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Continuous-flow/stopped-flow system for determination of ascorbic acid using an enzymatic rotating bioreactor

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Abstract

The high sensitivity that can be attained using an enzymatic system and mediated by hydroquinone, has been verified by on-line interfacing of a rotating bioreactor 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 catalyses the oxidation of hydroquinone to *p*-benzoquinone, whose electrochemical reduction back to hydroquinone is detected on glassy carbon electrode (GCE) surface at −0.15 V. Thus, when L-ascorbic acid is added to the solution, this acid is reduced chemically (*p*-benzoquinone to hydroquinone) and acts as mediator of HRP, decreasing the peak current obtained proportionally to the increase of its concentration. The recovery of L-ascorbic acid from four samples ranged from 99.09 to 101.10%. This method could be used to determine L-ascorbic acid concentration in the range 12 nM–3.5 μM ($r = 0.998$). The determination of L-ascorbic acid was possible with a limit of detection of 6 nM in the processing of as many as 25 samples h^{-1}. The method was successfully applied for the analysis of L-ascorbic acid in pharmaceutical formulations.

Keywords: L-ascorbic acid; Glassy carbon; Biosensor; Peroxidase; Hydroquinone; FIA

1. Introduction

The L-ascorbic acid (AA) is of vital importance in processes of oxidation and reduction in humans, participating in several metabolic reactions. AA has also been used for the prevention and treatment of the common cold, mental illness, infertility, cancer, and AIDS [1]. Thus, the determination of AA content is particularly important in the pharmaceutical and food industry, where it is used extensively as an antioxidant.

Many analytical methodologies have been proposed for the determination of AA taking into account its chemical role, including spectrophotometry, chemiluminiscence, titrimetry, enzymatic analysis, capillary electrophoresis, and electrochemistry [2–13]. The electrochemical techniques received much interest because they are more selective, sensitive, and less time consuming than those based on other colorimetric or spectrophotometric methods.

Several mediators for determination of AA in modificated electrodes have been reported using nickel hexacyanoferrate [14], copper hexacyanoferrate [15], ferricyanide [16–20] Prussian blue [21]. In spite of this, there are only a few reports for determination of AA using hydroquinone (H_2Q) as enzymatic mediators [22].

In this work, a bioreactor is proposed for determining AA in pharmaceutical formulations. Horseradish peroxidase (HRP) in the presence of H_2O_2 catalyses the oxidation of H_2Q to *p*-benzoquinone (Q) [23] whose electrochemical reduction back to H_2Q was obtained at peak potential of −0.15 V. Thus, when AA is added to the solution, this acid can chemically reduce Q to H_2Q and/or can act as mediator of HRP, decreasing the peak current obtained proportionally to the increase of AA concentration.

In this paper, we show that the limit of detection for AA can be lowered considerably, if the redox enzyme systems mediated by H_2Q are utilized using rather recently introduced modificated electrodes [21,22,24,25]. Our aim is to develop a bioreactor, able to analyze pharmaceuticals formulations, avoiding, or minimizing the number of steps needed to assess the concentration of the analytes. The re-

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sults were compared with those obtained by capillary zone electrophoresis (CZE).

Such strategy allows for an effective use of immobilized active centers and the effects of interferents can be also reduced by the low potential used [26]. It has to be pointed out that a large number of samples can be processed by means of the proposed method, which show adequate sensitivity, low cost, versatility, simplicity, and effectiveness.

2. Experimental

2.1. Reagents and solutions

All used reagents were of analytical reagent grade. The enzyme horseradish peroxidase, [EC 1.11.1.7] Grade II, were purchased from Sigma Chemical Co., St. Louis, glutaraldehyde (25% aqueous solution) and hydrogen peroxide were purchased from Merck, Darmstadt. 3-Aminopropyl-modified controlled-pore glass (APCPG) with 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. H_2Q and AA were purchased from Sigma Chemical Co., St. Louis, and all other reagents employed were of analytical grade and were used without further purifications. Aqueous solutions were prepared using purified water from a Milli–Q system and the samples were diluted to the desired concentrations using a 10-ml Metrohm E 485 burette.

2.2. Horseradish peroxidase immobilization

The rotating disk reactor (bottom part) was prepared by immobilizing HRP on 3-aminopropyl-modified controlled-pore glass. 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 1 month of daily use.

