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Analysis of L-dopa in pharmaceutical preparations and of total phenols content in urine by means of an enzyme–amperometric sensor*

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Abstract: An enzyme–amperometric method is proposed for the analysis of total phenols and L-dopa; the method is based on the enzyme tyrosinase, which is immobilized in a Nylon membrane and coupled to an oxygen gas-diffusion amperometric electrode. The method was applied to the determination of total phenols in urine and to L-dopa in formulations and was evaluated as a promising alternative to currently adopted methods, e.g. to a classical spectrophotometric technique, chosen as a reference method.

Keywords: *Enzyme sensor; tyrosinase; L-dopa; phenols; analysis.*

Introduction

It is well known that L-dopa (levodopa) is the most important drug contained in commercial pharmaceutical preparations employed in the treatment of Parkinson's disease [1]. The therapeutic activity of L-dopa in Parkinsonism derives from its conversion to dopamine in the brain, so that it is able to alleviate a good number of typical symptoms shown by patients affected by this chronic neurological disease [2].

The determination of urinary total phenolic compounds that are tyrosine analogues or metabolites is frequently required in clinical chemistry, particularly in cases of hyperthyroidism, diabetes mellitus, nephrosis, obesity, hypertension, or catecholamine-producing tumours, because this determination appears useful as a screening test for these disorders [3]; overall measurement of urinary catecholamine metabolites seems to be a good diagnostic aid in patients with suspected phaeochromocytoma and neuroblastoma [4].

For many years the authors' research group has been investigating the assembly, characterization and application of electrochemical biosensors both in standard solutions and in real matrices [5], in particular those of pharmaceutical and bioclinical interest [6]. Recently a useful enzyme sensor has been developed for phenol and phenol derivative analysis both in standard aqueous solutions and in authentic different matrices [7], based on a Clark oxygen gas-diffusion electrode coupled with the tyrosinase enzyme, chemically immobilized on Nylon functionalized membranes.

Because this sensor seems to be able to determine compounds containing phenol, as in the case of L-dopa, or the total phenolic compounds contained in real samples [7], tests were performed to find an enzymatic amperometric method which could be used to solve the above analytical problems. Data obtained in the analysis of commercial formulations containing L-dopa and results for urinary total phenolic compounds are presented and compared with results obtained by the classical spectrophotometric method [3].

Experimental

Reagents

Tyrosinase (EC 1.14.18.1, obtained from mushrooms 2400 U mg^{-1}), the dialysis membrane (code D-9777), 1-ethyl-3(3-dimethylaminopropyl)carbodi-imide and 3(3,4-dihydroxyphenyl)-L-alanine (i.e. L-dopa), were supplied by Sigma (St Louis, MO, USA); phenol and phenolic derivatives were supplied

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by Fluka AG (Buchs, Switzerland); all other reagents were of analytical grade and were obtained from Carlo Erba (Milano, Italy). The Pall Biodyne functionalized membranes (Nylon 6.6, porosity 0.45 mm) with carboxylic groups on the surface, were supplied by Pall Biodyne s.r.l. (Milano, Italy). The kit for the spectrophotometric measurements (code 7076) was supplied by Poli Industria Chimica S.p.A. (Milano, Italy).

Apparatus

An Orion oxygen gas-diffusion electrode (model 97-08), an Orion Ionalyzer (model 901), a Julabo thermostat (model 20B) and an Amel recorder (model 868) were used for the enzymatic–amperometric measurements.

Spectrophotometric measurements were carried out using a Lambda 15 Perkin–Elmer spectrophotometer and 1-cm pathlength quartz cells.

Samples

Three different commercial pharmaceutical preparations containing L-dopa were analysed; two were marketed as tablets and one as capsules. The contents of five capsules, or five tablets, were powdered with caution and homogenized in a mortar. An aliquot of the powdered drug was dissolved in distilled water to obtain 250 ml of a 10^{-2} mM solution.

Two urine samples obtained from a healthy subject at different times of the day were also directly analysed.

Enzyme chemical immobilization in Nylon membranes

Tyrosinase was immobilized in a Nylon Pall Biodyne membrane, with carboxylic groups on the surface, using classical methods [8]. For this purpose 1 mg of lyophilized tyrosinase was stratified on the Nylon membrane (0.6-cm diameter) pretreated as follows. A membrane disk was soaked, with stirring, in 0.5 M phosphate buffer (pH 4.8); a weighed amount of 1-ethyl-3(3-dimethylamino-propyl)carbodi-imide was gradually added to the buffer solution in order to obtain a final concentration of 0.1 M. Then the solution containing the Nylon membrane was left, with stirring, at room temperature, for 40 min. The membrane was then washed with a phosphate buffer solution (pH 7.0) and the enzyme stratified on the membrane, as described above. Then this membrane was inserted between the gas-permeable membrane of the electrode and a dialysis membrane. All three membranes were then fixed on the head of the Clark electrode by means of a rubber O-ring (Fig. 1).

