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Enzymatic oxidation of *tert*-butylcatechol in the presence of sulfhydryl compounds: Application to the amperometric detection of penicillamine

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Abstract

The high sensitivity that can be attained using an enzymatic system and mediated by 4-*tert*-butylcatechol (4-TBC) has been verified by on-line interfacing of a rotating biosensor and continuous flow/stopped-flow/continuous-flow processing. Horseradish peroxidase, HRP, [EC 1.11.1.7], immobilized on a rotating disk, in presence of hydrogen peroxide catalyzed the oxidation of 4-TBC, whose back electrochemical reduction was detected on glassy carbon electrode surface at −150 mV. Thus, when penicillamine (PA) was added to the solution, these thiol-containing compounds participate in Michael type addition reactions with 4-TBC to form the corresponding thioquinone derivatives, decreasing the peak current obtained proportionally to the increase of its concentration. The highest response for PA was obtained around pH 7. This method could be used to determine PA concentration in the range 0.02–80 μM ($r = 0.998$). The determination of PA was possible with a limit of detection of 7 nM, in the processing of as many as 50 samples per hour. The HRP-rotating biosensor was successfully applied to the determination of PA in pharmaceutical formulations.

Keywords: Penicillamine; Glassy carbon; Biosensor; Horseradish peroxidase; 4-*tert*-Butylcatechol

1. Introduction

Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) (PA) is an unphysiological sulfur-containing amino acid that belongs to the aminothiols family. This compound is derived from hydrolytic degradation of penicillin [1,2] but it does not have antibiotic activity. It exists in D- and L-enantiomeric forms that show different biological and toxicological properties. D-PA (*S*-isomer) is a highly potent therapeutic agent used for many years in the treatment of various illnesses. It is the drug of first choice for patients with Wilson's disease [3], an autosomal recessive disorder of copper transport [4]. It is able to enhance the urinary excretion of others heavy metals such as lead, arsenic, mercury and zinc and therefore, is used as an oral chelating agent to treat conventional heavy metals intoxication [5]. It is also used as antifibrotic agent to treat scleroderma [6] and as antirheumatic drug to treat patients with active rheumatoid arthritis [7]. D-PA reduces excess of cystine excretion in cystinuria, another rare inherited disease affecting the active transport of the diamino acids cystine, ornithine, lysine and arginine across the renal tubule and the small intestine [8]. Mechanisms of action of D-PA in mentioned diseases are various. It is a reductive chelating agent [9], it appears to inhibit collagen deposition by interfering with the intramolecular cross-linking of mature collagen [10], it has immunomodulatory-anti-inflammatory properties [11–13] and it is able to make dimers through disulfide interchange or direct reaction with other physiological thiols [14]. For example, in cystinuria, D-PA-cysteine disulfide formation, which is a dimer more soluble than cystine, prevents the clinical manifestation of the pathology [14]. This suggests that it may also represent a potential drug for the hyperhomocyst(e)inemia and hypercyst(e)inemia treatment, two independent risk factors for atherosclerosis and cardiovascular disease [15,16].