2.3. Flow-through reactor/detector unit

The main body of the cell was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the rotating enzyme reactor and the detector system. Glassy carbon electrode (GCE) is on the top of the rotating reactor. The rotating reactor 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 reactor disk carried 1.4 mg of controlled-pore glass on its surface. Rotation of the lower reactor was affected with a laboratory magnetic stirrer (Metrohm E649

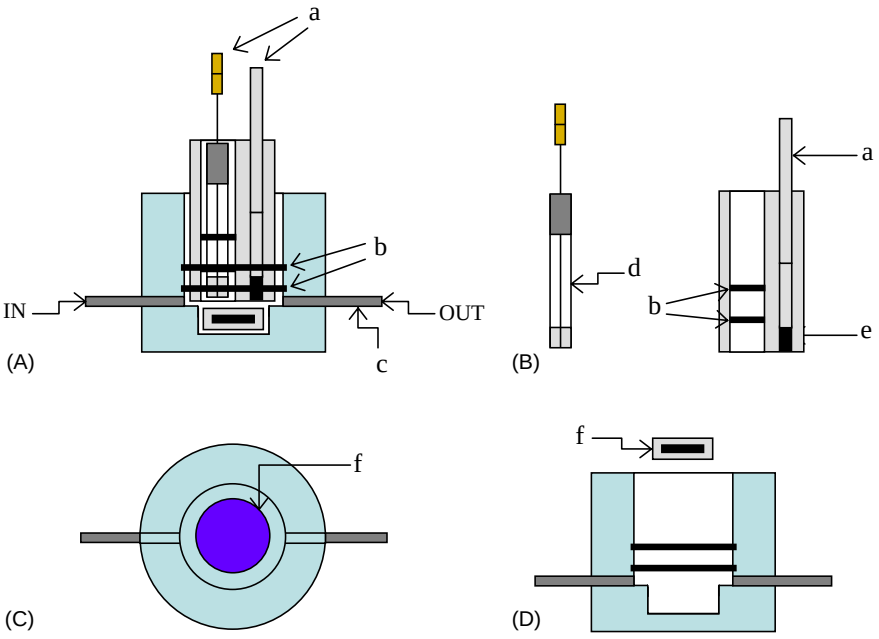


Fig. 1. Schematic representation of components in the bioreactor flow cell. A: Assembled reactor. B: Upper cell body. C: Top view of lower cell body; f denotes rotating bioreactor (with immobilized HRP). D: Lower cell body. a: electrical connection. b: O-ring. c: counter electrode. d: Ag/AgCl, 3.0 M NaCl reference electrode BAS RE-6. e: GCE.

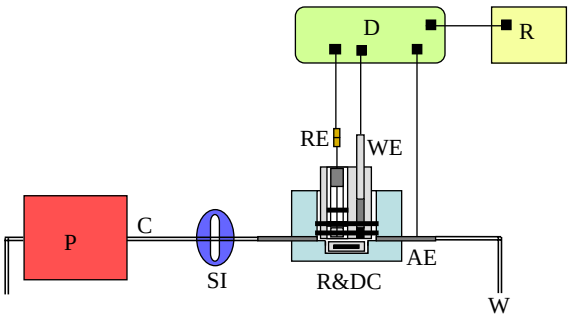


Fig. 2. Block diagram of the continuous-flow system and detection arrangement. P: pump. C: carrier buffer line. SI: sample injection. W: waste line. R & DC: reactor and detector cell. WE: GCE. RE: reference electrode. AE: auxiliary electrode. D: potentiostat/detection unit. R: recorder.

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 CV27 and BAS 100 B (electrochemical analyzer Bioanalytical System, West Lafayette, IN) was used for voltammetric determinations. The potential applied to the GCE for the functional group detection was −0.15 V 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. Fig. 2 schematically illustrates the components of the single-line continuous-flow setup. 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).

The capillary zone electrophoresis (CZE) experiments were performed with a Beckman P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising of IBM personal computer and P/ACE System MDQ software. Detection was performed at 265 nm. The fused silica capillaries were obtained from MicroSolv Technology Corporation, which had the following dimensions: 67 cm total length, 50 cm effective length, 75 μm i.d., 375 μm o.d. The temperature of the capillary and the samples was maintained at 25 °C.

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., Cambridge, MA).

2.4. Analysis of pharmaceutical samples

Ten tablets or the contents of 10 tablets were weighed from each dosage forms and were powdered. Amount equivalent to one tablet was weighed and transferred to a 100-ml volumetric flask. The flask was sonicated for 10 min and filled with 0.1 M phosphate–citrate buffer, pH 5.05. Appropriated solutions were prepared by taking suitable aliquots of the clear supernatant and diluting them with 0.1 M phosphate–citrate buffer, pH 5.05, and injected in to sample loop by means of a peristaltic pump.

