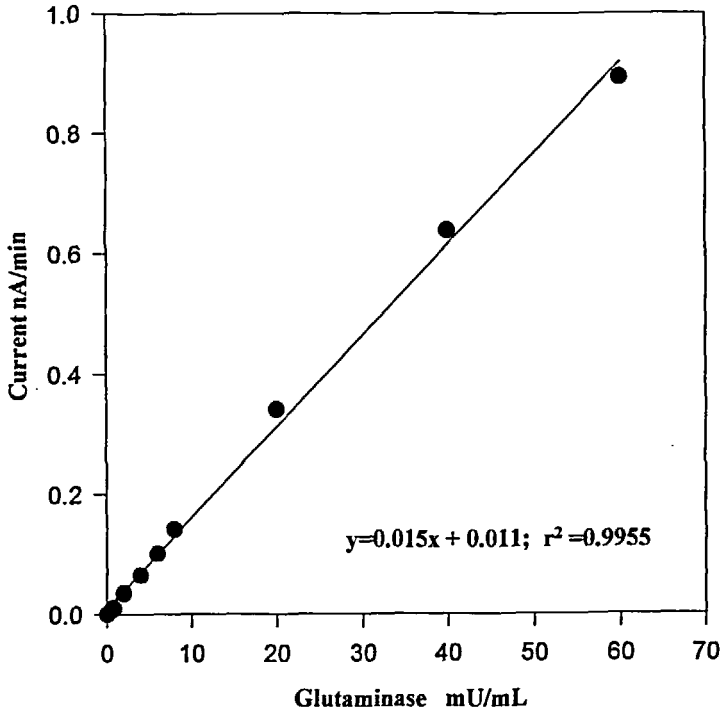


Fig. 2: Calibration curve of the glutaminase enzyme activity at 37°C. Acetate buffer pH 5.4.

samples have been performed in phosphate buffer 0.1 mol/L at physiological pH and in presence of 0.030 mol/L of glutamine.

Unfortunately the human glutaminase is not commercially available, so controls of its activity have been carried out with the enzyme obtained from *E. coli*. The latter catalyses the same reaction, but it shows maximum activity at pH 5.4¹² with a rapid decrease at pH higher than 6. A calibration curve of the enzyme activity at pH 5.4 is shown in Fig. 2. Results gave a detection limit of 0.1 mU/ml and linear range 0-60 mU/mL.

If we compare the calibration curve of glutamate attained the same day in the same conditions, we can convert the ordinate nA/min in $\mu\text{mol/L}$ of glutamate

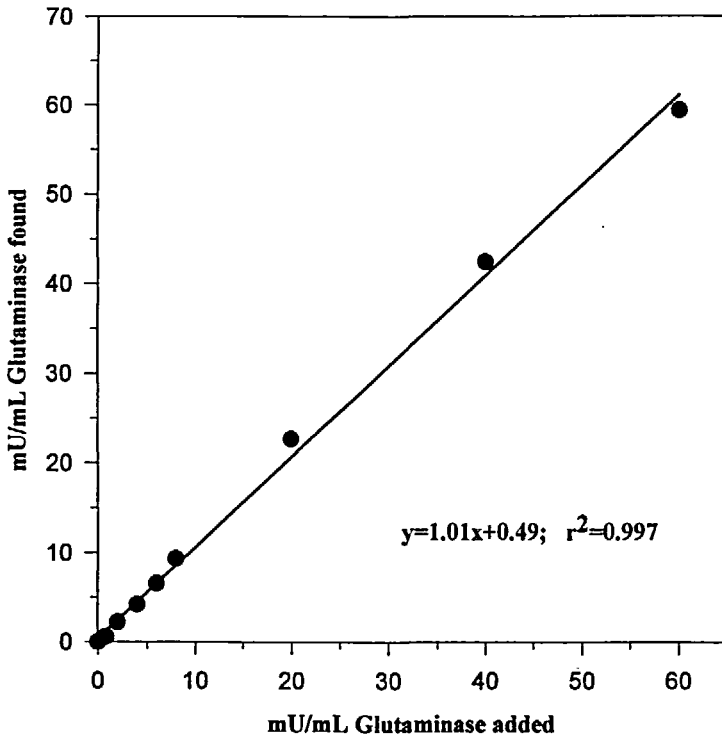


Fig. 3: Comparison between the nominal values of the activity of the glutaminase added to the solution and the activity values found by the proposed method. Glutaminase from *E. coli*, Acetate buffer pH 5.4, T=37°C.

produced per min and then convert it in values of enzyme activity. As reported in Fig. 3, the ordinate represents the experimental values and the abscissa reports the nominal values of the additions. Good agreement was obtained in such a plot. These experiments have been repeated several times.