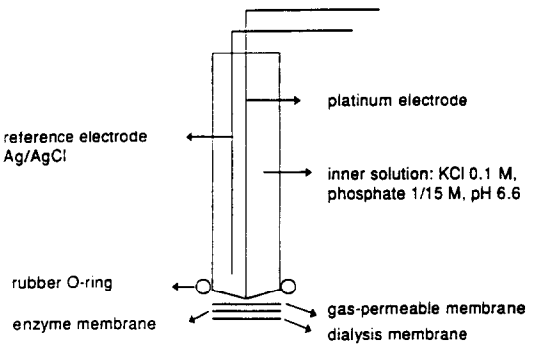

Figure 1
Tyrosinase enzyme electrode assembly.

Measurements of the immobilized enzyme specific activity

The immobilized enzyme specific activity of tyrosinase covalently bonded in Nylon membranes was measured using the same method as reported in a previous paper [9]; the enzymic membrane was accurately washed with buffer solution and then soaked in a thermostatted (25°C) cell containing 15 ml of Tris buffer (pH 8.5) and with an oxygen sensor dipping into it, allowing the sensor signal to reach a steady state. A suitable amount of substrate was then added; the rate of the enzyme reaction was determined by recording the oxygen decrease rate related to the enzyme activity; the activity value obtained for the Nylon membranes was found to be 22.5 nmol $(\text{min} \times \text{cm}^2)^{-1}$.

Method

The analytical method is based on the following enzyme reaction:

$$\text{phenol} + O_2 \text{ ------ tyrosinase ---> } o\text{-quinone} + H_2O.$$

The Clark oxygen electrode, coupled to the tyrosinase enzyme, immobilized as described above, was dipped into the Tris buffer solution (pH 8.5), with stirring, in a thermostatted cell, its signal being allowed to stabilize until a steady state was reached (about 15 min in the best conditions); then fixed volumes of the standard solutions of the substrate (phenol or L-dopa) were successively added to the buffer solution and, after each addition, the decrease of the current, related to the oxygen con-

sumption, was recorded until a new steady state was practically reached (generally about 2 min in the working conditions used). A calibration graph was obtained by plotting the recorded current decrease (as Δppm O_2) versus the substrate concentration values.

Analysis of real samples can be performed in the same way by adding a known volume of the sample, instead of the standard solution, to the buffer solution and recording the oxygen decrease. The unknown concentration of the tested analyte in the sample can be obtained, by the direct method, by means of the calibration curve for L-dopa in drug analyses and for phenol in urine analyses; for example, a suitably small volume of the solubilized drug or urine sample was added directly, with stirring to the Tris buffer solution thermostatted in the glass cell and the signal variation of the amperometric biosensor dipping into the buffer was recorded.

Spectrophotometric method

Spectrophotometric measurements were performed using the 4-aminoantipyrine-ferricyanide method [3]; the absorbance of the sample was measured after extraction in an isobutanol–dichloromethane mixture at λ = 460 nm in a 1-cm pathlength quartz cell against a reagent blank.

Results

The experimental working conditions, optimized as described in a previous paper [7], and the electroanalytical characterization of the enzyme sensor for standard phenol aqueous solutions are summarized in Table 1.

Table 2 shows the linearity range and the reproducibility of the calibration graph for standard L-dopa aqueous solutions, obtained using the enzyme sensor under the same experimental conditions as set out in Table 1.

The results of the L-dopa analysis in formulations and precision data, using the enzyme sensor, are shown in Table 3 and compared with the nominal values declared by the manufacturers of the analysed commercial drugs. Results obtained in the analysis of pharmaceutical preparations containing L-dopa in respect of the accuracy of measurements (such as recovery by the standard addition method) found using the enzyme sensor, are reported in Table 4.