In injectable preparations and the oral-drop preparations, the entire content was directly put into a 100-ml volumetric flask, and the procedure described above was followed.

All experiments were performed at room temperature in a 0.1 M phosphate–citrate buffer, pH 5.05 solution containing 1.0×10^{-3} mol l^{-1} H_2O_2 and 1.0×10^{-3} mol l^{-1} H_2Q. Aliquots of pharmaceutical samples were added into a 15-ml thermostated glass cell, homogenized with the aid of a magnetic stirrer, degassed with nitrogen for 1 min and the amperometric measurements were performed at −0.15 V, and the resulting cathodic current was displayed on the *x–y* recorder.

2.5. Preparation of synthetic tablet samples

Synthetic tablet samples were prepared into a 100-ml calibrated flasks by spiking a placebo (mixture of tablet excipients) with accurate amount of AA, at a concentration similar to formulation concentration (0.2–2 g). Then, the procedure described above was followed.

2.6. Sample preparation for CZE assay

Ten tablets or the contents of 10 tablets were weighed from each dosage form and were powdered. Amount equivalent to one tablet was weighed and transferred to a 100-ml volumetric flask. The flask was degassed by ultrasound for 10 min and filled with 0.20 mM phosphate buffer, pH 8.0. Appropriated solutions were prepared by taking suitable aliquots of the solution and diluting them with phosphate buffer, pH 8.0. The solution was mixed and filtered through a 0.45-μm membrane.

The electrolyte solution was prepared daily and filtered through a 0.45-μm titan syringe filters (Sri Inc.). At the beginning of the day, the capillary was conditioned with 0.1 mol l^{-1} NaOH for 5 min, followed by water for 5 min and then with running electrolyte for 10 min before sample injection. To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with sodium hydroxide for 2 min, followed by water for 2 min, then equilibrated with the running buffer for 4 min. Samples were pressure injected at the anodic side at 0.5 psi for lengths of time 3–7 s. A constant voltage was used for all the experiments.

2.7. Dosage forms of AA

(1) Redoxon 2 g effervescent tablets (Roche). (2) Redoxon 1 g effervescent tablets (Roche). (3) Redoxon masticatory tablets (Roche). (4) Redoxon oral drops (Roche). (5) Vitamin C injectable (Labimar S.A.). (6) Tanvimil C tablets (RAY-

MOS). (7) Tanvimil C oral drops (RAYMOS). (8) Cewin® (Sanofi∼synthelabo).

3. Results and discussion

3.1. Study of the enzymatic process

Reactions catalyzed by enzymes have long been used for analytical purposes in the determination of different analytes, such as substrates, inhibitors and also the enzymes. Bioreactors, which combine the selectivity of enzymes with the high sensitivity of electrochemical measurements, provide an excellent tool for analytical chemistry [27].

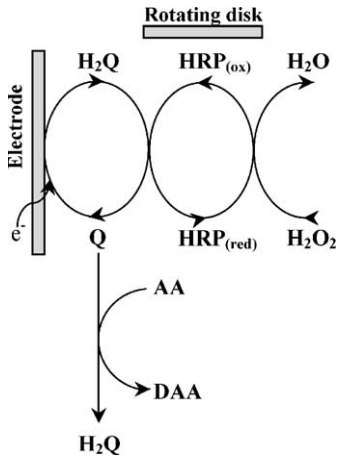
The mechanisms of HRP catalyzed reactions can be represented as follows [28]:

$$\mathrm{HRP(Fe^{3+})} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{Compound\ I} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{Compound\ I} + \mathrm{AH_2} \xrightarrow{k_2} \mathrm{Compound\ II} + \mathrm{AH} \quad (2)$$

$$\mathrm{Compound\ II} + \mathrm{AH_2} \xrightarrow{k_3} \mathrm{HRP(Fe^{3+})} + \mathrm{AH^*} + \mathrm{H_2O} \quad (3)$$

The first step (reaction (l)) involves the two-electron oxidation of the ferrihaeme prosthetic group of the peroxidase, HRP (Fe^{3+}), by H_2O_2. This reaction results in the formation of an unstable intermediate, compound I, consisting of oxyferryl iron (Fe^{4+}=O) and a porphyrin π cation radical. Next (reaction (2)), compound I loses one oxidizing equivalent during one-electron reduction by an electron donor AH_2 and forms compound II, which in turn accepts an additional electron during the third step (reaction (3)), whereby HRP is returned to its native resting state HRP (Fe^{3+}).



