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Penicillamine determination using a tyrosinase micro-rotating biosensor

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Abstract

Tyrosinase [EC 1.14.18.1], immobilized on a rotating disk, catalyzed the oxidation of catechols to *o*-benzoquinone, whose back electrochemical reduction was detected on glassy carbon electrode surface at −150 mV versus Ag/AgCl/NaCl 3 M. Thus, when penicillamine (PA) was added to the solution, this thiol-containing compound participate in Michael type addition reactions with *o*-benzoquinone to form the corresponding thioquinone derivatives, decreasing the reduction current obtained proportionally to the increase of its concentration. This method could be used for sensitive determination of PA in drug and human synthetic serum samples. A linear range of 0.02–80 μM ($r = 0.999$) was obtained for amperometric determination of PA in buffered pH 7.0 solutions (0.1 M phosphate buffer). The biosensor has a reasonable reproducibility (R.S.D. < 4.0%) and a very stable amperometric response toward this compound (more than 1 month).

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

1. Introduction

Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) (PA) is a non-physiological 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. Penicillamine is a medication that has been used for many years in the treatment of various rheumatic diseases, most commonly rheumatoid arthritis. It is also classified as a metal binding (or chelating) agent used in the treatment of Wilson's disease, a genetic disease that results in excessive copper deposits in the body tissues. Increased amounts of PA can cause rashes early in treatment. Rashes may be associated with itching, which can often be controlled by simply adding antihistamine medication. Rashes associated with fever and joint pain usually require discontinuation of the treatment. It can also cause loss of appetite, nausea, abdominal pain and loss of the sense of taste. Penicillamine can also cause bone marrow suppression and serious kidney disease. All patients who take penicillamine require regular blood and urine testing for monitoring.

Several methods have been proposed for the determination of D-penicillamine including high performance liquid chromatography (HPLC) [3–5], calorimetry [6], fluorometry [7,8], spectrophotometry [9–11], chemiluminescence [12], capillary electrophoresis [13,14] and NMR spectroscopy [15]. One of the important limitations of HPLC techniques is the fact that this thiol lacks sufficient UV absorption so a pre or post-column derivatization procedure is normally required [12]. Electrochemical methods are an alternative for the PA determination because they are cheap, simple, fast and sensitive. Mercury and mercury amalgam [16,17] have been extensively used for thiol-compound determination, however, mercury has limitations due to its toxicity and the rapid deterioration of the electrode response [18]. A chemically modified electrode [19] has

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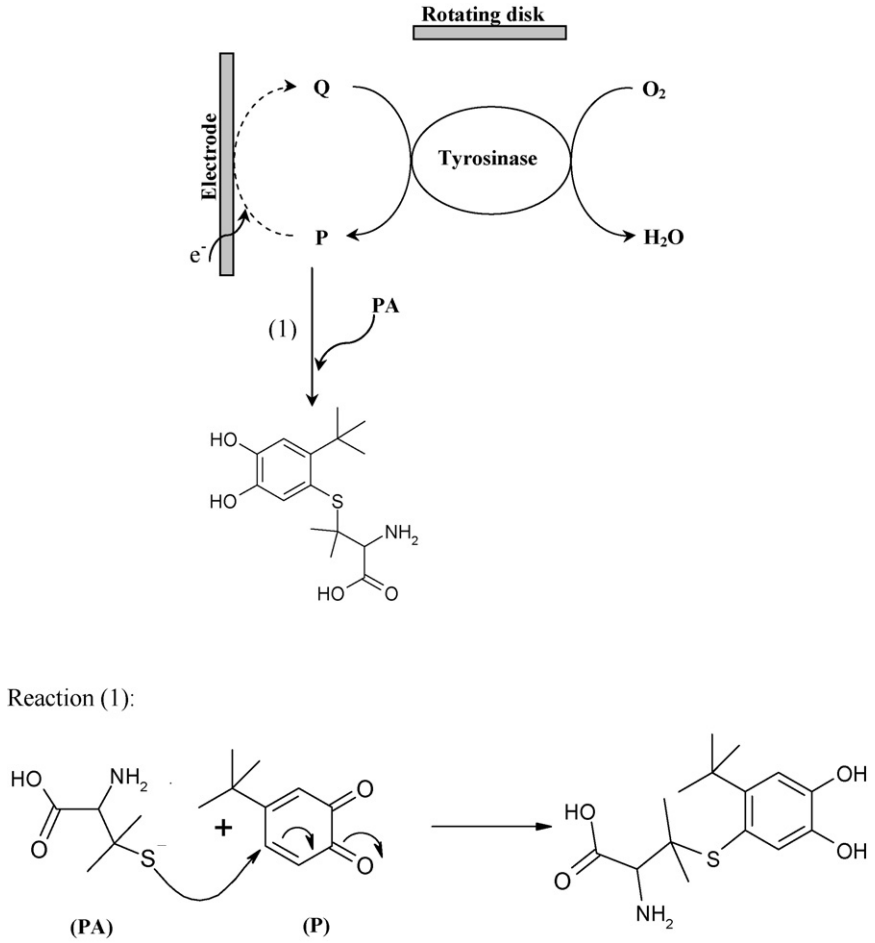