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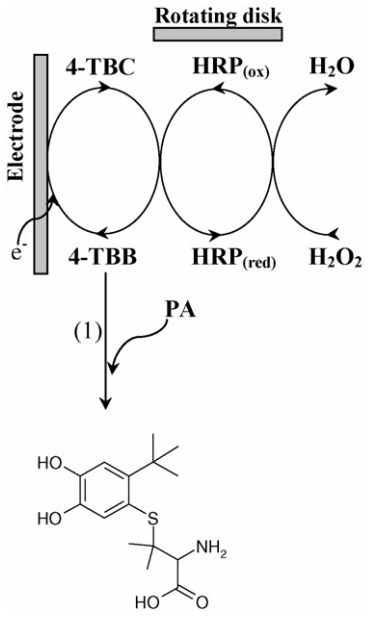
Several methods have been proposed for the determination of D-penicillamine including high performance liquid chromatography with pre or post column derivatization [17–19], calorimetry [20], fluorometry [21,22], spectrophotometric [23–25], chemiluminescene [26], capillary electrophoresis [27,28] and NMR spectrometry [29]. One of the important limitations of LC techniques is the fact that this thiol lacks sufficient UV absorption so a pre or post-column derivatization procedure is normally required [26]. Electrochemical methods are an alternative for the D-penicillamine determination because they are cheap, simple, fast and sensitive. Mercury and mercury amalgam [30,31] have been extensively used for thiol-compound determination, however, mercury has limitations due to its toxicity and the rapid deterioration of the electrode response [32]. A chemically modified electrode [33] has been also used to determine D-penicillamine but its use in flow injection analysis is limited because it needs particular mechanical and chemical stability towards the flowing solution. Recently, the boron-doped diamond thin film (BDD) electrode has emerged as a unique electrode material for several electrochemical applications, especially in electroanalysis [34].

To the best of our knowledge, no study with enzymatic biosensor behavior for PA has been reported. Thus, in this paper, we present and discuss for the first time the electrochemical and enzymatic reaction for PA determination, resulting in a single, fast and inexpensive analytical method as well as very sensitive devise based on HRP-rotating biosensor systems. The measuring principle of this biosensor for the detection of PA is shown in Scheme 1. Horseradish peroxidase (HRP) in the presence of H_2O_2 catalyses the oxidation of 4-*tert*-butylcatechol (4-TBC) [35] whose electrochemical reduction back was obtained at peak potential of −150 mV. Nevertheless, when thiol-containing compound is added to the solution, readily undergo reaction with quinone derivative (4-*tert*-butylbenzoquinone, 4-TBB), through the Michael type addition, decreasing the peak current obtained proportionally to the increase of thiol-containing compound concentration.



Scheme 1.

The initial reaction in the sequence (4-TBC⇆4-TBB) is well established [36–38] with NMR, pulse radiolysis and a number of electrochemical techniques used to probe the mechanism. A substantial body of research has also been compiled which documents the possible physiological consequences of such reactions [39–41]. The potential analytical utility offered by the second step (1) as a method of detecting PA is explored in this paper.

2. Experimental

2.1. Reagents and solutions

All reagents used, were of analytical reagent grade. The enzyme horseradish peroxidase, HRP, [EC 1.11.1.7] Grade II, were purchased from Sigma Chemical Co., St. Louis. The concentration of HRP was determined spectrophotometrically using the Soret extinction coefficient of 102 $mM^{-1}\,cm^{-1}$ at 403 nm (181 IU mg^{-1}). Glutaraldehyde (25% aqueous solution) and hydrogen peroxide were purchased from Merck, Darmstadt. 3-Aminopropyl-modified controlled-pore glass, 1400 Å mean pore diameter and 24 $m^2\,mg^{-1}$ surface area, was from Electro-Nucleonics (Fairfield, NJ) and contained 48.2 $\mu mol\,g^{-1}$ of amino groups. 4-*tert*-butylcatechol and penicillamine were purchased from Sigma Chemical Co., St. Louis. 4-*tert*-butylcatechol (0.1 M) and penicillamine (0.01 M) were prepared through the dissolution of the appropriate salt in deionized water and generally used within 1 h. All other reagents employed were of analytical grade and used without further purifications. All solutions were prepared with ultra-high-quality water obtained from a Barnstead Easy pure RF compact ultra pure water system, and the samples were diluted to the desired concentrations using a 10 ml Metrohm E 485 burette.