Scheme 1. Schematic representations of the reduction wave of the enzymatic process between hydroquinone (H_2Q), *p*-benzoquinone (Q), hydrogen peroxide (H_2O_2), L-ascorbic acid (AA), dehydroascorbic acid (DAA) and peroxidase (HRP).

For the reduction wave, H_2O_2 oxidizes the native form of HRP in a single two-electron process resulting in the oxidized form of HRP (denoted as compound I) and water [29]. Scheme 1 shows the enzymatic process between H_2Q, H_2O_2, AA, and HRP. H_2Q is enzymatically oxidized to Q, which at a potential of −0.15 V was electrochemically reduced to H_2Q providing a peak current related to its concentration [30]. Following this reduction wave scheme, when AA solution is added to the solution, this acid can reduce chemically Q to H_2Q [31] and/or act as mediator of HRP with dehydroascorbic acid (DAA) formation [32] (not shown in this Scheme) decreasing the peak current proportionally to the increase of AA concentration.

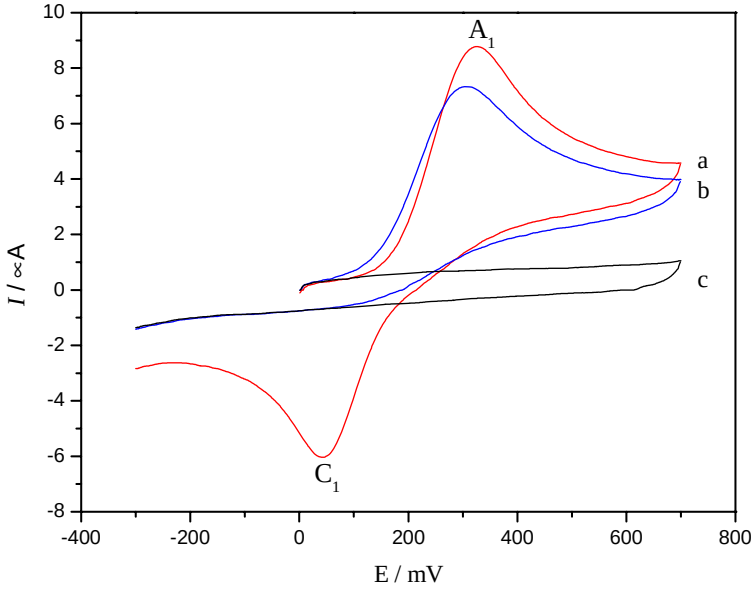


Fig. 3. Cyclic voltammogram in aqueous solution containing 0.1 M phosphate–citrate buffer (pH 5.05) of (a) H_2Q 0.5 mM, (b) AA 0.5 mM, (c) supporting electrolyte; scan rate: 30 mV s^{-1}, *T*: 25 ± 1 °C.

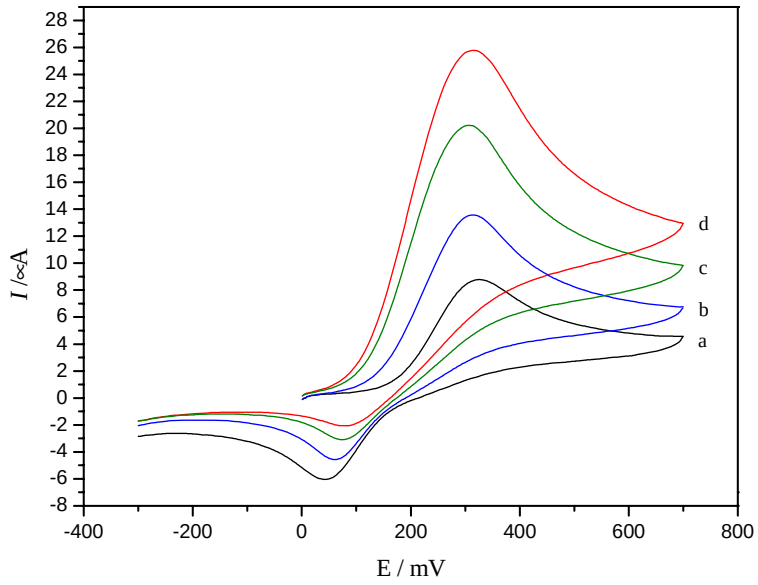


Fig. 4. Typical voltammograms of 5×10^{-4} M H_2Q at a GCE (3 mm diameter) in aqueous solution containing 0.1 M phosphate–citrate buffer (pH 5.05) at various AA concentrations, C_{AA}: (a) 0.0; (b) 4.54×10^{-4}; (c) 1.13×10^{-3}; (d) 1.81×10^{-3} mol l^{-1}; scan rate: 30 mV s^{-1}, T: 25 ± 1 °C.

