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A New Laccase-Based Biosensor for Epinephrine Determination

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ABSTRACT

In this paper, a novel amperometric epinephrine (adrenaline) biosensor with immobilization of laccase on polypyrrole–polyvinylsulphonate (PPy-PVS) film has been a complished. Laccase enzyme were immobilized on PPy-PVS film by cross-linking with glutaraldehyde. Determination of epinephrine (EP) was carried out by the reduction of enzymatically produced epinephrinequinone at - 0.220 V vs. Ag/AgCl. The effects of pH and temperature were investigated. There are two linear parts in the region between 0.1 - 1.0 µM and 1.0 - 10.0 µM. The storage stability and operation stability of the enzyme electrode were also studied. Interference effects were investigated on response of the biosensor.

Keywords: Epinephrine, laccase, biosensor, polypyrrole (PPy), polyvinylsulphonate (PVS)

1. INTRODUCTION

Epinephrine (EP) is an important catecholamine neurotransmitter in the mammalian central nervous system [1]. Epinephrine is a potent agonist of cardiac α and β-receptors, which are biosynthesized in the adrenergic medulla and sympathetic nerve terminals, and secreted by the suprarenal gland, along with norepinephrine [2]. Although a large portion of plasma epinephrine arises from the adrenal medulla, several investigations have suggested that local cardiac epinephrine (adrenaline) storage and synthesis play important roles in regulating cardiac function [3], for example, restoring the cardiac rhythm in cardiac patients [4]. Many life phenomena are related to the concentration of EP in blood. Medically, EP has also been used as a common emergency healthcare medicine [5]. Thus, epinephrine determinations can be very useful for physiological studies.

Usually, the determination of EP is carried out by using liquid chromatography (LC) [6, 7], visible spectrophotometry [8] and fluorimetric flow injection [9]. EP is an electroactive molecule, its electrochemical behaviors have been studied [10-12] and some methods have also been reported for its electrochemical determination [13-15]. However, these methods suffer from complicated pretreatments. An amperometric biosensor based on an enzyme, laccase, has been considered to be a promising method because of its effectiveness and simplicity [16–18]. Laccase (EC 1.10.3.1, *p*-benzenediol: oxygen oxidoreductase) belongs to a family of multicopper oxidases that catalyze the oxidation of a range of inorganic and aromatic compounds (particularly phenols) with the concomitant reduction of molecular oxygen to water [18, 19–21].

In this study, we report a new laccase based amperometric biosensor for determination of EP which is prepared by

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immobilization of laccase onto a PPy-PVS film cross-linking with glutaraldehyde. The determination of epinephrine is performed by the reduction of enzymatically generated epinephrinequinone. The optimum working conditions of biosensor with respect to the substrate concentration, the pH and temperature were investigated. The storage stability and operation stability of the biosensor were also investigated.

2. EXPERIMENTAL

2.1. Equipment and Reagents

The electrochemical studies were carried out using CHI 1230B electrochemical analyzer using a three-electrode cell. The working electrode was a platinum (Pt) plate (0.5 cm^2). The auxiliary and the reference electrodes were Pt wire and Ag/AgCl electrode (3 M KCl), respectively. The pH values of the buffer solutions are measured with an ORION Model 720A pH/ion meter. Temperature control was achieved with Grant GD 120 thermostat. Laccase (EC 1.10.3.1, purified from the *Rhus Vernicifera*, activity of 990 unit/mL) was purchased from Sigma. Epinephrine was purchased from Sigma. Pyrrole and sodium polyvinylsulphonate (PVS) were supplied by Fluka and from Aldrich, respectively. All other chemicals were obtained from Sigma. All the solutions were prepared with the use of distilled water.