2.2. Flow-through sensor/detector unit

The main bodies of the amperometric-rotating bioreactor were made of Plexiglas. The design of the flow-through chamber containing the rotating enzyme biosensor and the detector system was described previously [42]. Briefly, glassy carbon electrode (GCE) is on the top of the rotating biosensor. The rotating biosensor is a disk of Teflon in which a miniature magnetic stirring bar (Teflon-coated Micro Stir bar from Markson Science, Inc. Phoenix, AZ) has been embedded. Typically, a sensor disk carried 1.4 mg of controlled-pore glass on its surface. Rotation of the lower sensor was effected with a laboratory magnetic stirrer (Metrohm E649 from Metrohm AG Herisau, Switzerland) and controlled with a variable transformer with an output between 0 and 250 V and maximum amperage of 7.5 A (Waritrans, Argentina.). Amperometric detection was performed using a BAS LC-4C potentiostat and BAS 100 B/W (electrochemical analyzer Bioanalytical System, West Lafayette IN) was used to voltammetric determinations. The potential applied to the GCE for the functional group detection was −150 mV versus Ag/AgCl/3.0 M NaCl reference electrode BAS RE-6, and a

Pt wire counter electrode. At this potential, a catalytic current was well established.

A pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics, Inc. Middleton, WI) was used for pumping, sample introduction, and stopping of the flow. The pump tubing was Tygon (Fisher AccuRated, 1.0 mm i.d., Fisher Scientific Co., Pittsburgh, PA) and the remaining tubing used was Teflon, 1.00 mm i.d. from Cole–Parmer (Chicago, IL).

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.).

2.3. Horseradish peroxidase immobilization

The rotating disk biosensor (bottom part) was prepared by immobilizing HRP on 3-aminopropyl-modified controlled-pore glass (APCPG). The APCPG, smoothly spread on one side of a double-coated tape affixed to the disk surface, and was allowed to react with an aqueous solution of 5% w/w glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After washing with purified water and 0.10 M phosphate buffer of pH 7.00, the enzyme (10.0 mg of enzyme preparation in 0.50 ml of 0.10 M phosphate buffer, pH 7.00) was coupled to the residual aldehyde groups in phosphate buffer (0.10 M, pH 7.00) overnight at 5 °C. The immobilized enzyme preparation was finally washed with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C between uses. The immobilized HRP preparations were perfectly stable for at least one months of daily use.

2.4. Sample preparation for HRP-rotationg biosensor assay

A mass of powder of one capsule of penicillamine (Cuprimine 250 mg, Sidus Lab.; Cupripen 250 mg, Interpharm Lab.) was transferred to 100 ml volumetric flask and dissolved in 0.1 M phosphate buffer (pH 7). The flask was sonicated for 2 min and filled with 0.1 M phosphate buffer, pH 7.0. A small amount of non-dissolving excipients settled at the bottom of the flask. Then, suitable aliquots of the supernadant was further diluted with 0.1 M phosphate buffer (pH 7) to obtain a final concentration of 2.5 μM.

2.5. Preparation of synthetic capsule samples

Synthetic capsule samples were prepared in 100 ml calibrated flasks by spiking a placebo (mixture of capsule excipients) with accurately calculated amount of PA. Hereafter, the procedure described for the sample preparation was followed.

3. Results and discussion

3.1. Electrooxidation of 4-tert-butylcatechol in the absence and presence of PA

Cyclic voltammetry of a 1 mM solution of 4-*tert*-butylcatechol (4-TBC) in an aqueous solution containing 0.10 M phosphate buffer pH 7.0 as supporting electrolyte, shows one anodic (A_1) and a corresponding cathodic peak (C_1) which corresponds to the transformation of 4-TBC to *o*-benzoquinone and vice-versa within a quasi-reversible two-electron process (Fig. 1, curve a). A peak current ratio (Ip^{C_1}/Ip^{A_1}) of nearly unity, particularly during the repetitive recycling of potential, can be considered as a criterion for the stability of *o*-quinone produced at the surface of the electrode under the experimental conditions. In other words, any hydroxylation [43–46] or dimerization [47,48] reactions are too slow to be observed on the time scale of cyclic voltammetry. The oxidation of 4-TBC in the presence of PA as a nucleophile was also studied. Fig. 1, curve b, shows the cyclic voltammogram obtained for a 1 mM solution of 4-TBC in the presence of 2.5 mM PA. The voltammogram exhibits an anodic peak at 220 mV versus Ag/AgCl/3M NaCl, and the cathodic counterpart of the anodic peak A_1 has disappeared.