3.2. Cyclic voltammetry of AA and H2Q on GCE

Cyclic voltammetry (first cycle) of 5.0×10^{-4} mol l^{-1} of H_2Q in aqueous solution containing 0.1 M phosphate–citrate buffer (pH 5.05), shows one anodic (A_1) and corresponding cathodic peak (C_1), which correspond to the transformation of H_2Q to Q and vice versa within a quasi-reversible two-electron process (Fig. 3, curve a). A peak current ratio (I_{C1}/I_{A1}) of nearly unity, particularly during the recycling of potential, can be considered as criteria for the stability of Q produced at the surface of electrode under the experimental conditions. In other words, any hydroxylation [33–36] or dimerization [37,38] reactions are too slow to be observed in the time scale of cyclic voltammetry. The oxidation of H_2Q in the presence of AA as sample in aqueous solution containing 0.1 M phosphate–citrate buffer (pH 5.05), was studied in some details. It is seen that proportional to the augmentation of AA concentration, parallel to the decrease in height of C_1, the height of A_1 peak increases (Fig. 4 curves a–d). A similar situation is observed when the H_2Q concentration is decreased, while with decrease in height A_1, the height of C_1 peak decreases more sharply. The peak current ratio (I_{C1}/I_{A1}) versus concentration for a mixture of H_2Q and AA confirms the reactivity between both appearing as a decrease in the height of the cathodic peak C_1 at higher concentration of AA (Fig. 4).

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

The implementation of continuous-flow/stopped-flow programming and the location of two facing independent reactors (Fig. 1) 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 (see Table 1). The main advantages of this system are its simplicity and the ease with which it can be applied to the determination of AA at low levels.

If the reactor in the cell is devoid of rotation, there is practically no response. If a rotation of 900 rpm is imposed on the reactor located at the bottom of the cell (with immobilized HRP), the signal is dramatically enlarged. As shown in traces *d* in Fig. 5, if the lower reactor is devoid of rotation, the response is lower because diffusional limitations control the enzyme-catalyzed reaction. 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 initial rate is constant, and chemical kinetics controls the overall

Table 1
Values of K'_M (apparent Michaelis–Menten constant), determinated as discussed in the text (temperature: 20 ± 1 °C)

Rotation velocity (rpm)	K'_M [a] (mM)	Linear regression, standard deviation
170	22.03	±0.42
240	15.31	±0.30
420	10.81	±0.70
840	3.67	±0.10
900	2.98	±0.68
Estimated free enzyme in solution.	280.03	±0.33

[a] Each value of K'_M based on triplicate of six different substrate concentrations.