2.2. Preparation of Epinephrine Biosensor

The surface of the Pt plate electrode was cleaned [22]. It was covered with polypyrrole by the electropolymerization of pyrrole [23]. Immobilization of laccase was carried out by cross-linking with glutaraldehyde. The Pt plate electrode was immersed in a 10 mL solution of 0.1 M pyrrole and 2.5 mL (25%) of sodium polyvinylsulphonate. The solution was purged with argon in order to remove the oxygen. The electropolymerization of pyrrole was performed upon the electrode surface by the cyclic voltammetric scans between -1.0 and + 2.0 V at a scan rate of 50 mV/s (vs Ag/AgCl electrode (3 M KCl)) and 2 cycles. After electropolymerization onto the PPy-PVS film, the electrode was rinsed with deionized water to remove the unreacted pyrrole monomer. 50 µL of laccase enzyme (990 Unit/mL), 1 mg of bovine serum albumin, 30 µL of 0.1 M phosphate buffer (prepared at pH 5.0 and pH 8.0) and 20 µL of 2.5% glutaraldehyde were dropped upon PPy-PVS film. The electrode was dried at room temperature and washed with 0.1 M phosphate buffer solution (prepared at pH 5.0 and pH 8.0) several times in order to remove the non-immobilized enzyme and glutaraldehyde. Immobilized enzyme electrode was kept in a refrigerator at the 4 °C in phosphate buffer when not in use.

2.3. Amperometric Measurements

The Pt/PPy-PVS electrode was immersed into the 0.1 M phosphate buffer (prepared at pH 5.0 and pH 8.0). The solution contained 0.1 M potassium chloride as a supporting electrolyte. The electrode was brought to equilibrium by keeping it at – 0.220 V (vs. Ag/AgCl electrode (3 M KCl)). Steady-state current (i_a) was recorded. Epinephrine was added to the cell from stock solution and the system was stirred. The currents (i_b) obtained at – 0.220 V were recorded. The current values ($\Delta i=i_b-i_a$) were plotted against the epinephrine concentration in order to determine the working range of the electrode.

3. RESULTS AND DISCUSSION

In this study, we prepared a new epinephrine biosensor with cross-linking with glutaraldehyde of laccase on PPy-PVS film. The parameters affecting the performance of the biosensor were investigated. The determination of EP is performed by the reduction of enzymatically generated epinephrinequinone.

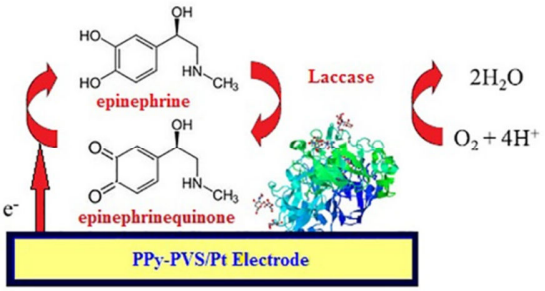


Fig. 1. Reaction scheme for epinephrine determination.

Figure 1 shows a scheme of the enzymatic processes between epinephrine (adrenaline) and laccase. Laccase catalyses the oxidation of epinephrine to epinephrinequinone Then, the epinephrinequinone are reduced on the electrode surface at potential of – 0.220 V. The resulting cathodic currents correlate directly with the concentration of each epinephrine in the sample solution.

3.1. The Determination of Working Potential

After the Pt/PPy-PVS electrode was prepared, different concentrations of epinephrine solution (0.05 mM, 0.1 mM, 0.5 mM, 1 mM) was added to the solution which contains 50 µL enzyme, 0.1 M phosphate buffer. DPV measurements of enzymatically produced epinephrinequinone was obtained (Figure 2). When Figure 2 is examined, it is seen that epinephrinequinone was reduced at nearly -0.220 V. -0.220 V was taken a working potential in the following studies.

