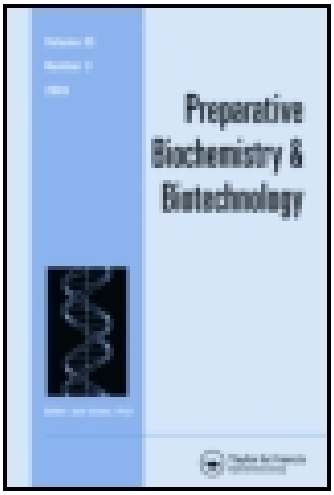

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A BIOSENSOR UTILIZING QUINCE (CYDONIA VULGARIS) TISSUE HOMOGENATE FOR DOPAMINE DETERMINATION IN PHARMACEUTICAL PREPARATIONS

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□ *Dopamine is a hormone and neurotransmitter occurring in a wide variety of animals, including both vertebrates and invertebrates. Chemically, it is a phenethylamine. Dopamine can be supplied as a medication that acts on the sympathetic nervous system, producing effects such as increased heart rate and blood pressure. However, since dopamine cannot cross the blood-brain barrier, dopamine given as a drug does not directly affect the central nervous system. To increase the amount of dopamine in the brains of patients with diseases such as Parkinson's disease and Dopa-Responsive Dystonia, L-DOPA (levodopa), which is the precursor of dopamine, can be given because it can cross the blood-brain barrier.*

In this study, a biosensor based on quince tissue homogenate was constructed for determination of dopamine. For the best results, some optimization studies such as the amount of quince tissue homogenate, gelatin and glutaraldehyde percentage, optimum temperature and pH were carried out. A linear range from 5 μM to 200 μM dopamine was obtained. Moreover, repeatability, and operational and storage stability of the biosensor were determined. Finally, the biosensor was applied to a real drug sample for the determination of dopamine content.

Keywords biosensor, clark electrode, dopamine, phenolic compounds, tissue biosensors

INTRODUCTION

Phenolic compounds determination is becoming important in the area of medicine, since some of these compounds belong to the neurotransmitter class. Dopamine, epinephrine (adrenalin), and seretonin are compounds called cathecholamines, having great clinical and pharmaceutical interest.[1] Cathecholamines are compounds that consist of amines attached to a benzene ring bearing two hydroxyl groups (catechol). As one of the cathecholamines, dopamine (3,4-dihydroxyphenylethylamine) is a central neurotransmitter, particularly important in the regulation of movement and possesses important intrinsic pharmacological properties.[2]

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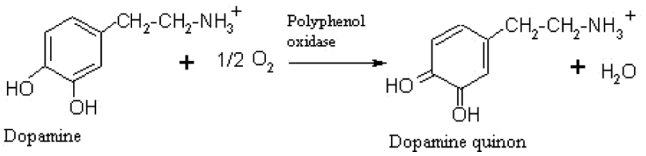

SCHEME 1 The reaction scheme for polyphenol oxidase occurred in the bioactive layer of the biosensor.

Polyphenol oxidase (E.C. 1.14.18.1) catalyses the ortho-hydroxilation of phenols and the oxidation of cathechols to ortho-quinone in the presence of O_2.[3] It is present in fruits such as mushroom tissue, apple tissue, banana pulp, and quince tissue and it is responsible for the dark color occurring after clipping. It is also found in microorganisms, animals, and plants.

Several methods have been employed to determine dopamine. United States Pharmacopoeia (USP) recommends the use of a high-performance liquid chromatography with spectrophotometric detection at 230 nm.[3] Other alternative methods for the quantification of this compound in formulations involved techniques such as chromatography,[4,5] molecular fluorescence,[6] chemiluminescence,[7,8] potentiometry,[9,10] amperometry,[11,12] and spectrohotometry.[13,14] Although all these methods are available for dopamine determination, most of them are complicated, expensive, and time-consuming protocols.

In this paper, we aimed to develop a quince tissue homogenate based biosensor for quantifying dopamine by using an amperometric Clark-type oxygen electrode. The biocatalytic activity of this electrode depends on polyphenol oxidase (E.C.1.14.18.1) from quince tissue being immobilized on the electrode with gelatin by the help of glutaraldehyde. The principle of the measurement is to determine the amount of oxygen consumed during the conversion of dopamine to dopamine-quinine and H_2O by polyphenol oxidase. The oxygen consumption is proportional to the dopamine concentration and it was determined using an YSI 54A model oxygen meter according to the reaction illustrated in Scheme 1.