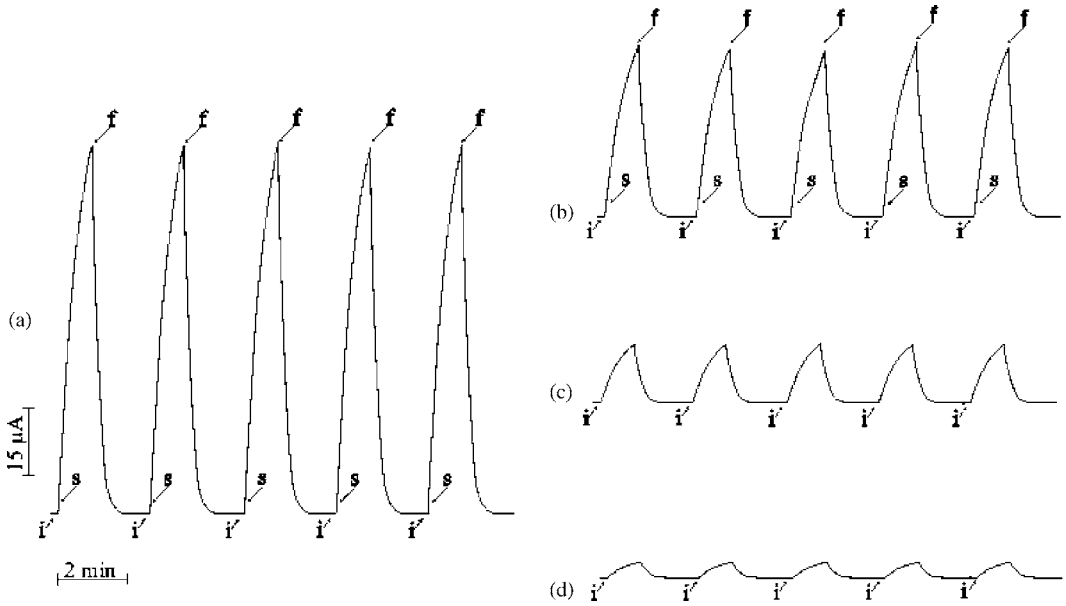


Fig. 5. Effect of reactor rotation under continuous and stopped-flow conditions. a: stopped flow with rotation. b: continuous flow with rotation. c: stopped flow without rotation. d: continuous flow without rotation. Flow rate, 1.00 ml min^{-1}, cell volume was 300 μl, 0.1 M phosphate–citrate buffer, pH 5.05 solution containing 1.0×10^{-3} mol l^{-1} H_2O_2, and 1.0×10^{-3} mol l^{-1} H_2Q. The flow was stopped for 60 s during measurement.

process. As observed earlier [39], although the mass transfer is being realized under conditions similar to a thin-layer bounded diffusion with imposed turbulence, the dependence seems to agree better with the response at a rotating disk electrode. Fig. 5 shows the effect of rotation under continuous and stopped flow conditions. Response to 1.0 mM H_2Q under continuous flow is relatively small but comparatively larger, if the reactor is rotated (compare traces (b) and (c) in Fig. 5). A significant signal that increases almost linearly as time develops when the disk is rotated. Under stopped flow conditions, there is a response but smaller than that with rotation. These responses indicate that the utilization of the biocatalytic action of the immobilized enzyme preparations is better under rotation of the reactor at the bottom of the cell.

The current developed at the detector should be directly proportional to the concentration of analyte in the bulk of solution and should increase with increasing rotation velocity. If the flow is stopped when the sample plug transported by continuous flow reaches the center of the reactor, detection take place under conditions similar to those of batch detection [40,41].

3.4. Effect of cell volume and sample size

Depending on the volume of the cell in contact with the reactors, 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 300 μl to 1 ml by removing the O-rings between the upper and lower half of the cell. The rate of response, 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 300 μl was adopted for further studies.

The rate of response increased linearly with sample size up to 200 μl in a cell with a volume of 300 μl. For convenience, a sample size of 200 μl was used to evaluate other parameters. Sensitivity is almost tripled in the range between 50 and 200 μl (Table 2).

3.5. The apparent Michaelis–Menten constant

As noted, rotation is expected to decrease the values of the apparent Michaelis–Menten constant, K'_M, since the catalytic efficiency is increased [42]. Michaelis constant differs substantially from that measured in homogeneous

Table 2
Effect of sample size

Sample size (μl)	i (μA)	Standard deviation, linear regression
50	7.03	±0.50
75	14.05	±0.28
100	20.86	±0.38
125	31.44	±0.30
150	42.68	±0.12
175	51.68	±0.62
200	59.66	±0.43
225	66.77	±0.18
250	70.13	±0.72
300	75.41	±0.42

The initial rate was measured under stopped-flow conditions, each value of initial rate based on triplicate of six determinations. In both cases, flow rate was 1.00 ml min^{-1}; cell volume was 300 μl; 0.1 mol l^{-1} phosphate–citrate buffer; pH 5.05; solution containing 1.0×10^{-3} mol l^{-1} H_2O_2, 1.0×10^{-3} mol l^{-1} H_2Q and 1.0×10^{-6} mol l^{-1} AA. The flow was stopped for 60 s during measurement.

solution and is not an intrinsic property of the enzyme but of the system. This constant characterizes the reactor, not the enzyme itself. It is a measure of the substrate concentration range over which the reactor response is linear [43]. Table 1 summarizes the values of K'_M for the system obtained at five different rotation velocities and stopping the flow for 60 s during measurement. The calculation of K'_M was performed under conditions in which [substrate] $\gg K'_M$, as a consequence, the following applies (Eq. (1)), assuming that the Briggs and Haldane scheme [44] is in operation:

$$\frac{1}{S} = \frac{m}{AA} + n \quad (1)$$

where S, m, and n are rate of response, slope, and intercept. The slope and intercept are obtained from the plot of $1/S$ versus $1/[AA]$ (Eq. (1)). This is a graphical approach similar to the Lineweaver–Burk plot.

3.6. Effect of pH, hydrogen peroxide, and hydroquinone concentration

The rate of response, under stopped-flow conditions, dramatically decreased (almost doubled) from pH 4.0 to 7.00 and continued a moderate decrease up to pH 8.00, the highest value tested. The pH used to evaluate the variables was 5.05, provided by a 0.1 M phosphate–citrate buffer.