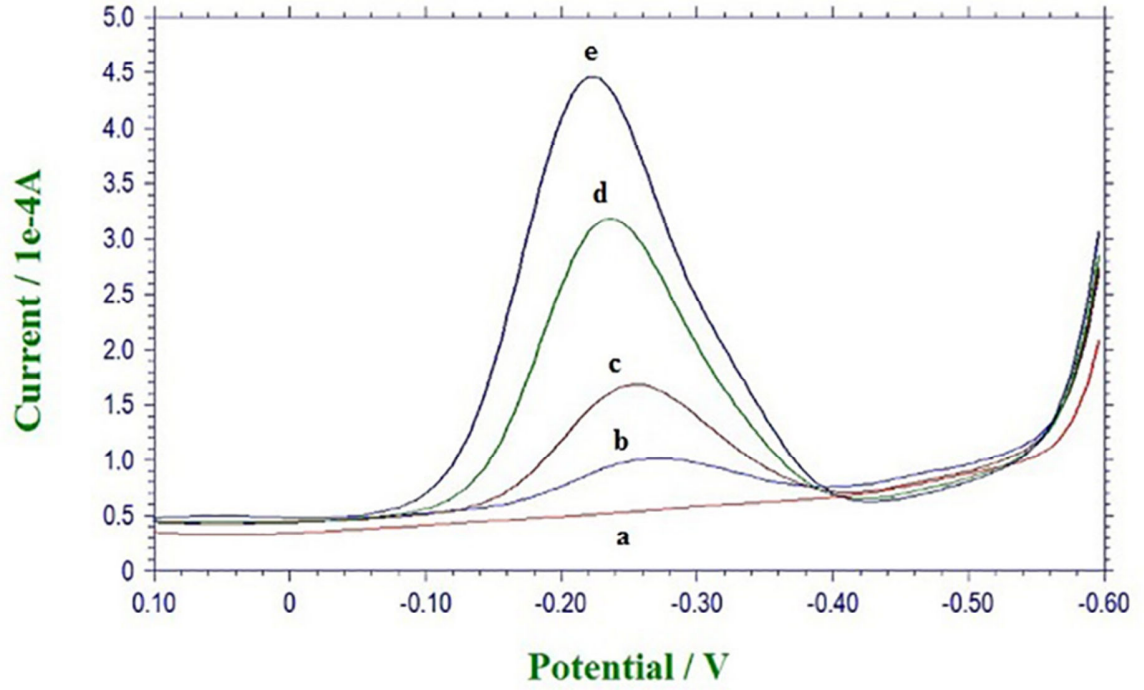


Fig. 2. DPV measurements of (a) PPy-PVS film in phosphate buffer, pH 7.0; in the presence of different concentrations of EP in 50 µL enzyme in phosphate buffer, pH 7.0 (b) 0.05 mM EP, (c) 0.1 mM EP, (d) 0.5 mM EP, (e) 1 mM EP.

3.2. Effect of Amount of Glutaraldehyde on the Response of Biosensor

In the construction of the biosensor, one of the suitable enzyme immobilization methods is cross-linking using glutaraldehyde [24]. To improve the performance of the biosensor, various factors influencing the response of the sensor, such as amount of glutaraldehyde, were investigated. Different amounts of glutaraldehyde solutions (10, 20, 30 µL for glutaraldehyde 2.5% glutaraldehyde solution) were used for immobilization of laccase. The results demonstrated that the activity of the cross-linking system increased when the amount of glutaraldehyde was increased from 10 to 20 µL (Figure 3). But when the glutaraldehyde amount was increased 20 to 30 µL, activity of the cross-linking system decreased. These results were explained by the amount of immobilized laccase. When the concentration of glutaraldehyde was low, the amount of immobilized protein was low [25]. For this reason, the current response of the epinephrine biosensor was decreased when the low glutaraldehyde concentration was used. But if the concentration of glutaraldehyde was higher, the immobilization film was thicker, causing a higher diffusion barrier [25]. In this study, 20 µL of glutaraldehyde, which was observed to give maximum activity with reasonable mechanical stability, was selected in the further characterization experiments.

