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Determination of L-glutamate and L-glutamine in pharmaceutical formulations by amperometric L-glutamate oxidase based enzyme sensors

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Abstract: An amperometric biosensor for the direct determination of L-glutamate was developed by chemical bonding of L-glutamate oxidase (GAO) on a carboxylic Nylon membrane with polyazetidine prepolymer (PAP), and using a hydrogen peroxide electrode as indicating sensor. The biosensor is specific for L-glutamate and the peculiar analytical properties (linearity range, reproducibility, accuracy) were experimentally determined. Furthermore, the same basic biosensor was also modified to be used and characterized for the direct determination of L-glutamine. This L-glutamine biosensor was obtained by coimmobilizing, on two separate membranes, glutamic acid oxidase and glutaminase (GMN) on the same biosensor. The two sensors were then used for the determination of glutamate and L-glutamine contained in pharmaceutical formulations and the results were compared with those obtained by other analytical methods.

Keywords: *L-glutamate; L-glutamine; enzyme electrodes; drug analysis.*

Introduction

Many analytical methods for the determination of L-glutamate and L-glutamine in pharmaceutical and nutritional formulations can be found in the literature. Besides the traditional chromatographic [1–7] and enzymatic–spectrometric [8–9] techniques, an increasing number of electrochemical biosensors selective to L-glutamate and L-glutamic acid have been described in recent years [10–30]. A particular class of GA-sensing bioelectrodes is represented by the amperometric L-glutamic acid oxidase (GAO) electrodes [23–30]. These biosensors are based on the coupling of a commercially available H_2O_2-sensing amperometric electrode with a recently purified enzyme, L-glutamic acid oxidase (E.C. 1.4.3.11) [23]. This enzyme catalyses the oxidation reaction of glutamate to α-ketoglutarate and hydrogen peroxide ($K_M = 2 \times 10^{-4}$ M at pH 7.4):

$$\text{L-Glutamate} + O_2 \overset{\text{GAO}}{\rightleftarrows} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2. \quad (1)$$

The concentration of L-glutamate is therefore easily determined since H_2O_2 is a readily detected compound [reaction (2)] by means of the commercial amperometric H_2O_2 electrode:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-. \quad (2)$$

These GAO-based amperometric biosensors represent a marked improvement, in terms of detection limits and of reproducibility of the experimental results, with respect to potentiometric biosensors based on the use of glutamate decarboxylase (GAD) and glutamate dehydrogenase (GDH) [10–22].

The purpose of the present work is to present a GAO biosensor to be used both for the direct determination of L-glutamate in pharmaceutical samples and as the sensing element of a bienzymatic GAO-glutaminase (GMN) biosensor for the direct determination of L-glutamine [25]. In this latter case an additional membrane, containing an adequate amount of immobilized GMN, is fixed on the tip of the glutamic acid biosensor. The concentration of L-glutamine in the sample is propor-

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tional to the amount of L-glutamate formed at the electrode interface according to reaction (3) ($K_M = 4 \times 10^{-4}$ M at pH 5.0):

$$\text{L-glutamine} + H_2O \overset{\text{GMN}}{\rightleftarrows} \text{L-glutamic acid} + NH_3. \quad (3)$$

The results obtained by means of the two biosensors are compared with those obtained by a traditional enzymatic–spectrometric technique [9].

Experimental

Reagents

L-Glutamate oxidase, from *Streptomyces* sp. X-119-6, was supplied by Yamasa Shoyu (Tokyo, Japan). L-Glutamate dehydrogenase (EC 1.4.1.3), ADP, NAD, glutaminase (EC 3.5.1.2), and all the amino acids used in the experiments were supplied from Sigma (St Louis, MO, USA).

The supports used for the immobilization of GAO (Nylon membrane with carboxylic groups on the surface) and GMN (cellulose acetate dialysis membrane) were supplied respectively by Pall Italia s.r.l. (Milano, Italy) (Biodyne Transfer membrane, Nylon 6.6, porosity 0.2 μm) and by Sigma (St Louis, MO, USA) (0.001 in. thick; molecular cut-off = 12000). The polyazetidine prepolymer solution (Hercules Polycup 172, 12% solids in water), used for the physico-chemical immobilization of GAO and GMN on the respective membranes, was obtained from Hercules Inc. (Wilmington, DE, USA).