The effect of varying H_2O_2 concentration from 7.0×10^{-4} to 5.0×10^{-3} mol l^{-1}, for 1.0×10^{-3} mol l^{-1} H_2Q solution and the effect of varying H_2Q concentration from 1.0×10^{-4} to 3.6×10^{-2} mol l^{-1}, for 1.0×10^{-3} mol l^{-1} H_2O_2 solution on the bioreactor response was evaluated. The optimum H_2O_2 and H_2Q concentration found were 1.0×10^{-3} mol l^{-1}, respectively. Those concentrations were then used.

Table 3
Specificity results of the amperometric measure[a]

Sample no.	Pure sample 1.0 (μM)	Synthetic tablet sample ($n = 5$) X (μM)
1	1.070	1.056
2	0.988	0.992
3	1.060	1.058
4	0.994	0.990
5	1.010	1.013
X	1.024 ± 0.017	1.022 ± 0.015
S.D.	0.039	0.034

[a] X (μM), mean ± standard error; S.D., standard deviation.

A linear relation (Eq. (2)) was observed between the rate of response and the AA concentration in the range of 12 nM and 3.5 μM (rotation 900 rpm).

$$\text{Rate of response } (\mu A/\text{min}) = 69.19 - 7.92\,[C_{AA}] \quad (2)$$

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 H_2Q solution. In this study, the minimal difference of concentration of AA was 6 nM. Reproducibility assays were made using repetitive standards ($n = 5$) of 1.0 μM; the percentage standard error was less than 4%.

The stability of the bioreactor was tested for nearly 3 h of continuous use in the FIA system. The long-term stability of the enzymatic system to pharmaceutical formulations was studied. In this experiment, after every four samples, a standard of 1.0 μM AA is injected to test the electrode response. In the FIA system using an enzymatic reactor, there

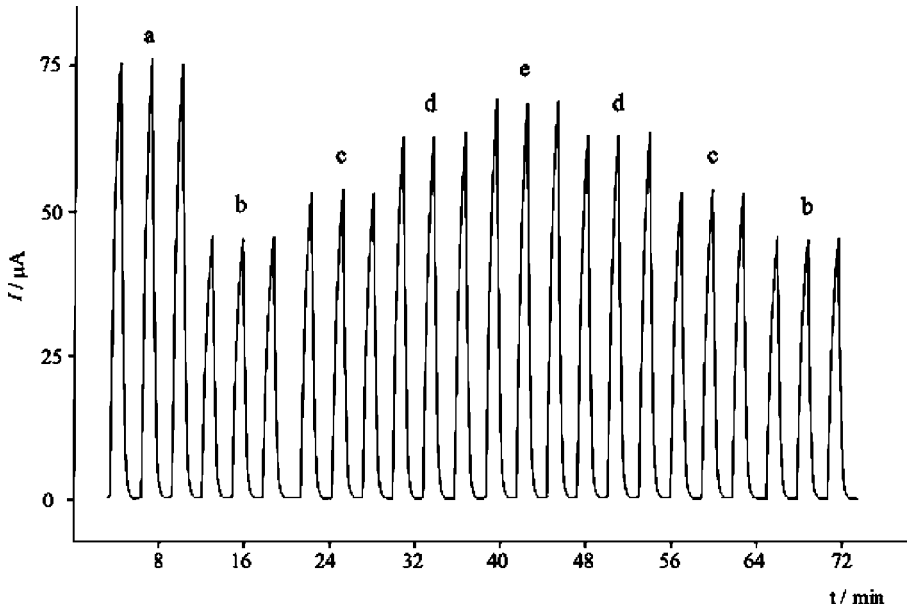


Fig. 6. Response of the microbioreactor for AA determination. (a) H_2Q 1.0×10^{-3} mol l^{-1}, and H_2O_2 1.0×10^{-3} mol l^{-1}, without addition of AA solution. From (b) to (e) is shown the response for several AA concentrations: (b) 2.0×10^{-6}, (c) 3.0×10^{-6}, (d) 7.0×10^{-7}, (e) 1.0×10^{-7} mol l^{-1}. The flow was stopped for 60 s during measurement.