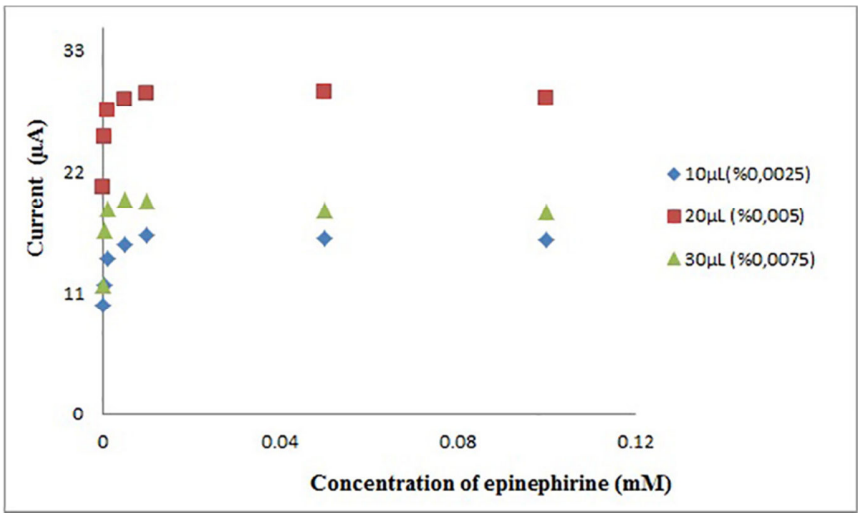


Fig. 3. The effect of glutaraldehyde amounts on the response of the biosensor (at 25 °C, at – 0.220 V operating potential).

3.3. The Effect of pH on Amperometric Response of Biosensors

Buffers solutions (0.1 M phosphate buffer) at various pH values were tested to investigate the effect of pH on amperometric response of biosensors. The pH values of the buffer solutions varied between 3.0 and 9.0. The measurements were performed at solutions containing 0.1µM – 0.1 mM EP. Furthermore, apparent $K_{m(app)}$, $I_{max(app)}$ values obtained at different pH are given in Table 1. From Table 1, it was seen that the most suitable decreasing $K_{m(app)}$, increasing $I_{max(app)}$ values were obtained from phosphate buffer. *Km* value for an enzyme shows the affinity of enzyme to a substrate. For EP biosensor; there are two optimum pH values, 5.0 and 8.0. There are pH values different than 5.0 and 8.0 like pH 7.0; 6.5 in literature [18, 26]. The difference in pH values was attributed to the fact that the used polymer and the type of immobilization were different. Because, the changings in environment of enzyme active site can cause different interactions between enzyme and immobilization materials [27-29].

Table 1. $K_{m(app)}$, $I_{max(app)}$ values obtained at different pH

pH Range (phosphate buffer, 25 °C)	$Km_{(app)}$ Value (µM)	$Imax_{(app)}$ Value(µA)
3	0.052	25.84
4	0.085	14.24
5	**0.015**	24.94
6	0.082	20.41
7	0.042	21.23
8	**0.011**	18.66
9	0.089	14.84

3.4. The Effect of Temperature

The amperometric response of the prepared biosensor was investigated at different temperatures using constant EP concentration of 10.0 µM (Figure 4). Epinephrine biosensor had two optimum pH values, 5.0 and 8.0. Temperature studies were made in both two pH values. In Figure 4, it was seen that optimum temperature for both pH values is 30°C. No response was observed in temperatures above 45°C. For the EP biosensor, temperature values different than 30°C were employed in the literature 55°C [30]. This was attributed to the fact that the used polymer and the type of immobilization were different. The study was carried out at 25°C due to the facilities.