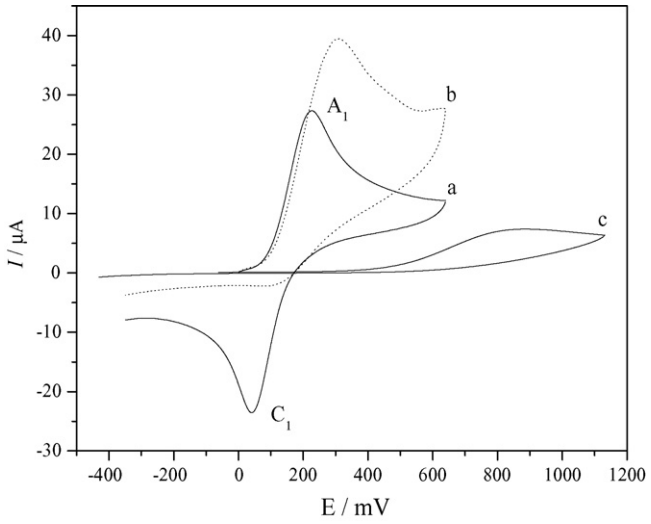


Fig. 1. Cyclic voltammograms of 1 mM 4-TBC: (a) in the absence; (b) in the presence of 2.5 mM PA; (c) 1 mM PA in the absence of 4-TBC, at glassy carbon electrode (3 mm diameter) in aqueous solution containing 0.1 M phosphate buffer (pH 7.00). Scan rate: 100 mV s^{-1}; *T*: 25 ± 1 °C.

The influence of increasing PA concentration on the electrochemical behavior of 4-TBC was investigated and the responses recorded are shown in Fig. 2. The height of the oxidation peak was found to increase with increasing additions of PA with the loss of the corresponding reduction peak consistent with the ECE type mechanism proposed in Schemes 1 and 2. The increase in the oxidation peak height is attributed to the oxidation of 4-TBC–PA adducts that have arisen through the electrochemically initiated reaction previously detailed in Scheme 1. The successive decrease in the height of the 4-TBC reduction peak can be ascribed to the fact that increasing concentration of PA serve to scavenge the oxidized form of 4-TBC such that on the reverse sweep there is little available for electro-reduction.

Given that the direct oxidation of this thiol at the electrode does not occur within the potential window studied (Fig. 1, curve c), the increase in the magnitude of the 4-TBC oxidation peak can be attributed solely to the re-oxidation of the 4-TBC–PA adduct.

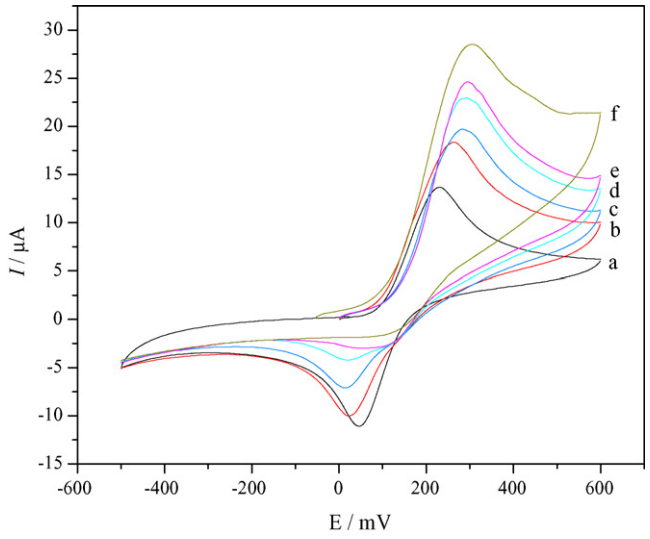


Fig. 2. Typical voltammograms of 0.5 mM 4-TBC at a glassy carbon electrode (3 mm diameter) in aqueous solution containing 0.1 M phosphate buffer (pH 7.00) at various PA concentrations, C_{PA}: (a) 0.0; (b) 0.09; (c) 0.17; (d) 0.29; (e) 0.37; (f) 0.67 mM. Scan rate: 100 mV s^{-1}, T: 25 ± 1 °C.