Scheme 1. Schematic representations of the reduction wave of the enzymatic process between catechol (Q), benzoquinone (P), penicillamine (PA) and tyrosinase.

been also used to determine PA 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 [20].

Tyrosinase a two copper-containing enzyme, catalyses the *o*-hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols (Q) to *o*-quinones (P) (diphenolase activity) [21–23]. Over the past decades several reports on the tyrosinase action mechanism have been published [24–27], although major advances in understanding this mechanism have only been obtained by studying the nature of the copper site [27–29]. This enzyme has been used extensively in the development of biosensors for the detection of phenolic compounds [30–32]. The inhibition of tyrosinase activity was utilized in the determination of toxic pollutants in environmental and biological samples [33,34]. Tyrosinase has also been used in combination with a PQQ-dependent dehydrogenase for the determination of alkaline phosphatase [35,36] and in combination with a hydroxylase for NADH and NADPH measurements [37,38].

The measuring principle of this biosensor for the determination of thiol-containing compounds is shown in Scheme 1. First, the tyrosinase immobilized on a rotating disk converts Q to P [39,40], and then the quinones are reduced back to Q at the electrode surface (at ca. −150 mV versus Ag/AgCl/NaCl 3 M). Second, the detection of the PA is accomplished by suppressing the substrate recycling process between tyrosinase and the electrode (denoted by the dotted arrow). Therefore, the detection principle is similar to biosensors based on substrate competition [41–43].

In this paper, we apply a tyrosinase biosensor for a highly sensitive determination of PA in pharmaceutical preparations and also for a recovery test of the drug spiked in human synthetic serum.

2. Experimental

2.1. Reagents and solutions

All reagents used were of analytical grade. The enzyme tyrosinase (from mushroom, EC 1.14.18.1, 2000 U mg^{-1}), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The enzyme concentration was determined taking the value of M_r as 120,000. Glutaraldehyde (25% aqueous solution) was 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, USA) and contained 48.2 μmol g^{-1} of amino groups. Catechol, 4-*tert*-butylcatechol (4-TBC), caffeic acid, penicillamine, were purchased from Sigma Chemical Co. D,L-Alanine, L-arginine, L-aspartic acid, glycine, L-histidine, L-phenylalanine, L-serine, D,L-tryptophan, L-cysteine, glutathione, D,L-methionine, L-lysine, ascorbic acid and citric acid for preparation of synthetic human serum were purchased from Sigma–Aldrich and Merck. All solutions were prepared with ultra-high quality water obtained from a Barnstead Easy pure RF compact ultra-pure water system.

For pharmaceutical sample preparation, a mass of powder of one capsule of PA (Cuprimine 250 mg, Sidus Laboratory (brand A); Cupripen 250 mg, Interpharm Laboratory (brand B)) was transferred to 100 mL volumetric flask and dissolved in 0.1 M phosphate buffer (pH 7.0). The flask was sonicated for two minutes and filled with 0.1 M phosphate buffer, pH 7.0. A small amount of non-dissolving excipient settled at the bottom of the flask. Then, a suitable aliquot of the supernatant was further diluted with 0.1 M phosphate buffer (pH 7) to obtain a final concentration of 2.5 μM.