Table 1
Experimental conditions and enzyme sensor characterization in standard solutions in phenol

indicating electrode	oxygen amperometric sensor
immobilized enzyme	tyrosinase
immobilization method	in Nylon membrane
buffer	Tris 0.1 mM
pH	8.5
response time	5 min
% response after 2 min	73–87%
lifetime	>60 days
activity loss after 60 days	about 20%
working temperature	25°C
regression line	$y = 3.0 \times 10^{-2} x - 0.07$ (x = μM; y = Δppm O_2)
correlation coefficient	0.9992
linearity range	5.0–190 μM
minimum detection limit	1.0 μm
precision on standard solutions (RSD)	8.0%
inaccuracy on standard solutions by direct method	−2.4–7.8%

Table 2
Slope, intercept, correlation coefficient and reproducibility of four calibration graphs, by enzyme sensor, in an aqueous solution of L-dopa

Calibration no.	Linearity range (μM)	Slope (Δppm O_2/μM)	Intercept (Δppm O_2)	Correlation coefficient (r)
1	10–850	0.011	−0.079	0.9999
2	10–850	0.011	−0.072	0.9999
3	10–850	0.012	−0.074	0.9998
4	10–850	0.011	−0.068	0.9997
Mean	10–850	0.011 RSD = 3.8%	−0.073 RSD = 5.4%	0.9998

Table 3
Repeatability of L-dopa determination, in commercial preparations, using the enzyme sensor and comparison of results with nominal values

Drug no. and its pharmaceutical form	Nominal value (as % by weight) (*a*)	Value found (as % by weight) by enzyme sensor	Mean value (*b*)	RSD%	$\frac{b-a}{a}$ %
1 (tablets)	65.8	62.4 67.0 67.3	65.6	3.4	−0.3
2 (tablets)	66.7	63.4 65.2 67.3	65.3	2.4	−2.1
3 (capsules)	66.7	67.4 67.7 69.3	68.1	1.2	+2.1

Table 4
Recovery of L-dopa, in commercial preparations, using the enzyme sensor. Reported values are the final concentrations, after appropriate dilution, so that the concentration falls within the linearity range

Drug no. and its pharmaceutical form	L-dopa found in the sample by enzyme sensor (mg l^{-1})	L-dopa added (mg l^{-1})	Total value (found + added) by enzyme sensor (mg l^{-1})	Recovery (%)
1 (tablets)	39.8	26.3	65.6	99.2
	39.8	39.4	80.0	101.0
	39.8	47.3	86.2	99.0
2 (tablets)	39.2	26.3	65.0	99.2
	39.2	39.4	79.4	101.0
	39.2	47.3	85.5	98.8
3 (capsules)	32.7	26.3	58.2	98.6
	32.7	39.4	72.3	100.3
	32.7	47.3	79.6	99.5

Table 5
Comparison of precision and accuracy data for L-dopa analysis in commercial preparations, using the enzyme sensor or spectrophotometric method, and correlation of values found by both the two analytical methods

	Enzyme sensor	Spectrophotometric method
Precision (as RSD)	1.2–3.4%	2.8–3.1%
Inaccuracy (as recovery) by standard addition method	−1.4–+1.0%	−0.9–+2.7%

Comparison of values found by both analytical methods. Each value is the mean of three determinations

Drug no. and its pharmaceutical form	Value found by enzyme sensor (as % by weight) (*a*)	Value found by spectrophotometric method (as % by weight) (*b*)	$\frac{b-a}{a}$ %
1 (tablets)	65.5 (RSD = 3.4%)	61.8 (RSD = 3.0%)	−5.6
2 (tablets)	65.3 (RSD = 2.4%)	65.1 (RSD = 2.8%)	−0.3
3 (capsules)	68.2 (RSD = 1.2%)	70.0 (RSD = 3.1%)	+2.6

In Table 5 the main results of the formulation analysis, precision and accuracy data using the enzyme sensor, are compared with those obtained by the spectrophotometric method.

Tables 6 and 7 show, respectively, the results obtained in the analysis of total phenols in two different urine samples by both analytical methods.

A comparison of data obtained using the two methods and the repeatability of measurements are shown in more detail in Table 6. The accuracy by the standard addition method, using the enzyme sensor and the spectrophotometric methods, is reported in Table 7.

Finally, the relative activity of the sensor for different phenols and L-dopa and for other common substances, tested for their possible

Table 6
Comparison of repeatability of determination of total phenols in human urine samples, using the enzyme sensor and the spectrophotometric method

Sample no.	Found values by enzyme sensor (mg l^{-1})	Mean (mg l^{-1})	RSD%
1	68.5 67.9 60.8	65.7	5.3
2	41.6 44.8 38.8	41.7	5.9
Sample no.	Found values by spectrophotometric method (mg l^{-1})	Mean	RSD%
1	65.0 65.3 64.0	64.8	0.9
2	40.5 38.9 39.7	39.7	1.6

Comparison of results for determination of total phenols in human urine samples by both analytical methods. Each value is the mean of three determinations

Sample no.	Value by enzyme sensor (mg l^{-1}) (a)	Value by spectrophotometric method (mg l^{-1}) (b)	$\frac{a-b}{a}$ %
1	65.7	64.8	+1.4
2	41.7	39.7	+4.8