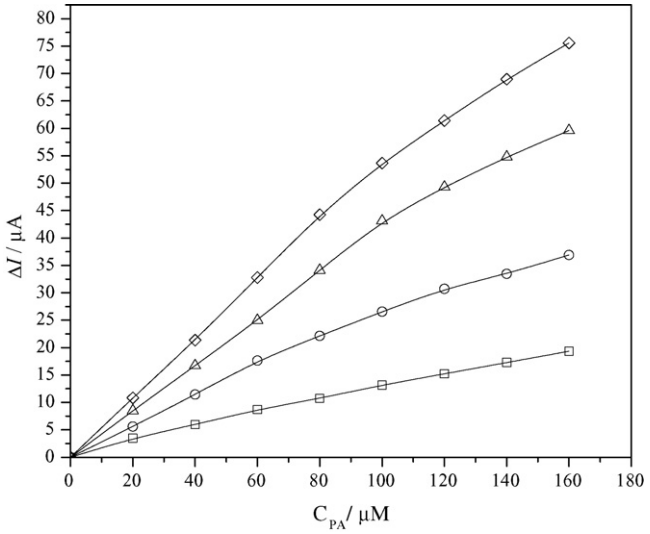
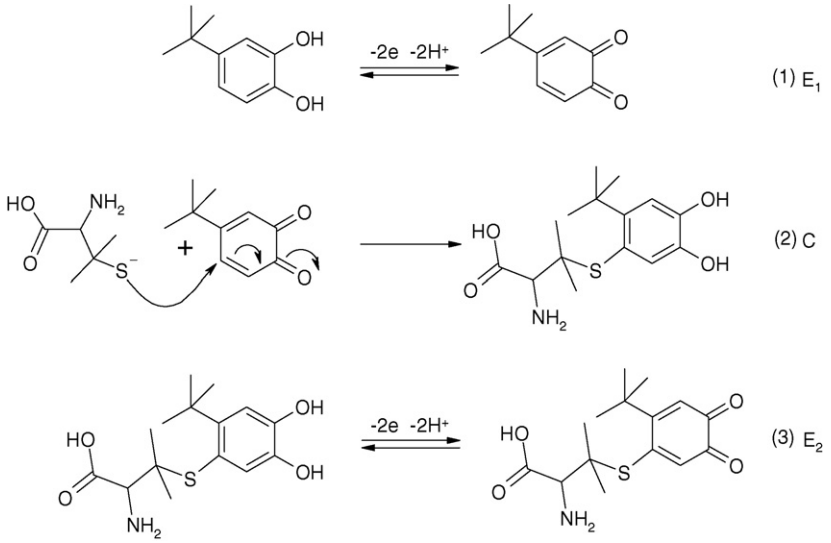


Fig. 3. Influence of pH on the magnitude of the oxidation signal for 1.00 mM 4-TBC in the presence of several penicillamine concentrations, using phosphate buffer (□) pH 4, (◯) pH 5, (△) pH 6 and (◊) pH 7.

