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A biosensor based on urate oxidase–peroxidase coupled enzyme system for uric acid determination in urine

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Abstract

A new amperometric biosensor based on urate oxidase–peroxidase coupled enzyme system for the specific and selective determination of uric acid in urine was developed. Commercially available urate oxidase and peroxidase were immobilized with gelatin by using glutaraldehyde and fixed on a pretreated teflon membrane. The method is based on generation of H_2O_2 from urine uric acid by urate oxidase and its consuming by peroxidase and then measurement of the decreasing of dissolved oxygen concentration by the biosensor. The biosensor response depends linearly on uric acid concentration between 0.1 and 0.5 μM. In the optimization studies of the biosensor, phosphate buffer (pH 7.5; 50 mM) and 35 °C were obtained as the optimum working conditions. In addition, the most suitable enzyme activities were found as 64.9×10^{-3} U cm^{-2} for urate oxidase and 512.7 U cm^{-2} for peroxidase. And also some characteristic studies of the biosensor such as reproducibility, substrate specificity and storage stability were carried out.

Keywords: Uric acid; Urate oxidase; Peroxidase; Coupled enzyme systems; Biosensors

1. Introduction

The development of biosensors is most promising in the progress of analysis of biologically active compounds. In the past two decades biosensors had emerged from laboratories and some cases became conventional devices for routine analysis [1–3] owing to the advantages biosensors possess, such as simple measurement procedure, short response time, sensitivity and selectivity. As a result of these important factors biosensors can be widely used for the analysis of many metabolites [4–6]. Traditional monitoring methods are often slower and call for expensive equipment which makes them unsuitable for real time control and this also makes biosensors more attractive [7].

Uric acid is the primary end-product of purine metabolism and its excreted in the urine is derived from purines arising from the catabolism of dietary and endogenous nucleic acid stem from increased catabolism dysfunction of one of the shunt pathways which leads to increased urate production. Excessive production of uric acid may lead to precipitation in the kidney and lower extremities [8]. Moreover, one of the biggest problems about the uric acid metabolism is the

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Gout disease. Gout is caused by an excess of uric acid in the body. And this excess can be caused by an increase in production by the body, via a decrease in elimination of uric acid by the kidneys, or increasing of intake of foods containing purines which are metabolized to uric acid in the body. Also, it has been suggested that one function of uric acid in human body fluids is to act as an antioxidant [9]. Consequently, the measurement of uric acid for the diagnosis and treatment of various disorders is very important.

For the uric acid determination in serum and urine several study about biosensors had been reported [10–14]. A reagentless amperometric urate biosensor based on uricase and peroxidase enzymes immobilized in carbon paste without the addition of an electron transfer mediator have been described [15].

In the present study, we report a new amperometric biosensor based on the coupling two enzymes, which are urate oxidase (E.C 1.7.3.3) and peroxidase (E.C 1.11.1.7), for high sensitive, not time consuming and specific measurement of uric acid.

Measurements were carried out by standard curves which were obtained by the determination of consumed oxygen level, related to uric acid concentration in the enzymatic reactions, given below.

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{Urate oxidase}} \text{Allantoin} + CO_2 + H_2O_2 \quad (1)$$

$$\text{Uric acid} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Oxide donor} + 2H_2O \quad (2)$$

In the first reaction, the oxygen consumption depending on the substrate concentration, i.e. uric acid, was monitored. However, the product, H_2O_2 undergoes a non-enzymatic decomposition and produces oxygen which contributes the total concentration of dissolved oxygen (DO) at the electrode surface. Therefore, resulting ΔDO values were found much lower than expected that for a certain uric acid concentration.

If the two reactions are coupled by using both enzyme, the product of the first reaction, H_2O_2, is subsequently consumed by the second reaction. In this case, the contribution to the total oxygen concentration from the non-enzymatic decomposition is eliminated. Therefore, the biosensor based on urate oxidase–peroxidase coupled enzyme system serves as a tool which allows to obtain more sensitive results than those with the biosensor containing urate oxidase enzyme [16].

2. Experimental

2.1. Chemicals

Urate oxidase (E.C 1.7.3.3) from porcine liver and peroxidase (E.C 1.11.1.7) from horse radish used in the biosensors construction, uric acid monosodium salt, uric acid kit, calf skin gelatin (225 bloom), glutaraldehyde (25%) and all other chemicals were purchased from Sigma Chemical Co., USA. All solutions used in the experiments were prepared just before their use.