EXPERIMENTAL

Reagents and Solutions

All chemicals were of analytical grade, used without further purification. Buffer solutions we prepared using distilled water. The quince used was purchased from a local grocery. 25×10^{-3} μM dopamine stock solution used as substrate was daily prepared in phosphate buffer (pH 7.5, 50 mM).

Dopamine, glutaraldehyde (25%), gelatin (Type 3, 225 Bloom), SDS were supplied from Sigma, St. Louis, ABD. Phenol, catechol, pyrogallol, 8-hydroxyquinone, 1-naphthol were acquired from E. Merck, Germany.

Instrumentation

A YSI model 54A oxygen meter, YSI 5739 Model dissolved oxygen (DO) probes (YSI Co., Yellow Spings, USA), highly sensitive Teflon membrane (0.0005 in thick) for oxygen, thermostat (Haake FJ, Germany), magnetic stirrer (IKA-Combimag, RCO) and, for preparing buffer solutions, a pH meter (WTW pH 538, Germany) was used.

Biosensor Preparation

A dissolved oxygen probe was covered with highly-sensitive Teflon membrane by using an O-ring and then the membrane selective for oxygen was pretreated with 0.5% sodium dodecyl sulfate (SDS) in phosphate buffer (pH 7.5, 50 mM) to reduce the tension on the membrane surface.

For preparation of the bioactive layer, 300 mg quince tissue was homogenized in 800 µL phosphate buffer (pH 7.5, 50 mM). 30 mg gelatin was weighed and added to a test tube containing 600 µL quince tissue homogenate. This mixture was incubated at 38°C until the gelatin dissolved.

200 µL of gelatin-quince tissue homogenate mixture was dispersed over the dissolved oxygen probe membrane surface and allowed to dry at 4°C, for about an hour. The probe carrying the bioactive layer was immersed into 2.5% (v/v) glutaraldehyde solution (prepared in 10 mL, pH 7.5, 50 mM phosphate buffer) about 4 minutes for cross-linking. At the end of this time, after washing the biosensor, it was ready to use.

After using the biosensor, it was stored in a flask having distilled water at 4°C. The biosensor shouldn't contact with this water. It is just important for providing a moisture medium so that it is possible to protect the bioactive layer from dryness.

Measurement Procedure

The quince-tissue homogenate biosensor was put into the thermostatic reaction cell containing working buffer (phosphate buffer pH 7.5, 50 mM) and the stirrer was fixed at 1000/min. speed. We waited until the concentration of the dissolved oxygen between working buffer and dissolved oxygen probe was equilibrated. At that time, a sample or standard solution was added to the thermostatic reaction cell. It was seen that the DO concentration changed due to the dopamine concentration. Measurements were

carried out by standard curves which were obtained by determination of consumed oxygen level by the catalytic reaction of polyphenol oxidase in the bioactive layer of the biosensor. In all measurements, phosphate buffer solutions were used. The pH value of the phosphate buffer could be adjusted to a desired value easily. Also, it is one of the most used buffer compounds in the range of pH 6–8. Moreover, it is one of the cheapest buffer materials.

RESULTS AND DISCUSSION

Optimization Studies of the Biosensor

Effect of the Quince Tissue Homogenate Amount

For the determination of the effect of tissue homogenate amount, different amounts of quince tissue homogenate (100 mg, 200 mg and 300 mg) were dispersed on the DO (dissolved oxygen) probe one by one while the gelatin and gluteraldehyde amount kept constant.

It is clear that increasing the amount of tissue used could increase the enzyme pholyphenol oxidase which catalyses the reaction. Consequently, the signals probably increase. As shown in Figure 1, biosensor response increases proportionally with an increase of tissue amount.

However, more increase in the amount of tissue didn't increase the biosensor signals. With 400 mg quince tissue, because of the presumptive compact diffusion effects, a meaningful signal could not be obtained. As a result, 300 mg quince tissue homogenate was obtained as the optimum amount.