The proposed procedure (phosphate buffer 0.1 mol/L pH 7.4, glutamine 0.030 mol/L) was then applied to measure the glutaminase activity in human serum samples. In this matrix, the interference of endogenous glutamate and glutamine could arise. Nevertheless, the endogenous glutamate concentration in

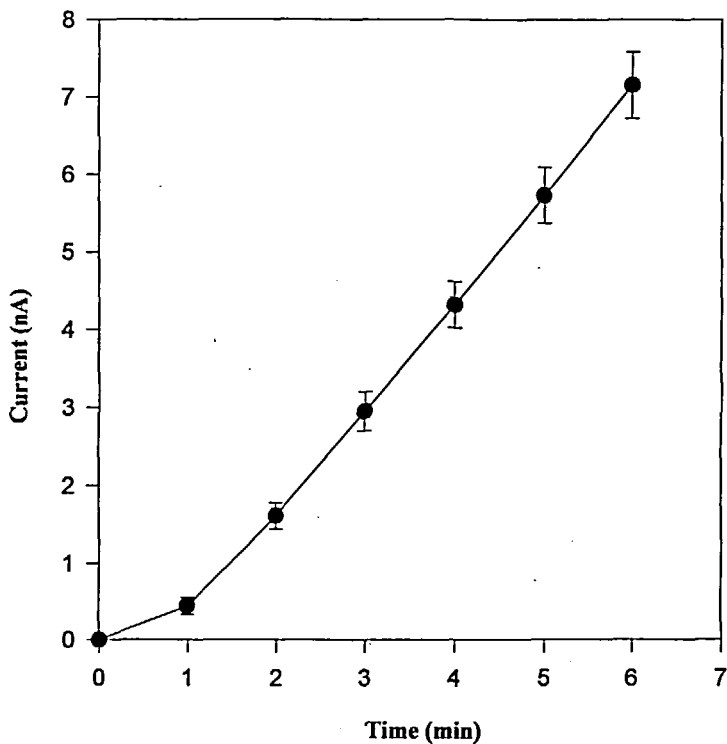


Fig. 4: Measurement of the glutaminase activity of 20 mg of porcine kidney tissue. Mean \pm SD, $n=4$, Phosphate buffer 0.1 mol/L, pH 7.4, and glutamine 0.030 mol/l.

serum is about 10 $\mu\text{mol/L}$, the samples were diluted at least 10 times, and a constant current value is eventually obtained. The measurement of the enzyme activity is based on the evaluation of the current variation during time; therefore a constant low value of glutamate should not interfere. Moreover the presence of the high concentration of glutamine substantially eliminated any interference of endogenous glutamine which is present in serum at a concentration of $3\text{-}7 \times 10^{-5}$ mol/L after 10 times dilution.

TABLE 1: Glutaminase activity measurements (as nmol of glutamate produced *per min per mg* of tissue) on real samples. (Mean \pm SD).

Sample Source	Quantity (mg)	n	Activity (nmol /mg *min)
Porcine Liver I	2.9 \pm 0.2	5	2.3 \pm 0.4
Porcine Liver II	10 \pm 1	5	1.5 \pm 0.2
Porcine Liver III	21 \pm 1	8	1.2 \pm 0.2
Porcine Kidney I	3.1 \pm 0.3	4	7.4 \pm 0.8
Porcine Kidney II	10 \pm 1	4	6.9 \pm 0.7
Porcine Kidney III	20 \pm 1	4	5.3 \pm 0.4
Human Kidney I	18 \pm 1	6	9.0 \pm 0.8
Human Kidney II	25 \pm 1	5	5.4 \pm 0.6
Human Kidney III	18 \pm 1	4	3.9 \pm 0.5
Human Kidney IV	16 \pm 1	3	4.1 \pm 0.5

Attempts to measure the glutaminase activity in serum have been found in literature in 1978²⁶. In that case the authors applied a spectrophotometric method with a detection limit of 10 mU/ml. Our method is 100 times more sensitive than the previous one, but both their and our attempts to measure the glutaminase activity in serum were unsuccessful, confirming the hypothesis of previous authors that the glutaminase activity is strictly associated within mitochondria and not detectable in serum.

Several tissue samples from human kidney and from pork liver and kidney were assayed for enzyme activity; the experiments gave measurable values and results are reported in Fig. 4 and summarised in Table I.

Reproducible values have been obtained for the same samples, when similar amounts were assayed, provided that a minimum amount of 3 mg of tissue sample was used.

These results showed that glutaminase enzyme activity measurement could be performed in a rapid, simple and reproducible way, allowing a new diagnostic tool for physiological and clinical studies.

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