2.2. Apparatus

In this study, a YSI Model 57 oxygenmeter, YSI 5739 Model DO probes (with YSI 5740 model cable) (YSI Co., Yellow Springs, USA), high sensitive teflon membranes (0.0005 in thick) for oxygen and Ultra-thermostat (Colora, FRG) were used.

2.3. Preparation of the biosensor

To construct the biosensor DO probe was covered with high-sensitive teflon membrane by using an O-ring and then teflon membrane which is selective for oxygen was pretreatment with 0.5% SDS (sodiumdodecylsulphate) in phosphate buffer (50 mM, pH 7.5) to reduce the tension on the membrane surface. After this step, urate oxidase (110 U), peroxidase (600 U) and gelatin (20 mg) were mixed in phosphate buffer (300 μl) at 38 °C for a few minutes to dissolve the mixture. Subsequently 200 μl of the solution was spread over the DO probe membrane surface and allowed to dry at 4 °C for 1 h. At the end of this time the biosensor was crosslinked with glutaraldehyde (2.5%) in

phosphate buffer (50 mM, pH 7.5) and a bioactive layer was formed on the DO probe so a new biosensor based on urate oxidase–peroxidase coupled enzyme system which was sensitive and specific for uric acid determination was developed.

2.4. Measurements

To determine the concentration of uric acid, oxygen consumption which occured in the reactions catalyzed by the coupled enzyme system was detected. The measurements with the biosensor developed were done at steady-state conditions. There is an interval surface between bioactive layer and Teflon membrane of DO probe. ΔDO express distinction of DO concentration when the substrate is not in the medium and after addition of substrate to the medium until steady-state. ΔDO is not DO concentration of reaction cell (or the medium). But it expresses the differences of DO concentration, related to uric acid concentration, which occur in the interface between the bioactive layer and teflon membrane of DO probe. All the measurements were done at 35 °C by using a thermostatic reaction cells [17] and the oxygen saturated phosphate buffer (50 mM, pH 7.5) was used in all experiments.

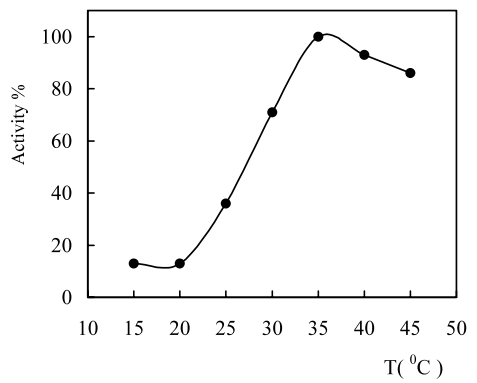

Fig. 1. The effect of temperature on the biosensor response. (Phosphate buffer; pH 7.5, 50 mM. The activity of urate oxidase, peroxidase, the percentage of glutaraldehyde and gelatine amount were kept constant at 64.9×10^{-3} U cm^{-2}, 512.7 U cm^{-2}, 1.25% and 11.8 mg cm^{-2}, respectively.)

2.5. Application

For the determination of uric acid level in urine the biosensor developed and uric acid kit (Sigma Co., USA) methods were used. For this purpose uric acid concentration in urine sample diluted 7500-fold was determined by using both methods. After determining uric acid concentration in urine sample (a), the results obtained by both methods were checked by using standard addition method. In the experiments known uric acid amount (b) was added to urine sample and total uric acid concentration in urine sample (a+b) was determined by using the biosensor and the kit method.

3. Results and discussion

3.1. Influence of experimental parameters

3.1.1. Effect of pH

To determine the effect of pH value on the biosensor response different buffer systems were investigated. For this purpose 50 mM concentration of citrate (pH 5.5–6.5), phosphate (pH 7.0–8.5) and glycine (pH 9.0–10.0) buffers were used in the experiments. The optimum pH value was obtained as 7.5.

3.1.2. Effect of temperature

For determination of temperature effect on the biosensor response, the experiments were carried out between 15 and 45 °C. Results obtained were given in Fig. 1. As illustrated, the highest biosensor response was observed at 35 °C. Below and above 35 °C, decreases in the biosensor response resulted from changes in tertiary structures of the enzymes were recorded.