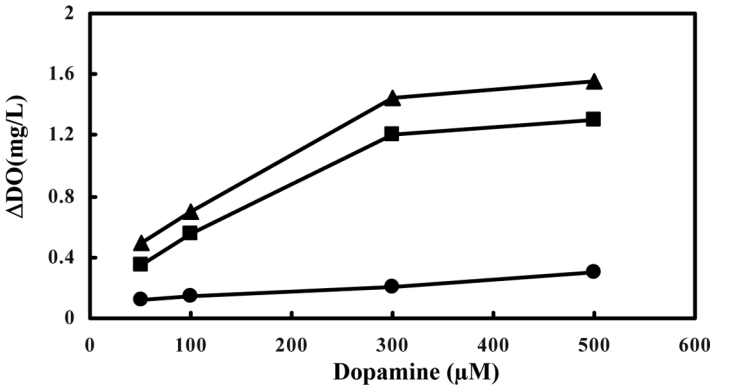

FIGURE 1 The effect of quince tissue homogenate amount on the activity of the biosensor [-●-●-: 100 mg, -■-■-: 200 mg, ▲-▲: 300 mg. The amount of glutaraldehyde and gelatin were kept constant at 2.5% and 10 mg, respectively. Working conditions: Phosphate buffer, 50 mM, pH 7.5, T:35°C].

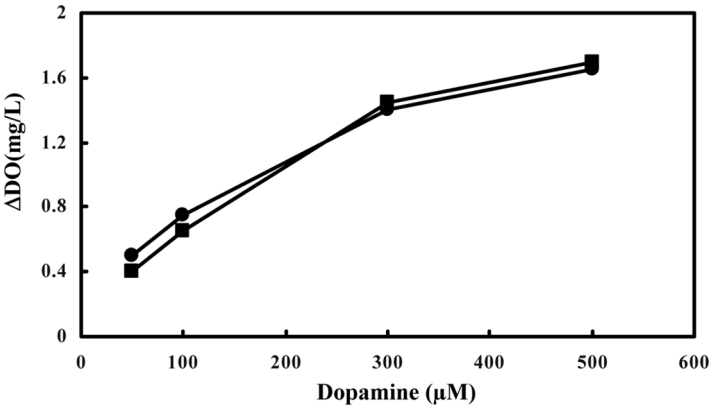

FIGURE 2 The effect of gelatin amount on the biosensor [-■-■-: 10 mg, -●-●-: 20 mg. The amount of quince tissue homogenate and glutaraldehyde were kept constant at 300 mg and 2.5%, respectively. Working conditions: Phosphate buffer, 50 mM, pH 7.5, T:35°C].

Effect of Gelatin Amount

Gelatin amounts differing as 10 mg and 20 mg was used to determine the effect of gelatin amount.

As shown in Figure 2, the biosensor response obtained with 10 mg and 20 mg gelatin was closed to each other. Besides, using lower amounts of gelatin caused an unstable bioactive layer. As a result, 10 mg was determined as the optimum amount of gelatin.

Effect of Glutaraldehyde Amount

In an investigation of the effect of differing glutaraldehyde amounts on the dissolved oxygen probe surface, experiments were carried out by

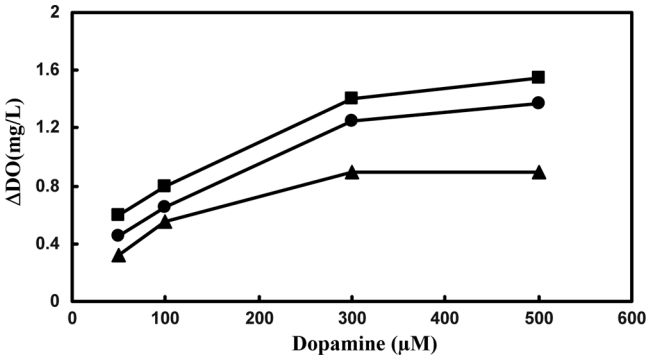

FIGURE 3 The effect of glutaraldehyde percentage on the activity of the biosensor [-●-●-: 1.25%, -■-■-: 2.5%, ▲-▲: 5%. The amount of quince tissue homogenate and gelatin were kept constant at 300 mg and 10 mg, respectively. Working conditions: Phosphate buffer, 50 mM, pH 7.5, T:35°C].

keeping the amount of quince tissue homogenate and gelatin constant while changing the amount of glutaraldehyde as 1.25%, 2.5%, and 5%.

As is obvious from Figure 3, the highest biosensor response was observed when 2.5% glutaraldehyde was used.