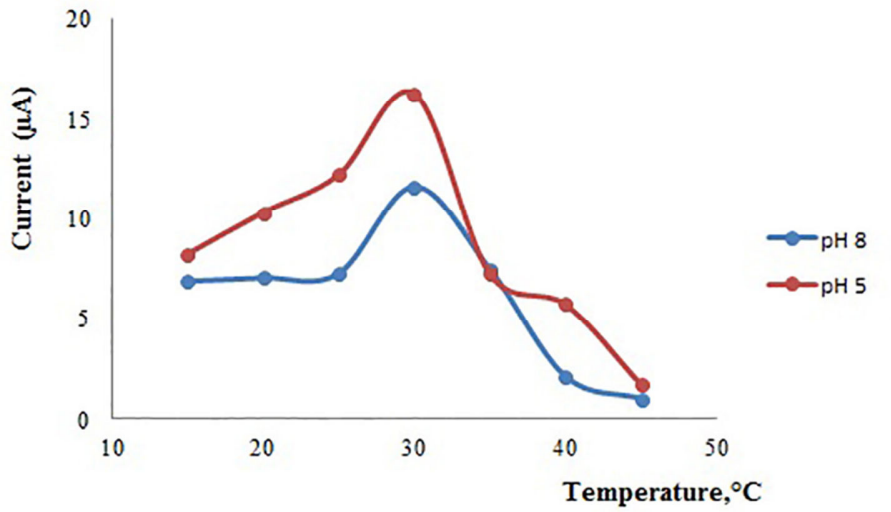


Fig. 4. The effect of temperature on the response of the biosensor (10.0 µM EP at –0.220V operating potential, phosphate buffer).

3.5. Substrate Concentration and Calibration Curves

In order to determine the linear working range of the EP biosensor, the current values obtained for solutions containing 0.1 µM - 0.1 mM EP at pH 5.0 and pH 8.0 were recorded (Figure 5). There are two linear parts in the region between 0.1- 1.0µM (R^2= 0.998) (Figure 6 (a)) and 1.0 – 10.0 µM (R^2= 0.995) (Figure 6 (b)) at pH 5.0. The graph for the lower concentration range is given in Figure 6 (a). It has been shown that the linearity of this graph is highly satisfactory and it can be used for the quantitative determination of EP. The low detection limit of the biosensor was found to be 0.01 µM and the response time of the biosensor was 200 s. There is linear part in the region between 0.1-1.0 µM at pH 8.0. The $K_{m(app)}$constant, a specific parameter of enzymes, was found out through the use of 1/[EP]- 1/Δi graph (Lineweaver - Burke plot). $K_{m(app)}$ and I_{max} values were found to be 0.027 µM and 27.3 µA at pH 5.0, respectively. $K_{m(app)}$ and I_{max} values were found to be 0.039 µM and 19.61 µA at pH 8.0, respectively. Following studies was made at pH 5.0 because that $K_{m(app)}$ value was low and there was large concentration range at pH 5.0

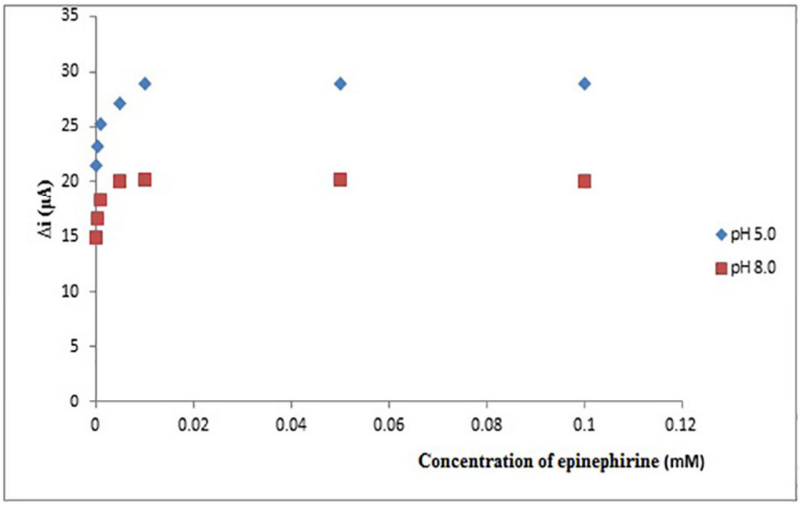
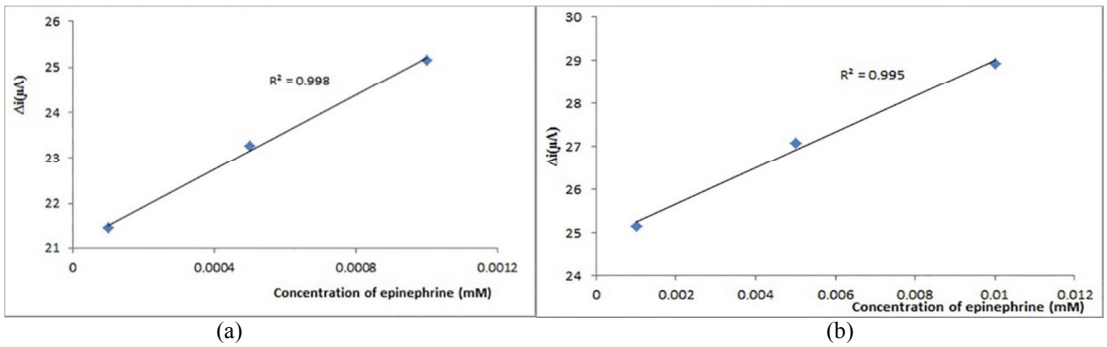