Preparation of L-glutamate and L-glutamine biosensors

(a) Immobilization of L-glutamate oxidase. The immobilization of the enzyme was carried out by chemical bonding, based on an available Nylon 6.6 membrane with carboxyl groups on the surface and of a prepolymer, polyazetidine. GAO and PAP (1 mg GAO/10 μl PAP) were spread uniformly on a disk of the membrane (0.8 cm diameter; density of enzyme = 0.135 mg cm^{-2}). The enzyme membrane was left for 24 h at 4°C, washed with phosphate buffer (0.1 M, pH 7.0) and stored in the same buffer containing sodium azide (0.01 M) at 4°C.

(b) Immobilization of L-glutaminase. On a dialysis membrane (0.8 cm diameter) 10 μl of the prepolymer solution, as obtained, was mixed with 2 mg of enzyme and spread uniformly. The membrane was then left for 24 h at 4°C, washed with phosphate buffer (0.1 M, pH 7.0) and stored in the same buffer containing sodium azide (0.01 M) at 4°C.

(c) Assembly of the sensors. The glutamate oxidase sensor was assembled by placing on the platinum surface of a commercial H_2O_2 sensing electrode (Universal Sensors Inc., Los Angeles, CA, USA) three different membranes, in the following order: first, a cellulose acetate membrane (to eliminate interferences from electroactive substances, such as, for example, ascorbic acid and uric acid); then, the GAO membrane (prepared as described above), finally a dialysis membrane (to prevent microbial attack of the enzyme and leaking of the enzyme itself from the membrane). A rubber O-ring was used to fix the three layers on the tip of the hydrogen peroxide sensor.

The glutamate oxidase–glutaminase sensor was assembled following the same procedure, but with the difference that the outer dialysis membrane was substituted by the GMN membrane, with the enzyme immobilized on the inner surface of the dialysis membrane.

A schematic representation of the general assembly of the sensors is given in Fig. 1.

Measurements

(a) Samples of pharmaceutical formulations. The reliability of the proposed approach has

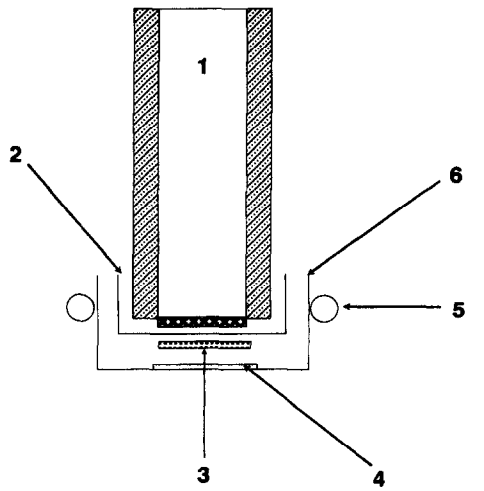

Figure 1
Schematic representation of the GAO and of the GAO–GMN sensors: (1) H_2O_2 electrode; (2) cellulose acetate membrane; (3) GAO membrane; (4) GAO–GMN membrane (only in the GAO–GMN sensor); (5) O-ring; (6) dialysis membrane (only in the GAO sensor).

been evaluated by comparing the results obtained by the enzymatic GAO-based biosensor with those obtained by a traditional spectrometric procedure [9]. The amount of L-glutamate and of L-glutamine has been determined on eight pharmaceutical formulations (hereafter indicated as samples 1–8) having the following nominal compositions:

L-Glutamate containing samples 1–3. Sample 1: (a) L-glutamate = 34.0%; (b) corn starch = 12.3%; (c) magnesium stearate = 3.5%; (d) lactose = 45.0%; (e) cellulose = 2.4%; (f) titanium oxide = 0.3%; and (g) polyethylene glycol = 2.1%. Sample 2: (a) L-glutamate = 17.0%; (b) lactose = 22.2%; (c) talc powder = 15.3%; (d) magnesium stearate = 5.1%; (e) polyvinylpyrrolidone = 22.1%; (f) rice starch = 12.6%; (g) cellulose = 4.5%; (h) arabic gum = 1.2%. Sample 3: (a) L-glutamate = 28.5%; (b) rice starch = 14.2%; (c) magnesium stearate = 5.2%; (d) sucrose = 15.9%; (e) talc powder = 35.6%; (f) titanium oxide = 0.6%.