Effect of pH

The effect of pH in the enzymatic activity was studied in the range of 6.5–8. The dependence of the response of the biosensor is shown in Figure 4 and, according to the figure, biosensor activity was determined to be maximum at pH 7.5. Previous papers with PPO of other plant sources like banana, pear, and mango have shown that the optimum pH for the PPO activity is close to pH: 7.5, so this value was accepted as the optimum pH.

Effect of the Buffer Concentration

In order to determine the effect of the buffer concentration, phosphate buffers with differing concentrations (25, 50, 100, 150, 200, and 250 mM) at constant pH (7.5) were used. The maximum biosensor response was obtained when we worked with 50 mM phosphate buffer solution. For 100 mM buffer concentration, the biosensor signal was about 24% lower than that found at 50 mM and its value decreased for concentration values higher than 100 mM, respectively, 39.5%, 39.5%, and 46.1%. The biosensor response was dramatically decreased at a concentration value of 25 mM. This was probably caused from weak ionic strength effects on catalytic activity of the biosensor. At higher concentrations than 50 mM, the biosensor response was decreased, probably because of the

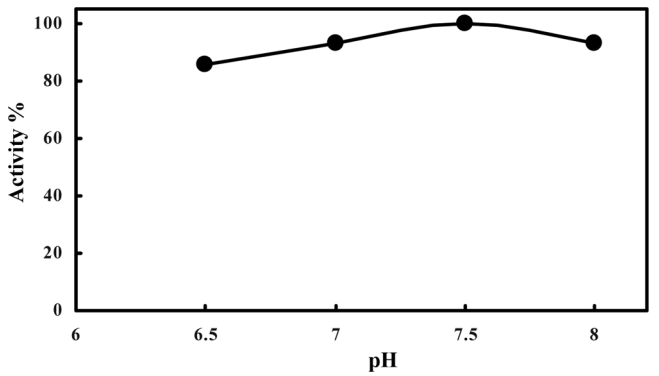

FIGURE 4 pH dependence of the biosensor [Phosphate buffers with varying pHs: 6.5, 7.0, 7.5 and 8, 50 mM. The amount of quince tissue homogenate, glutaraldehyde percentage and gelatin were kept constant at 300 mg, 2.5% and 10 mg, respectively. T:35°C].

negative effect of the high ionic strength of the working buffer. Moreover, this point could affect the substrate ionic structure. As can be understood from the results, the optimum buffer concentration was obtained as 50 mM.

Effect of Temperature

Temperature is a key factor for most enzymatic reactions. In most cases, the activity of the enzyme increases with increasing temperature till the definitive value. The effect of temperature on quince tissue homogenate biosensor response was examined at temperatures varying between 20–45°C. Results showed that the biosensor response was becoming higher proportional with temperature up to 35°C. The response decreased at temperatures above 35°C, probably due to the damage to the structure and metabolism of the plant, as well as denaturation of the enzyme in the cell (Figure 5).

As a result the highest biosensor response was obtained at 35°C as the optimum temperature.

Operational Stability

Determination of the operational stability was carried out under optimized conditions determined before.

For this investigation, the measurements were taken one after another in one hour's intervals. As is obvious from the figure, the activity of the biosensor kept nearly % 50 in 8 hours (Figure 6).

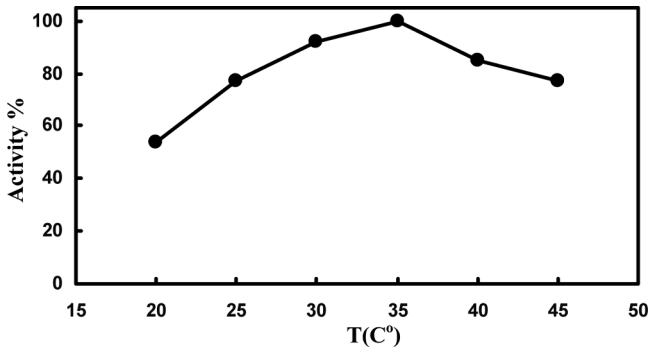

FIGURE 5 Optimum temperature of the biosensor [The amount of quince tissue homogenate, glutaraldehyde percentage and gelatin were kept constant at 300 mg, 2.5% and 10 mg, respectively. Working conditions: Phosphate buffer, 50 mM, pH 7.5].