3.2. *Effect of pH and H_2O_2 concentration*

The influence of pH on peak potential (Ep) of the reaction was assessed through examining the electrode response to 4-TBC–PA obtained in solutions buffered between pH 4 and pH 8. The position of the redox couple was found to be dependent upon pH with a shift of 61 mV pH^{-1} indicative of an n electron n proton behavior with n likely to be two [49]. A quantitative evaluation of the 4-TBC change peak current (ΔI) response to increasing additions of PA as a function of solution pH is highlighted in Fig. 3. The ΔI reported hereafter is the difference between the reduction current (from addition of 4-TBC) and the current due to the addition of PA (from addition of 4-TBC + PA). The response was found to decrease steadily as the acidity of the solution is increased. This can be attributed to the fact that as the solution pH is lowered, the thiol functionality will be increasingly protonated (PA RSH, pK_a ~7.9) and hence the nucleophilic character of the thiol moiety diminished. Increasing the pH clearly improves the response but an operational limit is reached once neutral conditions prevail. Alkaline solution severely compromises the enzyme stability as well as the response as the increased presence of nucleophilic hydroxyl ions compete with the less prevalent thiol. Therefore, the pH value used was 7.00 in 0.1 M phosphate buffer in concordance with the steadier pH of the enzyme.

The effect of H_2O_2 concentration may be observed easily varying H_2O_2 concentration from 2.5×10^{-5} to 5.0×10^{-3} M, for 20 µM 4-TBC solutions and several concentrations of HRP, whilst maintaining a constant 4-TBC concentration [42]. At low H_2O_2 concentration, 0.025 mM, a lineal relation can only be seen when the enzymes concentrations are low, losing this lin-



Scheme 2.

earity as the enzymatic concentration increases. This change occurs because the H_2O_2 concentration is insufficient to generate maximum catalytic activity. With 0.1 mM H_2O_2 concentration, a perfect linearity within the concentration range studied is obtained. With 0.5 mM H_2O_2 concentration, the linearity is lost to low concentrations. This is due to the fact the HRP is inactivated in excess of H_2O_2. At higher H_2O_2 concentration, such as 5 mM, inhibition by conversion of the initial enzyme by H_2O_2 into E_3 is observed throughout the entire HRP concentration range studied. In this case linearity is observed but the catalytic current obtained is less significant than in the optimal case.

3.3. Effect of biosensor rotation and continuous-flow/stopped-flow operation

The implementation of continuous-flow/stopped-flow programming and the location of two facing independent biosensors [42], permits: (a) utilization of relatively low enzyme loading conditions (b) instantaneous operation under high initial rate conditions, (c) easy detection of accumulated products, and (d) reduction of apparent Michaelis-Menten constant, K'_M. A more complete reagent homogenization is achieved, because the cell works as a mixing chamber by facilitating the arrival of substrate at the active sites and the release of products from the same sites. The net result is high values of current (Fig. 4). The main advantages of this system are its simplicity, and the ease with which it can be applied to the determination of PA at low levels.

As observed earlier [50], if the sensor in the cell is devoid of rotation, there is practically no response. If a rotation of 900 rpm is imposed on the sensor located at the bottom of the cell (with immobilized HRP), the signal is dramatically enlarged. The trend indicates that, up to velocities of about 900 rpm, a decrease in the thickness of the stagnant layer improves mass transfer to and from the immobilized enzyme active sites. Beyond 900 rpm, the current is constant, and chemical kinetics controls the overall process. Therefore, a rotation velocity of 900 rpm was used.

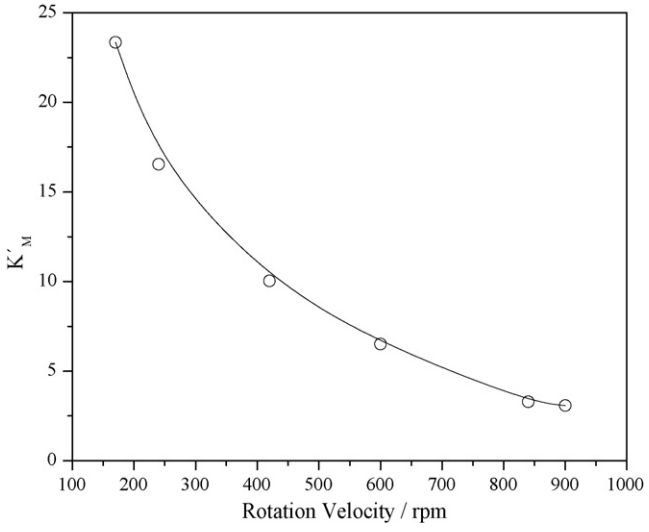


Fig. 4. The K'_M values for the system obtained at six different rotation velocities and stopping the flow for 1 min during measurement. The K'_M values were determined as discussed in the text; T: 20 ± 1 °C.