L-Glutamine containing samples 4–8. Sample 4: (a) L-glutamine = 50.0%; (b) cytidine = 35.4%; (d) rice starch = 13.0%; (e) magnesium stearate = 1.2%; (f) talc powder = 0.4%. Sample 5: (a) L-glutamine = 35%; (b) phosphoserine = 28%; (c) ferritin = 18%; (d) corn starch = 10.0%; (e) magnesium stearate = 4.0%; (f) talc powder = 5.0%. Sample 6: (a) L-glutamine = 42.3%; (b) ferritin = 12.4%; (c) rice starch = 11.1%; (d) lactose = 21.0%; (e) polyvinylpyrrolidone = 8.2%; (f) talc powder = 5.0%; (g) magnesium stearate = 3.8%. Sample 7: (a) L-glutamine = 15.2%; (b) L-phosphothreonine = 6.0%; (c) coenzyme B12 = 78.2%; (d) folinic acid = 0.6%. Sample 8: (a) L-glutamine = 25.4%; (b) L-phosphoserine = 23.1%; (c) L arginine = 26.65%; (d) L-phosphothreonine = 8.5%; (e) hydroxycobalamin = 0.05%; (f) mannitol = 15.3%; (g) polyethylene glycol 6000 = 0.8%; (h) precipitated silica = 0.2%.

(b) Amperometric experiments. Amperometric measurements were carried out by connecting the previously described bioelectrodes to an amperometric detector (ABD, Universal Sensors, Los Angeles, CA, USA). A constant potential of +650 mV was applied between the platinum anode and the Ag/AgCl cathode of the hydrogen peroxide electrode. The electrode jacket was filled with an internal filling solution of KH_2PO_4 and KCl, both 0.1 mol l^{-1}, pH 7.4. Experiments were carried out in 10 ml of phosphate buffer 0.1 mol l^{-1}, in a glass cell, thermostated at 37°C by forced water circulation. Magnetic stirring was used during the operation. The sensor was employed to determine L-glutamate concentration in commercial pharmaceutical preparations, by adding the sample, appropriately diluted if necessary, with the buffer solution.

Samples containing L-glutamine were assayed either directly, by means of the GAO–GMN biosensor, or indirectly, by means of the GAO biosensor, after a hydrolysis pretreatment necessary to convert L-glutamine to glutamate. In this latter case the L-glutamine samples were strongly basified with NaOH, and maintained at 90°C for 2 h, with magnetic stirring.

(c) Spectrometric experiments. The amperometric results were compared with those obtained by using a traditional enzymatic–spectrometric method employing glutamate dehydrogenase (GDH) [9]. Spectrometric measurements to detect NADH, formed by GDH activity in the presence of GA, were carried out with a Perkin–Elmer Lambda 15 UV–vis spectrometer at λ = 340 nm and in 1.0 cm path-length silica cell. Samples containing L-glutamate were diluted in 0.01 mol l^{-1} Tris–acetate buffer pH 8.0, containing NAD 0.4 g l^{-1}, GDH 2 × 10^{-4} g l^{-1} and EDTA 10^{-5} mol l^{-1}.

Samples containing L-glutamine were assayed spectrometrically after hydrolysis pretreatment required to convert glutamine into glutamate by following the same procedure described for amperometric experiments.

Results

The experimental work was carried out in two successive steps: first, GAO and GAO–GMN sensors were characterized for their main physico-chemical features; then, they were applied to the analysis of real samples, checking all the results with those obtained by other available analytical methods.

Table 1 reports the main electroanalytical characteristics of the GAO and of the GAO–GMN sensors, respectively.

The results of the study of the electrodes life times of operation are shown in Fig. 2.

Table 1
Analytical characterization of the GAO based biosensors in glutamate and glutamine standard solution

	GAO biosensor	GAO–GMN biosensor
Temperature analysis (°C)	37	37
pH	7.5	7.0
Buffer	Tris–HCl 0.1 mol l^{-1}	Phosphate 0.1 mol l^{-1}
Response time (min)	≤2	≤2
Equation of the calibration graph*	$Y = 10.7 + 0.52\ X$ (Y = nA; X = L-glutamate conc. in μmol l^{-1})	$Y = 8.3 + 0.35\ X$ (Y = nA; X = L-glutamine conc. in μmol l^{-1})
Linearity range	2.5×10^{-6}–1.4×10^{-3} mol l^{-1}	2.5×10^{-5}–1.0×10^{-3} mol l^{-1}
Correlation coefficient†	0.9996	0.9987
Lower detection limit (mol l^{-1})	1.0×10^{-6}	1.0×10^{-5}

*Calculated by the method of least squares.