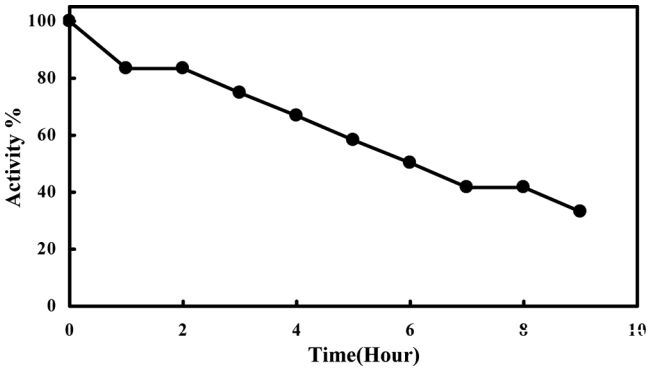

FIGURE 6 Operational stability of the biosensor [The amount of quince tissue homogenate, glutaraldehyde percentage and gelatin were kept constant at 300 mg, 2.5% and 10 mg, respectively. Working conditions: Phosphate buffer, 50 mM, pH 7.5, T:35°C, Dopamine concentration used as standard was 100 μM].

Characterization Studies of the Biosensor

Linear Range

A linear calibration curve was obtained over the range $5 \cdot 10^{-3}$–$2 \cdot 10^{-1}$ mM dopamine concentration at the conditions investigated by the studies above. The linear calibration curve of the biosensor is shown in Figure 7.

Repeatability

Repeatability studies were carried out with 0.1 mM dopamine concentration and 8 measurements were taken one after another. The average

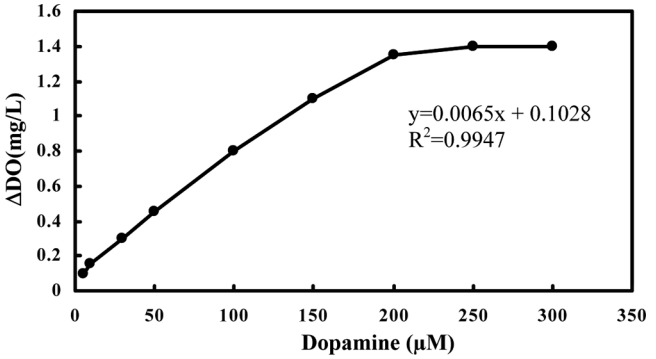

FIGURE 7 Calibration graph for dopamine [The amount of quince tissue homogenate, glutaraldehyde percentage and gelatin were kept constant at 300 mg, 2.5% and 10 mg, respectively. Working conditions: Phosphate buffer, 50 mM, pH 7.5, T:35°C].

TABLE 1 Substrate Specificity of the Biosensors

Substrate	Activity (%)
Dopamine	100
Phenol	92.3
Catechol	84.6
Pyrogallole	84.6
8-Hydroxyquinoline	46.1
1-naphtol	23

value (x), the standard deviation (S.D) and the variation coefficient (C.V) were calculated as 0.096, ±5.72 and % 5.95, respectively.

Storage Stability

The effect of storage stability on quince tissue homogenate based biosensor activity was also determined. The biosensor protected its activity in first 7 days and at the end of 28 days only % 14.3 of its activity lost. After 42 days it was still had nearly % 60 of its initial activity.

Substrate Specificity

In order to evaluate substrate specificities, interference effects of some phenolic compounds were studied. The concentrations of all compounds tested were 100 μM. Because of the group specificities of the polyphenol oxidases, all compounds tested were found to have interference effect in varying degrees on the determination of dopamine. Table 1 summarizes the relative interference effects.

The presented biosensor was constructed for dopamine determination in pharmaceutical preparations. Consequently, the interference effects of the substances in Table 1 were not important.

Real Sample Analysis

To demonstrate the applicability of the biosensor based on quince tissue homogenate for dopamine analysis, a commercial drug named Giludop was analyzed. These data are shown in Table 2.

TABLE 2 Dopamine Analysis in a Real Sample

Sample	Amount Added (μM)	Amount Found* (μM)	Average Recovery (%)
Giludop	20	40	100

*Average of three determinations.

CONCLUSION

In this work a biosensor based on quince tissue homogenate contained polyphenol oxidase for the detection of dopamine has been successfully developed. The optimum conditions of the biosensor parameters for the enzyme immobilization procedure and the use of optimum working conditions allowed the development of a biosensor possessing high response repeatability, good analytical characteristics, a wide linear response and a very good long-term operational stability. The biosensor was successfully used as an efficient screening tool to test the presence of dopamine in pharmaceuticals.

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