Synthetic capsule samples were prepared in 100 mL calibrated flasks by spiking a placebo (mixture of capsule excipients: povidone, starch, lactose, sodium starch glycollate, magnesium stearate, hydroxymethylpropyl cellulose, talc, titanium oxide and polyethylene glycol) with accurately calculated amount of PA. Hereafter, the same procedure described for the sample preparation was followed.

Synthetic serum samples were prepared by choosing its composition near to the normal level in real human serum [44].

2.2. Apparatus

Electrochemical experiments were performed in unstirred solutions using a BAS 100B electrochemical analyzer Bioanalytical System, West Lafayette IN, USA, using positive feedback routine to compensate the ohmic resistance. The three electrodes system consisted of a glassy carbon (GC) working electrode model BAS MF-2012, 3.0 mm diameter, 0.071 cm^2 geometrical area, a 3 M NaCl Ag/AgCl reference electrode BAS MF-2052 and a Pt wire counter electrode. Before each voltammogram, the working electrode was carefully polished PK-4 polishing kits, BAS MF-2060, and rinsed following the general guideline for polishing electrodes recommended for BAS Electrode Polishing and Care, BAS A-1302.

Amperometric detection was performed using a BAS LC-4C (Bioanalytical System). 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.

The main body of the amperometric-rotating bioreactor was made of Plexiglas. The design of the flow-through chamber containing the micro-rotating enzyme biosensor and the detector system was described previously [45]. Briefly, glassy carbon electrode (GCE) is on the top of the micro-rotating biosensor (detector system, upper part). The micro-rotating enzyme bioreactor is a Teflon disk (bottom part) in which a miniature magnetic stirring bar (Teflon-coated Micro Stir bar from Markson Science Inc., Phoenix, AZ, USA) has been embedded. Typically, a sensor disk carried 1.4 mg of controlled-pore glass on its surface. The cell volume and sample size were 150 μl, respectively. Rotation of the lower enzyme reactor was effected with a laboratory magnetic stirrer (Metrohm E649 from Metrohm AG Herisau, Switzerland) and controlled with a variable transformer (Waritrans, Argentina) with an output between 0 and 250 V and maximum amperage of 7.5 A.

A pump (Gilson Minipuls 3 Peristaltic Pump, Gilson Electronics Inc., Middleton, WI, USA) 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, USA) and the remaining tubing used was Teflon, 1.00 mm i.d. (Cole-Parmer, Chicago, IL, USA).

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

2.3. Tyrosinase immobilization

The micro-rotating disk bioreactor (bottom part) was prepared by immobilizing tyrosinase 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 a 5% (w/w) glutaraldehyde aqueous solution at pH 10.0 (0.20 M carbonate) for two hours at room temperature. After washed 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 with the residual aldehyde groups in phosphate buffer (0.10 M, pH 7.00) overnight at 4 °C. The Schiff bases were reduced with 20 mg mL^{-1} $NaBH_3CN$ solution in buffer phosphate pH 7.0, and the reaction was allowed to proceed for 1–2 h under stirring at room temperature. The immobilized enzyme preparation was then carefully washed and stored in phosphate buffer (pH 7.0) at 4 °C until use. The immobilized tyrosinase preparations were stable throughout at least 2 months of daily use.

3. Results and discussion

3.1. Cyclic voltammetric studies

Fig. 1, curve a, shows a typical cyclic voltammogram at the GC working electrode in an aqueous solution containing 0.10 M phosphate buffer pH 7.0 and 1 mM of 4-TBC, for a scan rate (v) of 100 mV s^{-1}. The cyclic voltammogram is characteristic of an electrochemically quasi-reversible reaction showing only one anodic peak (A_1, E_{pa} = 225 mV) and one cathodic peak (C_1, E_{pc} = 41 mV). The voltammogram profile does not change significantly even after 20 cycles. The ratio I_{pc}/I_{pa} = 0.914 confirms the reversibility of the system under these conditions. In other words, any hydroxylation [46–48] or dimerization [49] reactions are too slow to be observed on the time scale of cyclic voltammetry.