†Calculated as: $r = \dfrac{\sum_{i=1}^{n} X_i Y_i - n\bar{X}\bar{Y}}{\sqrt{\left(\sum_{i=1}^{n} X_i^2 - n\bar{X}^2\right)\left(\sum_{i=1}^{n} Y_i^2 - n\bar{Y}^2\right)}}$.

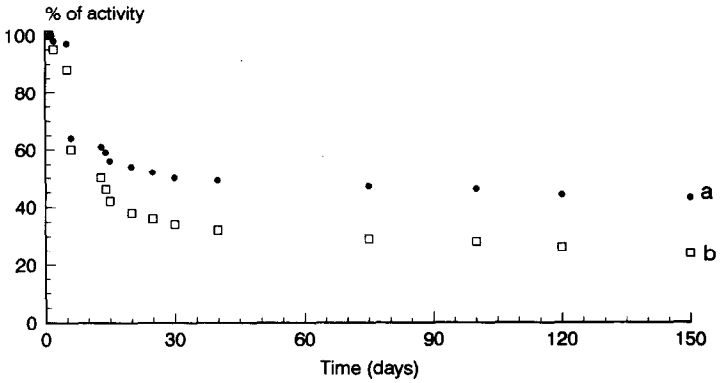

Figure 2
Life times of operation of the GAO sensor (a) and GAO–GMN sensor (b) in phosphate buffer, at 37°C. Data are expressed as a percentage of original electrode activity.

Table 2 shows the selectivity of the GAO sensor with respect to some different amino acids.

Tables 3 and 4 show the repeatability of the measurements, evaluated in standard solutions, for three different GAO sensors and three different GAO–GMN sensors, respectively.

Tables 5 and 6 show the results obtained by means of the GAO sensor in the analysis of pharmaceutical preparations (tablets) containing L-glutamate or L-glutamine (assayed as glutamate after alkaline hydrolysis). These

Table 2
Selectivity of the GAO sensor (analytes concentration 0.1 mM), evaluated at 37°C and pH 7.5 (Tris 0.1 M HCl)

Analyte	Response of the sensor (%)
Monosodium L-glutamate	100
L-glutamine	0*
D-glutamic acid	0
L-tyrosine	0
L-lysine	0
L-asparagine	0

*0% of response refers to a recorded electrical signal <1 nA, where the background signal recorded in the buffer solution is 0.5 nA.

Table 3
Analytical characterization of three different GAO biosensors, in L-glutamate standard solutions, at 37°C and pH 7.5 (Tris 0.1 M HCl). Values (in μmol l^{-1}) are the mean of four determinations. Biosensors indicated as 1, 2 and 3 were prepared and stored in the same way

Standard	Nominal value (*a*)	Biosensor 1 (*b*)	Biosensor 2 (*c*)	Biosensor 3 (*d*)	$\frac{b-a}{a}$ (%)	$\frac{c-a}{a}$ (%)	$\frac{d-a}{a}$ (%)
1	5.0	4.9 (RSD% = 0.8)*	5.1 (RSD% = 0.6)	5.0 (RSD% = 0.7)	−2.0	2.0	0.0
2	25.0	24.8 (RSD% = 1.0)	25.1 (RSD% = 0.9)	24.9 (RSD% = 1.0)	−0.8	0.4	−0.4
3	50.0	49.8 (RSD% = 1.2)	50.2 (RSD% = 1.1)	50.3 (RSD% = 1.0)	−0.4	0.2	0.6

*RSD = relative standard deviation.

Table 4
Analytical characterization of three different GAO–GMN biosensors, in L-glutamine standard solutions, at 37°C and pH 7.5 (Tris 0.1 M HCl). Values (in μmol l^{-1}) are the mean of four determinations. Biosensors indicated as 1, 2 and 3 were prepared and stored in the same way

Standard	Nominal value (*a*)	Biosensor 1 (*b*)	Biosensor 2 (*c*)	Biosensor 3 (*d*)	$\frac{b-a}{a}$ (%)	$\frac{c-a}{a}$ (%)	$\frac{d-a}{a}$ (%)
1	30.0	29.3 (RSD% = 1.1)	30.5 (RSD% = 0.9)	29.6 (RSD% = 1.0)	−2.3	+1.6	−1.3
2	50.0	50.6 (RSD% = 1.5)	50.7 (RSD% = 1.2)	49.7 (RSD% = 1.1)	+1.2	+1.4	−0.6
3	80.0	79.2 (RSD% = 1.4)	80.3 (RSD% = 1.6)	81.0 (RSD% = 1.4)	−1.0	+0.4	+1.2