Fig. 5. The effect of EP concentration on the response of the biosensor (at 0.1 M phoshate buffer, 25°C, -0.220 V operating potential).



(a) (b)

Fig. 6. The calibration curve of EP biosensor (a) 0.1- 1.0μM EP (b) 1.0 – 10.0 μM EP (at 0.1 M, pH 5.0 phoshate buffer, 25 ^{o}C).

3.6. The Operational Stability of the Epinephrine Biosensor

In order to test the operational stability of the enzyme electrode prepared, the current changes obtained after subsequent usage were plotted against the number of measurements. Operational stability of biosensor was studied for pH 5.0 . When Figure VII is examined, it is seen that operational stability is very good. The relative standard deviations obtained after 31 measurements at a constant epinephrine concentration of 5.0 μM were found to be 5.08 %.

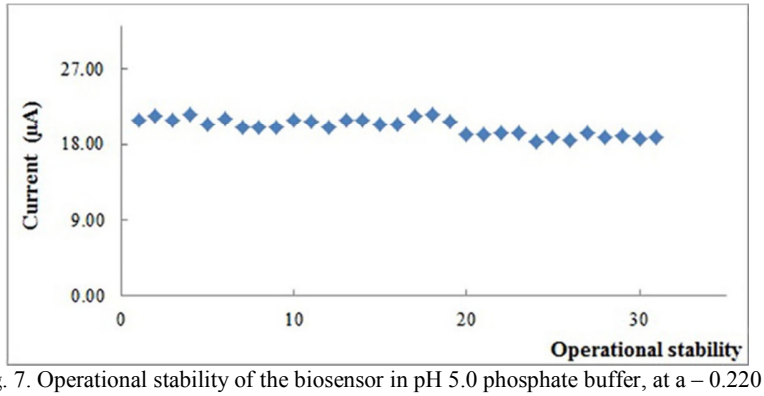


Fig. 7. Operational stability of the biosensor in pH 5.0 phosphate buffer, at a – 0.220 V operating potential, 25 °C

3.7. The Storage Stabilization of the Enzyme Electrode

The response of the enzyme electrode prepared under optimum conditions was measured for a period of 10 days at constant epinephrine concentration (5.0 μM). Results of nine measurements during this period are plotted in Figure 8. There was no change in the response during the first four days. But then the answer was a slight reduction in the response. The electrode showed 80.9% of the initial amperometric response at the end of ten day.