Table 1
Effect of sample size[a]

Sample size (μl)	I (μA)	Linear regression, standard deviation
50	5.82	±0.26
75	27.99	±0.19
100	50.92	±0.11
125	68.60	±0.26
150	72.35	±0.14
175	72.43	±0.29
200	72.60	±0.36

Solution containing 0.1 mM H_2O_2, 1.0 mM 4-TBC in 0.1 M phosphate buffer, pH 7.00. Flow rate, 1.00 ml min^{-1}.

[a] The current was measured under stopped-flow conditions. Each value of current is based on triplicate of six determinations.

Table 2
Intra- and Inter-day precision and accuracy (n = 5)

	Intra-day			Inter-day		
$C_{nominal}$ (μM)	0.60	5.20	21.80	0.60	5.20	21.80
Mean C_{found} (μM)	0.62	5.19	21.82	0.59	5.17	21.78
C.V. (%)	1.35	0.77	0.97	0.45	0.84	1.21
Bias (%)	3.22	0.19	0.10	1.70	0.60	0.10

Table 3
Specificity results of the HRP-rotating biosensor[a]

Sample no.	Pure sample 2.50 (μM)	Synthetic capsule sample (n = 5) X (μM)
1	2.51	2.51
2	2.48	2.48
3	2.51	2.50
4	2.49	2.48
5	2.52	2.51
6	2.48	2.48
X	$2.49 \pm 7 \times 10^{-3}$	$2.49 \pm 6 \times 10^{-3}$
S.D.	0.02	0.01
C.V. (%)	0.80	0.41

[a] X (μg ml^{-1}), mean ± S.E., standard error; S.D., standard deviation; C.V., variation coefficient.

Table 4
Determination by the developed method of amount of PA contained in commercial formulations

Sample No	Cuprimine (Sidus Lab.) (g/capsule)	Cupripen (Interpharm Lab.) (g/capsule)
1	250.10	249.91
2	249.95	249.87
3	250.05	250.10
4	249.90	250.15
5	249.87	249.89
6	249.98	250.04
7	250.12	249.94
8	250.09	250.08
X	250.01	249.98
S.D.	0.09	0.11

Table 5
A comparison of previous methods for determining penicillamine

Detection method	Matrix	Response time (min)	Linear range (μM)	Detection limit (μM)	Ref.
Square-wave voltammetry	Pharmaceutical preparations	<1	0.10–2.5	0.08	[51]
Capillary electrophoresis	Plasma	10	0.1–48	2×10^{-4}	[28]
Differential pulse voltammetry	Pharmaceutical preparations	<1	0.4–500	0.1	[52]
Spectrophotometry	Pharmaceutical preparations	<1	37.5–402	6.7	[53]
Amperometric	Pharmaceutical preparations	<1	0.5–50	0.01	[54]
Spectrophotometric	Pharmaceutical preparations	1.15	25–300	–	[23]
Amperometric	Pharmaceutical preparations	1	0.02–80	7×10^{-3}	Proposed method

3.4. Effect of cell volume and sample size

Depending on the volume of the cell in contact with the sensors, the overall process becomes controlled by diffusion (large volumes) or by the chemical kinetics of the enzyme-catalyzed reactions (small volumes). The cell volume was changed from 150 μl to 1 ml by removing the O-rings between the upper and lower half of the cell. The measured current, as expected, decreased linearly with an increase in cell volume, due to the dilution effect favored by rotation, and the fact that the measured current is directly proportional to bulk concentration. The smallest cell volume of 150 μl was adopted for further studies.