Table 5
L-glutamate determination in pharmaceutical formulations. Comparison of results obtained by the GAO sensor and by the spectrometric method. Values, as % by weight, are the mean of five determinations

Sample	Nominal value (*a*)	Amperometric method (*b*)	Spectrometric method (*c*)	$\frac{b-a}{a}$ (%)	$\frac{c-a}{a}$ (%)	$\frac{c-b}{b}$ (%)
1	34.0	34.6 (RSD% = 1.1)	33.5 (RSD% = 1.4)	1.8	−1.5	−3.2
2	17.0	17.1 (RSD% = 1.3)	16.8 (RSD% = 1.0)	0.6	−1.2	−1.7
3	28.5	28.5 (RSD% = 0.9)	28.3 (RSD% = 1.2)	0	−0.7	−0.7

Table 6
Determination of L-glutamine in pharmaceutical formulations; L-glutamine has been assayed as L-glutamate after a hydrolysis pretreatment. Results obtained by the GAO sensor are compared with those obtained by the spectrometric method. Values, as % by weight, are the mean of five determinations

Sample	Nominal value (*a*)	Amperometric method (*b*)	Spectrometric method (*c*)	$\frac{b-a}{a}$ (%)	$\frac{c-a}{a}$ (%)	$\frac{c-b}{b}$ (%)
4	50.0	50.5 (RSD% = 1.6)	49.2 (RSD% = 1.2)	1.0	−1.6	−2.6
5	35.0	35.7 (RSD% = 1.4)	34.5 (RSD% = 1.6)	2.0	−1.4	−3.4
6	42.3	43.0 (RSD% = 2.0)	41.8 (RSD% = 1.1)	1.6	−1.2	−2.8
7	15.2	15.4 (RSD% = 1.8)	14.9 (RSD% = 2.1)	1.3	−2.0	−3.2
8	25.4	25.8 (RSD% = 2.1)	24.9 (RSD% = 1.7)	1.6	−2.0	−3.5

Table 7
Recovery of L-glutamate by the standard addition method, by the GAO sensor in some pharmaceutical preparations, containing L-glutamate. Values, as % by weight, are the mean of five determinations

Sample	L-glutamate found in the sample	L-glutamate added to the sample	Total (nominal value) (*a*)	Total (found value) (*b*)	$\frac{b-a}{a}$ (%)
1	17.0	10.0	27.0	27.3 (RSD% = 0.9)	1.1
2	50.0	10.0	60.0	60.5 (RSD% = 1.2)	0.8
3	25.4	10.0	35.4	36.0 (RSD% = 1.0)	1.7

Table 8
Determination of L-glutamine in pharmaceutical formulations. Results obtained by the GAO sensor, after sample pretreatment, are compared with those obtained by the GAO–GMN sensor. Values, as % by weight, are the mean of five determinations

Sample	Nominal value (*a*)	GAO sensor (*b*)	GAO–GMN sensor (*c*)	$\frac{b-a}{a}$ (%)	$\frac{c-a}{a}$ (%)	$\frac{c-b}{b}$ (%)
4	50.0	50.5 (RSD% = 1.6)	49.4 (RSD% = 2.5)	1.0	−1.2	−2.2
5	35.0	35.7 (RSD% = 1.4)	34.4 (RSD% = 2.1)	2.0	−1.7	−3.6
6	42.3	43.0 (RSD% = 2.0)	41.7 (RSD% = 2.3)	1.6	−1.4	−3.0
7	15.2	15.4 (RSD% = 1.8)	14.9 (RSD% = 2.2)	1.3	−1.9	−3.2
8	25.4	25.8 (RSD% = 2.1)	25.1 (RSD% = 1.9)	1.6	−1.2	−2.7

data were also compared with those obtained by means of the spectrometric method.

Table 7 reports the accuracy, as recovery data, respective to the GAO-sensor.

The results listed in Table 8 refer to the direct analysis of pharmaceutical formulations containing L-glutamine, and are compared with the ones obtained by means of the GAO biosensor after the alkaline hydrolysis conversion of L-glutamine to L-glutamate.

Discussion

The experimental data prove the reliability of the results obtained by means of the two biosensors here proposed, when applied for the direct determination of L-glutamate and L-glutamine concentrations in pharmaceutical formulations.