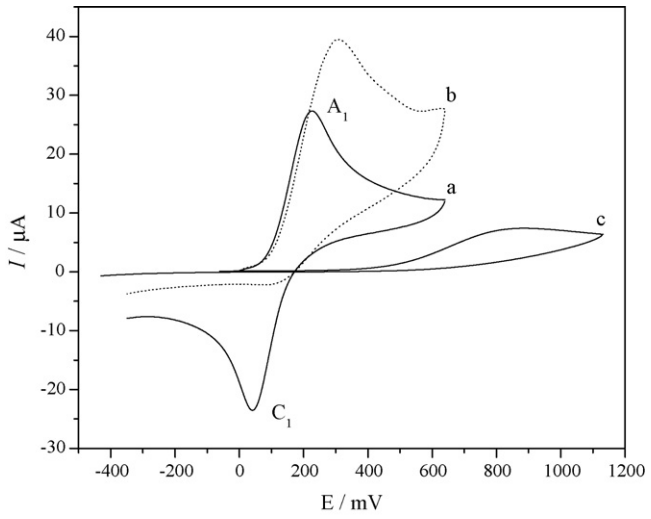

Fig. 1. Cyclic voltammograms of 1 mM 4-TBC: (a) in the absence, (b) in the presence of 2.5 mM PA and (c) 1 mM PA in the absence of 4-TBC, at GC electrode (3 mm diameter) in aqueous solution containing 0.1 M phosphate buffer (pH 7.00). Scan rate, 100 mV s^{-1}; T, 25 ± 1 °C.

The oxidation of 4-TBC to the corresponding quinone, 4-TBB, in the presence of PA as a nucleophile was also studied. The voltammogram (Fig. 1, curve b) exhibits an anodic peak at 313 mV versus Ag/AgCl/3 M NaCl, and the cathodic counterpart, C_1, has disappeared. 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 Scheme 1. Hence, the increase in the oxidation peak height is attributed to the oxidation of 4-TBC–PA adducts that arises through the electrochemically initiated reaction (Scheme 1). In fact, once 4-TBB is formed, could react with a variety of nucleophilic reagents, as those possessing sulfhydryl (–SH) groups [50]. Moreover, compounds with sulfhydryl groups appear to be far more reactive towards *o*-quinones, than amines [51]. For this reason, in the case of 4-TBC oxidation in the presence of PA, only the thioether (S-adduct) is formed but no N-adduct is observed [52].

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.

Furthermore, the consequent decrease on the height of the 4-TBB reduction peak can be ascribed to the fact that increasing concentration of PA scavenges the oxidized form of 4-TBC leaving little available for electro-reduction.

The influence of pH on peak potential (E_p) 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 results obtained were comparable to the cysteine study [53]. Therefore, the pH value used was 7.00 in 0.1 M phosphate buffer in concordance with the steadier pH of the enzyme.

Other catechol derivatives were examined as redox indicators. Caffeic acid (CAF) is the phenylpropenoid most encountered in nature and has proven medical properties, especially as an antioxidant agent [54]; therefore its properties as indicator was compared with 3-TBC and catechol. The compounds investigated are shown in Table 1 along with a summary of their electrochemical properties. As can be seen from this table, 4-TBC, shows greater sensibility and therefore is selected for this work.

Table 1
Electrochemical detection of PA using tyrosinase-rotating biosensor with different redox co-substrates

Co-substrate	E_{ox} (V)	Penicillamine		
		Sensitivity (μA mol^{-1})	Range (μM)	DL[a] (nM)
Caffeic acid	0.180	1.98	0.10–75	24
Catechol	0.480	0.87	0.04–65	10
4-*tert*-Butylcatechol	0.227	0.43	0.02–80	7

[a] 95% confidence interval; $n = 6$.

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

As observed earlier [55], 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 tyrosinase), 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.

3.3. PA measurement with tyrosinase micro-rotating biosensor

The working potential was selected using the same cyclic voltammogram showed before (Fig. 1, curve a) for the couple 4-TBB/4-TBC at the GC electrode in phosphate buffer pH 7.0. For potentials values below −150 mV, the cathodic current became independent of the applied potential; therefore, this value was chosen as the working potential. Furthermore, at this potential, less contribution of the electroactive interferences is expected.