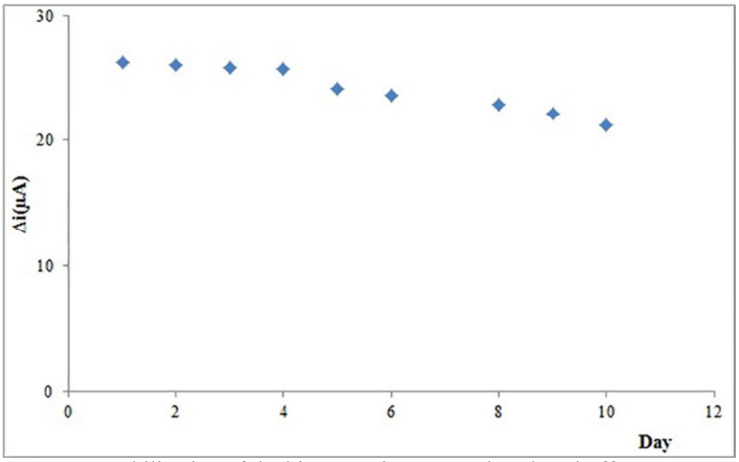


Fig. 8. Storage stabilization of the biosensor in pH 5.0 phosphate buffer, at a – 0.220 V operating potential, 25 °C

3.8. Interference Effect

The effect of several possible interfering substances (uric acid, ascorbic acid, dopamine, norepinephrine) on the epinephrine biosensor was investigated at – 0.220 V versus Ag/AgCl electrode in 0.1 M phosphate buffer solution (at pH 5.0). The concentration interferants were determined by 1000 times diluated. The influence of the interfering compound, reported in Table 2 as deviation of response percentage for a epinephrine concentration of 1 µM. Table 2 shows that uric acid, ascorbic acid and dopamine had no effect.

Table 2. Interference effects on response of the biosensor.

Interfering substances	Concentration (mM)	Epinephrine concentration (mM)	Interferences %
Uric acid	$1.0x10^{-3}$	$1.0x10^{-3}$	-
Ascorbic acid	$1.0x10^{-3}$	$1.0x10^{-3}$	-
Dopamine	$1.0x10^{-3}$	$1.0x10^{-3}$	-
Norepinephrine	$1.0x10^{-3}$	$1.0x10^{-3}$	0.32

3.9. Determination of Epinephrine in Sample Taken from Droxan

The determination of epinephrine in the epinephrine sample taken from droxan was performed on enzyme electrode utilizing a calibration method. The current response was determined in the 9.0 mL 0.10 M pH 5.0 phosphate buffer and 1.0 mL of 0.1 M potassium chloride solution, containing sample of 50 µ L (0.40 µM). Epinephrine amount in sample was determined from calibration graph as 0.39 µM ± 0.02 µM. The data points obtained with the enzyme electrode were the averages of the three measurements.

The results obtained with the biosensors showed a good correlation with those obtained by the reference method and allowed us to ascertain the practical applicability of the proposed biosensor.

4. CONCLUSION

Epinephrine biosensor prepared in this study is useable in a large concentration range 0.1- 1.0 µM (R^2:0.998) and 1.0-10.0 µM (R^2:0.995). It has a very low detection limit (0.01 µM) and an acceptable response time for a biosensor (200 s). Epinephrine biosensor gives perfect reproducible results at pH 5 after 31 measurements with 5.08 % relative standard deviation. Also, it has good storage stabilization gives 80.9% of the initial amperometric response at the end of the 10th day. Epinephrine biosensor had two optimum pH values, 5 and 8. Optimum temperature at pH 5.0 and pH 8.0 was found to be 30 °C. The *Km(app)* and

Imax(app) values (at pH 5) of laccase enzyme immobilized in PPy-PVS were 0.027 µM and 27.30 µA, respectively. Epinephrine biosensor prepared in this study is easy to prepare and is highly cost-effective.

Table 3. Optimum conditions and characteristics for epinephrine sensor

Parameters	Optimum conditions and characteristics
Response time	200 second
Working potential	- 0.220 V
Temperature	30 °C
pH	5.0 or 8.0
Working range	0.1-1µM, 1-10 µM (pH 5.0)
Detection limit	0.01 µM (pH 5.0)
Storage stabilization	Retained 80.9% of the initial amperometric response at the end of ten day (pH 5.0)
Operational stability	Retained 89.9% of the initial amperometric response at the end of 31 measurement (pH 5.0)
***Km*$_{(app)}$ and Imax$_{(app)}$**	0.027 µM, 27.3 µA (pH 5.0)

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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