Table 4
Accuracy and precision dates for AA obtained by amperometric measure

Added (g)	Found (g)	Recovery (%)	Precision (g)	Accuracy[a] (% relative error)
0.1	0.099	99.09	$X^{b} = 0.099 \pm 0.012$ $s^{c} = 0.005$ VC = 5.05%	−1
0.5	0.507	101.10	$X = 0.507 \pm 0.07$ $s = 0.009$ VC = 1.77%	1.4
1.00	1.017	100.70	$X = 1.017 \pm 0.05$ $s = 0.019$ VC = 1.87%	1.7
2.00	2.020	100.20	$X = 2.020 \pm 0.03$ $s = 0.028$ VC = 1.38%	1

[a] Accuracy = [(found − added)/added] × 100.
[b] X: mean.
[c] s: standard deviation.

is practically no decay in the catalytic current after 6 samples (Fig. 6).

3.7. Determination of AA in pharmaceuticals formulations

3.7.1. Specificity/selectivity

Specificity is the ability of the method to measure the analyte response in the presence of all the potential interference. For the specificity test, FIA of standard solution of tablet excipients were recorded at selected conditions. The response of the analyte with excipients, such as citric acid, glucose, lactose, magnesium stearate, oxalic acid, starch sucrose, tartaric acid, sodium chloride, and poly(ethylene glycol) 1500, were compared with the response of pure AA. It was found that assay results were not changed. Therefore, the excipients did not interfere with the quantization of AA as such in synthetic as well as in commercial tablet samples. In Table 3, the results are showed.

3.7.2. Recovery

Recovery studies were performed by adding a synthetic mixture prepared according to the manufacturer's batch formula to known amount of AA. The recovery was 101.4 %. The results are showed in Table 4.

3.7.3. Precision

The precision of a method is defined as the closeness of agreement between independent test results obtained under prescribed conditions. The precision around the mean value should not exceed 15% of the VC [45]. The precision for AA was 5.05% within the range 0.1–2.0 g. (Table 4).

Precision studies were performed by adding a synthetic mixture prepared according to the manufacturer's batch formula to known amount of AA.

3.7.4. Accuracy

The accuracy of a method is defined as the closeness of agreement between the test result and the accepted reference value. It is determined by calculating the percentage relative error between the measured mean concentrations and the added concentrations [45]. The accuracy for AA was 1.7% (Table 4).

3.7.5. Application to pharmaceuticals

The developed FIA-bioreactor method for the AA determination was applied to eight commercial preparations (Table 5). There is no need for any extraction procedure before FIA analysis. No change in the peak height in the presence of the excipients was observed. Table 3 gives the results of FIA analysis of commercial preparations. Table 6 gives

Table 5
Results obtained in the measurement of AA in pharmaceuticals formulations

Sample no.	Redoxon effervescent 2 g/tablet	Redoxon effervescent 1 g/tablet	Redoxon masticatory 0.5 g/tablet	Redoxon oral drops 0.2 g ml^{-1}	Vitamin C injectable 1 g/5 ml	Tanvimil C 0.5 g/tablet	Tanvimil C oral drops 0.2 g ml^{-1}	Cewin® 0.5 g/tablet
1	2.010	1.052	0.495	0.203	1.007	0.497	0.207	0.489
2	1.979	0.984	0.521	0.210	1.012	0.512	0.197	0.496
3	2.064	1.013	0.497	0.199	0.998	0.493	0.199	0.501
4	2.018	1.010	0.512	0.206	1.026	0.510	0.204	0.509
5	1.983	0.992	0.506	0.198	1.010	0.517	0.210	0.498
$X \pm$ S.D.	2.011 ± 0.034	1.010 ± 0.026	0.502 ± 0.008	0.203 ± 0.005	1.010 ± 0.010	0.504 ± 0.012	0.203 ± 0.006	0.496 ± 0.008

Table 6
Results for AA-containing commercial tablets analyzed by two techniques for brand A

Sample no.	Redoxon effervescent, 2 g/tablet	
	FIA-Bioreactor	CZE
1	2.010	2.016
2	1.979	1.985
3	2.064	2.055
4	2.018	1.988
5	1.983	2.049
$X \pm$ S.D.	2.011 ± 0.034	2.186 ± 0.032

the results obtained using the two methods for six separate determinations starting from different groups of tablets of AA. The results were compared, and there was no significant difference between the methods.

4. Conclusions

The usefulness of enzyme bioreactor used for the determination of very low concentrations of AA is demonstrated. In practice, the method is very simple and straightforward, it has the good applicability in pharmaceutical industry. The bioreactor developed in this work is able to operate as a fast, selective, and sensitive detection unit when incorporated into a FIA system, and it provides a fast and cost-effective solution for the realization of quantitative information at extremely low levels of concentrations.

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