For PA measurement in pharmaceutical and biological samples, the following procedure was used: (i) a baseline current was established with the buffer solution; (ii) a solution containing 1 mM 4-TBC was injected in the micro-rotating biosensor; (iii) 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 (iv) a solution containing 1 mM 4-TBC and several PA concentrations were injected in the micro-rotating biosensor; (v) the flow was detained and the disk was rotated to 900 rpm and the reduction current was measured. The addition of PA resulted in a current decrease (ΔI). After 1 min the flow was started again. A PA calibration plot was obtained by plotting ΔI versus PA concentration. The current versus time profiles obtained with this method of PA measurement is shown in Fig. 2. A linear relation,

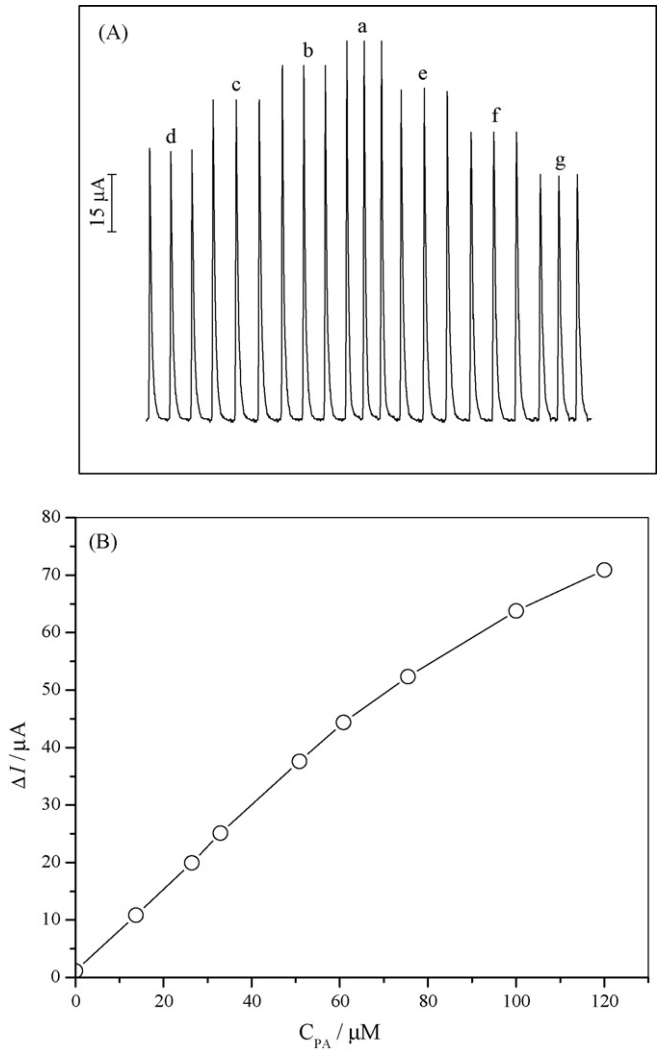

Fig. 2. (A) Response of the tyrosinase-rotating biosensor for PA determination in aqueous solution containing 0.1 M phosphate buffer (pH 7.00). (a) 4-TBC 1.0 mM and H_2O_2 0.1 mM, without addition of PA solution. (b–g) The response for several PA concentrations: (b) 13.74 μM, (c) 32.90 μM, (d) 60.85 μM, (e) 26.43 μM, (f) 50.88 μM and (g) 75.48 μM. Flow rate, 1.00 mL min^{-1}; cell volume was 150 μl; potential, −150 mV versus Ag/AgCl 3 M NaCl. The flow was stopped for 60 s during measurement. (B) Calibration plot obtained from (A).

$\Delta I\ (\mu A) = 6.8 \times 10^{-3} + 0.652\ [C_{PA}]$, was observed for PA concentrations in the range of 0.02 and 80 μM (rotation 900 rpm). The correlation coefficient for this type of plot was typically 0.999. Detection limit (DL) was calculated as the amount of PA required to yield a net peak that was equal to the pure 4-TBC signal minus three times its SD. 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 and 10 μM PA; the percentage standard error was less than 3%.