It is evident that the overall analytical performance of the GAO biosensor is better than that of the GAO–GMN biosensor, mainly in terms of sensitivity, range of linearity, correlation coefficient in the linearity range, and life time of operation. Indeed, when a bienzymatic biosensor is set up, a two-step reaction must take place at the electrode interface in order to produce the recordable signal. In our case, the response of the GAO–GMN biosensor in the presence of L-glutamine is the result of the hydrolysis of L-glutamine to L-glutamate, operated by GMN, and of the following oxidation of L-glutamate to α-ketoglutarate and H_2O_2, operated by GAO. Since the yield of two combined reactions is generally smaller than the yield of a single step reaction, the slope of the calibration graph respective to the GAO–GMN biosensor (i.e. the electrode sensitivity) is lower than that of the GAO biosensor (0.35 nA/μmol l^{-1} vs 0.52 nA/μmol l^{-1}). Moreover, the best matching of the operating conditions of the two enzymes takes place in a reduced range of concentrations, and it explains the narrower range of linearity (about two decades vs almost three decades of concentration), the lower correlation coefficient (0.9987 vs 0.9996) and the higher detection limit (10 μmol l^{-1} vs 1 μmol l^{-1}) of the GAO–GMN electrode with respect to the GAO electrode.

It should be noted that the use of an inner H_2O_2 electrode as the sensing element of the two biosensors presented in this work can markedly reduce, in our experimental conditions, the lack of reliability that could be encountered by employing an oxygen selective Clark electrode. Indeed, the possible presence of variable levels of dissolved oxygen in the samples could be the cause of several interferences. Also the use of ammonium or ammonia selective potentiometric electrodes, even if theoretically possible, should be avoided: potentiometric gas-sensing or ion-selective electrodes, in fact, present a lower analytical performance with respect to the more sensitive and rapid amperometric sensors.

The membrane used as the support for the immobilization of GAO plays a crucial role on the overall analytical performance of the L-glutamate sensitive biosensor, including stability features. At this purpose, a comparative study on the influence of the biocatalytic membrane on the analytical performance of L-glutamate bioelectrodes has been carried out in our laboratory [31]. The carboxylic membrane and the prepolymer of polyazetidine used in the present work ensure very good results when a combination among sensitivity, reproducibility and long-term stability after an extensive use on real samples is required.

To build up the GAO–GMN sensor, the additional GMN membrane was prepared by immobilizing GMN on a dialysis membrane: a dialysis membrane was selected to minimize the duration of analytical measurements. It is indeed well known that the thickness of the layer between the electrochemical sensor and the sample solution is directly proportional to the response time of the biosensor, so that thinner layers are always associated to shorter response times.

Both the L-glutamate and the L-glutamine biosensors presented remarkable features in terms of lifetime (up to 350 assays performed with a single GAO membrane, with a sharp reduction of membrane activity after 6 days from immobilization and constant activity for the following 14 weeks), ease of use and cost of operation.

Several standard solutions of the two different analytes, i.e. glutamic acid and glutamine, have been considered to have information on the performance of the two above mentioned biosensors.

The reproducibility of the obtained data was expressed as relative standard deviation (RSD); the one for GAO biosensor was 1.3%, while the one for GAO–GMN biosensor was 1.5%.

The accuracy for the GAO biosensor was 2.0% when determined by direct method and 1.0% when determined by standard addition method; while the accuracy for the GAO–GMN was −2.0% when determined by direct method and 2.7% when determined by standard addition method.

These characteristics have been revealed both on standard solutions and real pharmaceutical samples. Since calibration of the GAO sensor is very simple and its use extremely easy, the sensor could find practical application in all those analytical needs where a combination of fast response times and good accuracy is required. The same consideration can be extended also to the GMN sensor; in this latter case, there is in addition a marked improvement in the ease of operation, the sensor allowing the direct quantitative determination of substrate with no need of sample pretreatment.

As previously mentioned, to eliminate possible interferences from electroactive substances, such as ascorbic acid and uric acid, the cellulose acetate membrane (MW cut off 100–150 D) in contact with the internal solution, was used just to minimize such possible interferences, even if no electrooxidizable chemical species was present in the considered pharmaceutical formulations.

Research carried by our group have revealed the suitability of the proposed sensor also for the determination of the catalytic activity of L-glutamic acid decarboxylase [32] and for the study of other biometabolic processes involving L-glutamate or L-glutamine [33].

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