The measured current increased lineally with sample size up to 150 μl in a cell with a volume of 150 μl. For convenience a sample size of 150 μl was used. Sensitivity is almost tripled in the range between 50 and 150 μl (Table 1).

3.5. PA measurement with HRP-rotating biosensor

The working potential was selected using the same cyclic voltammogram showed before (Fig. 1a) for the couple 4-TBB/4-TBC at a GCE in phosphate buffer. For potentials values below −150 mV, the cathodic current became independent of the applied potential; therefore, this value was chosen as working potential. Furthermore, at this potential, a less contribution of the electroactive interferences is expected.

The performance of the HRP-rotating biosensor for the measurement of reduced thiols concentrations was characterized. For PA measurement, the following procedure was used: (a) a baseline current was established with the buffer solution; (b) a solution containing 1 mM 4-TBC and 0.1 mM H_2O_2 were injected in the rotating biosensor; (c) the flow was detained and the disk was rotated to 900 rpm, thus, a large reduction current was observed due to the quinone derivative and after 1 min, the flow was started again; then (d) a solution containing 1 mM 4-TBC, 0.1 mM H_2O_2, and several PA concentrations were injected in the rotating biosensor; (e) the flow was detained and the disk was rotated to 900 rpm and the reduction current was measurement. The addition of PA resulted in a current decrease. After 1 min, the flow was started again. A PA calibration plot was obtained by plotting ΔI versus PA concentration. A linear relation, $\Delta I\,(\mu A) = 8.7 \times 10^{-3} + 0.543$ [CPA], was observed between the ΔI and the PA concentration in the range of 0.02 and 80 μM (rotation 900 rpm). The correlation coefficient for this type of plot was typically 0.998. Detection limit (DL) is the minimal difference of concentration that can be distinguished from the signal of the pure 4-TBC solution. The DL was calculated as the amount of PA required to yield a net peak that was equal to three times the S.D. of the pure 4-TBC signal. In this study, the minimal difference of concentration of PA was 7 nM. Reproducibility assays were made using repetitive standards solutions ($n = 5$) containing 1.0 mM 4-TBC, 0.1 mM H_2O_2 and 10 μM PA; the percentage standard error was less than 3%.

The proposed method was applied to the determination of PA capsules. The precision of the method was obtained on the basis of intra-assay using standard addition. The intra- and inter-day precision (C.V.%) and accuracy (bias%) of the assay procedure were determined by the analysis of five samples at each lower, medium and higher QC concentration in the same day and one sample at each QC concentration in 5 different days, respectively (Table 2). There are no significant differences in the results, indicating that the analysis of PA capsules by the proposed method is reproducible.

Excipients frequently added to dosage forms are povidone, starch, lactose, sodium starch glycollate, magnesium stearate, hydroxymethylpropyl cellulose, talc, titanium oxide, and polyethylene glycol. The response of the analyte with excipients and PA was compared with the response of pure PA. It was found that assay results were not changed. Therefore, excipients commonly found in typical pharmaceutical preparations did not interfere with the quantization of PA present as active principle. In Table 3 the results are showed.

The developed method for the PA determination was applied to two different commercial preparations (Table 4). There is no need any extraction procedure before HRP-rotating biosensor analysis.

4. Conclusions

The usefulness of enzyme biosensor used for the determination of very low concentrations of PA was demonstrated. The biosensor developed in this work is found to be more sensitive than other techniques previously developed (Table 5). This type of detection (addition reaction on co-substrates) shows good promise in pharmacological sensing. Also, this biosensor is able to operate as a fast, selective and sensitive detection unit when is incorporated into a FIA system, and provides a fast and cost effective solution to the realization of quantitative information at extremely low levels of PA concentrations.

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