Table 7
Recovery of phenol in human urine samples, using the enzyme sensor and by the spectrophotometric method

Sample no.	Value found by the enzyme sensor (mg l^{-1})	Phenol added (mg l^{-1})	Total value (found + added) by enzyme sensor (mg l^{-1})	Recovery (%)
1	65.7	50.0 100.0	116.9 162.5	103.7 101.9
2	41.7	50.0 100.0	87.3 133.7	95.2 94.4
Sample no.	Value found by spectrophotometric method (mg l^{-1})	Phenol added (mg l^{-1})	Total value (found + added) by spectrophotometric method (mg l^{-1})	Recovery (%)
1	64.8	25.0 69.8	95.2 141.9	106.0 105.4
2	39.7	50.0 100.0	86.5 113.9	96.4 81.5

interference in the L-dopa or phenol analysis or their capability of changing the response of the sensor are listed in Table 8.

Discussion

It is not necessary to repeat here data already published [7] on the working conditions, the engineering of the sensor and the immobilization method used for the enzyme. Results obtained in phenol standard solutions are summarized in Table 1. The sensitivity of the enzyme probe (as the slope of the calibration graph) to L-dopa is about 60% with respect to the sensitivity to phenol, but the linearity range is wider and the reproducibility is good as can be seen from Table 2. The results contained in Tables 3–5, in which data

Table 8
Comparison of linearity range and relative activity (as % slope of the calibration curve, referred to the phenol slope, assumed to be 100%) for different phenols and for other common substances tested for their possible interference in the analysis using the tyrosinase enzyme sensor*

Substrate	Linearity range (mM)	Relative activity (as %)
phenol	5.0–190	100.0
o-cresol	25.0–580	16.2
m-cresol	5.0–680	67.8
p-cresol	5.0–580	90.3
hydroquinone	1.0–250	225.8
resorcinol	700–2.0×10^4	6.6
catechol	5.0–350	96.6
L-dopa	10.0–850	58.1
ascorbic acid	30.0–130	11.3
sulphide	30.0–150	3.3
sulphite	30.0–1.3×10^2	0.6

*For Cl^-, Br^-, I^-, F^-, NO_3^-, NO_2^-, SO_4^{2-}, CO_3^{2-}, PO_4^{3-}, $B_4O_7^{2-}$, acetate, citrate, benzoate, oxalate, relative activity = 0, but benzoate strongly lowers the response of the sensor to phenol, while SO_4^{2-} and I^- slightly enhance the response.

obtained from the analysis of three different commercial pharmaceutical forms containing L-dopa are presented, show that the analysis of pharmaceutical preparations, using the enzyme sensor, gives satisfactory results in respect of both reproducibility and accuracy but, above all, without any noteworthy pretreatment of the samples; also the correlation with the nominal values is good and the agreement with the spectrophotometric results is satisfactory. The high specificity of the enzyme sensor in the analysis of L-dopa in formulations, contribute to making this analytical method one of great practical interest; the number of substances that may interfere with the method is small, except for other possible phenol derivatives (see Table 8), so there is a possibility of directly analysing these authentic matrices, without pretreatment, even in turbid or coloured solutions.

The biosensor has also been employed in the determination of the total phenols content of urine samples; so far only two samples have been analysed from healthy people, taken at different times of the day. From an analytical point of view, the results are undoubtedly valid. Table 3 shows a good repeatability of measurements and the accuracy data (using the standard addition method) proved to be comparable or better than those obtained by the spectrophotometric method (Table 4).

Measurements of urine samples were performed on an empirical basis, as the sensor shows different affinities for different phenol derivatives (see Table 8). Moreover, the exact composition of the analysed samples as phenolic compounds was not known so the total phenols content found by the enzyme sensor was given as the phenol concentration in mg l^{-1}; however, it is encouraging to note that for both analysed samples, the results obtained using two different analytical methods proved to be in fairly good agreement. On the other hand, the spectrophotometric method, chosen as a reference method, is currently used in clinical chemistry to determine total phenols in urine samples [3] and, for this purpose, the concentration data must be given as mg l^{-1}, i.e. the same concentration units used in the proposed enzymatic–amperometric method.

It is evident that the correlation between the two methods must be further verified; for this purpose, a greater number of urine samples must be simultaneously analysed and, above all, samples both from healthy and sick people have to be examined.

Observation of the concentration values for the total phenols content of the two urine samples (Table 6) shows that the concentration of the second sample falls within the normal range of healthy subjects [3], while that of the first sample is a little higher. This difference is probably due to the particular time of day at which the sampling was performed (first urine in the morning), as the donor subject was perfectly healthy.

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