The response of the analyte with excipients and PA was compared with the response of pure PA. The 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 2, the results are showed.

Table 2
Specificity results of the tyrosinase micro-rotating biosensor

Sample number	Pure sample, 2.50 (μM)	Synthetic capsule sample ($n = 5$), X (μM)
1	2.49	2.52
2	2.48	2.48
3	2.51	2.51
4	2.52	2.48
5	2.51	2.50
6	2.49	2.49
X	$2.50 \pm 6 \times 10^{-3}$	$2.49 \pm 7 \times 10^{-3}$
S.D.	0.01	0.02
CV (%)	0.40	0.79

X (μg mL^{-1}), mean ± S.E., standard error; S.D., standard deviation; CV, variation coefficient.

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 (CV%) and accuracy (bias%) of the assay procedure were determined by the analysis of five samples at each lower, medium and higher concentrations in the same day and one sample at each concentration in 5 different days, respectively (Table 3). The results show no significant differences, indicating that the analysis of PA capsules by the proposed method is reproducible. Also the method was applied for the PA determination to two different commercial preparations (Table 4).

Results of the recoveries of the PA added to 10.0 mL of human synthetic serum re shown in Table 4. In these measurements, the composition of the synthetic serum was chosen near to its normal level in real human serum [44]. The results shown in entry 3 (Table 4) indicate that the constituents in the complex matrix of the serum sample interfere with the detection of PA. This fact can be avoided if standard addition method is used instead of calibration curve. This was checked using the standard addition method was carried out in the same biosensor system. Different known amounts of PA were added to the synthetic serum sample containing a known amount of PA prior to testing and injection; the recovery was 97.17%. Therefore, the interference was avoided, and then this amperometric method can be used as a very highly sensitive detection device for PA in biological and pharmacological preparations. The correlation coefficients of the calibration plots in the standard addition method were in the range of 0.998–0.999 ($n = 6$). The precision of the method was assessed by six repetitions of the analyses of the synthetic serum sample. In these experiments, when spiked with PA solution, the R.S.D. was between 3.1 and 3.6%.

Table 3
Analysis of PA capsules by the proposed method

	Intra-day			Inter-day		
$c_{nominal}$ (μM)	0.60	5.20	21.80	0.60	5.20	21.80
Mean c_{found} (μM)	0.59	5.19	21.77	0.57	5.16	21.76
CV (%)	1.69	0.77	0.37	1.72	0.58	1.21
Bias (%)	1.69	0.19	0.14	5.00	0.77	0.18

Intra- and inter-day precision and accuracy ($n = 5$).

Table 4
Results of analysis of PA capsules and recovery of PA added to synthetic serum samples

Experiment number	Sample preparation	Labeled value (mg/capsule)	Amount found (mg)[a]	Recovery (%)
1	Brand A	250.0	249.98 (±0.09)	–
2	Brand B	250.0	250.02 (±0.08)	–
3[b]	10 mL serum + 1.0 mg PA	–	1.29 (±0.06)	129.3
4[c]	10 mL serum + 1.0 mg PA	–	0.96 (±0.07)	96.5
5[c]	10 mL serum + 5.0 mg PA	–	4.89 (±0.18)	97.8
6[c]	10 mL serum + 10.0 mg PA	–	9.72 (±0.34)	97.2

[a] Values in parenthesis give the standard deviation based on six replicates. Results were obtained using a calibration plot. Amount found in entries 1 and 2 are mg/capsule.
[b] Human synthetic serum sample. Results were obtained using a calibration plot.
[c] Human synthetic serum sample. Results were obtained using standard addition method.

4. Conclusions

Based on the amperometric studies of PA, a very highly sensitive method is applied successfully for the determination of trace amounts of PA in clinical and pharmaceutical preparations. High sensitivity and a low detection limit, together with the very easy preparation and also long time stability and reproducibility makes the system discussed above useful in the construction of simple devices for the determination of PA.

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