3.1.3. Effect of the enzyme activity

Uric acid is a substrate for both urate oxidase and peroxidase and H_2O_2 is a product of the first reaction catalyzed by urate oxidase and at the same time it is also a co-substrate for peroxidase. In the presence of H_2O_2 the second reaction catalyzed by peroxidase occurs.

To investigate the effect of enzyme activities on the biosensor response, firstly the activity of

peroxidase in the bioactive layer of the biosensor was kept constant as 512.7 U cm^{-2}, however, the activity of urate oxidase was changed as 32.34×10^{-3}, 64.9×10^{-3} and 97.24×10^{-3} U cm^{-2}, respectively. From the experiments, better linear range and reproducible results were obtained when 64.9×10^{-3} U cm^{-2} urate oxidase activity was used. After this study, the activity of urate oxidase in the bioactive layer of the biosensor was kept constant as 64.9×10^{-3} U cm^{-2} and the activity of peroxidase was changed as 170.5, 342.2 and 512.7 U cm^{-2}. According to the results obtained, the best biosensor response was carried out with 512.7 U cm^{-2} peroxidase activity. The results obtained from the studies were given in Figs. 2 and 3.

3.2. Analytical characteristics

3.2.1. Linear range

The data obtained for the determination of detection limits for uric acid were given in Fig. 4. When we consider the Figure, the biosensor responses depend linearly on uric acid concentration between 0.1 and 0.5 μM.

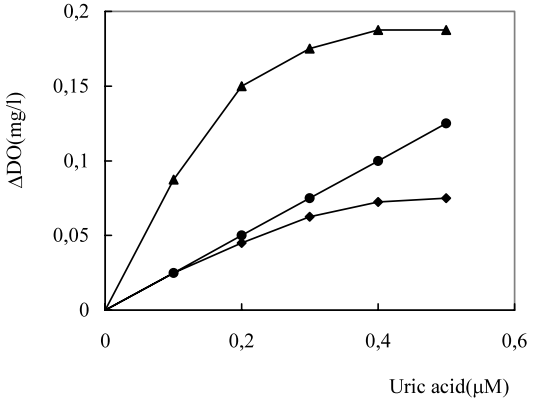

Fig. 2. The effect of urate oxidase activity on the biosensor response. (Phosphate buffer; pH 7.5, 50 mM; T 35 °C. The activity of urate oxidase; (◆-◆)32.34×10^{-3} U cm^{-2}, (●-●) 64.9×10^{-3} U cm^{-2}, (▲-▲) 97.24×10^{-3} U cm^{-2}. The activity of peroxidase, the percentage of glutaraldehyde and gelatine amount were kept constant at 512.7 U cm^{-2}, 1.25%, 11.8 mg cm^{-2}, respectively.)

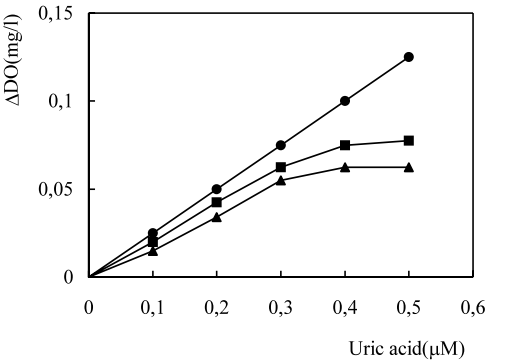

Fig. 3. The effect of peroxidase activity on the biosensor response. (Phosphate buffer; pH 7.5, 50 mM; T 35 °C. The activity of peroxidase; (▲-▲) 170.5 U cm^{-2}, (■-■) 342.2 U cm^{-2}, (●-●-) 512.7 U cm^{-2}. The activity of urate oxidase, the percentage of glutaraldehyde and gelatine amount were kept constant at 64.9×10^{-3} U cm^{-2}, 1.25% and 11.8 mg cm^{-2}, respectively.)

3.2.2. Reproducibility

The reproducibility of the biosensor was also investigated for 0.3 μM uric acid concentration ($n = 10$), the average value ($\bar{x}$), the standard deviation (S.D.) and variation coefficient (C.V.%) were calculated as 0.296 μM, ±0.009 μM and 2.96%, respectively.

3.2.3. Specificity

For determination of substrate specificity of the biosensor 0.3 μM of standards of various com-

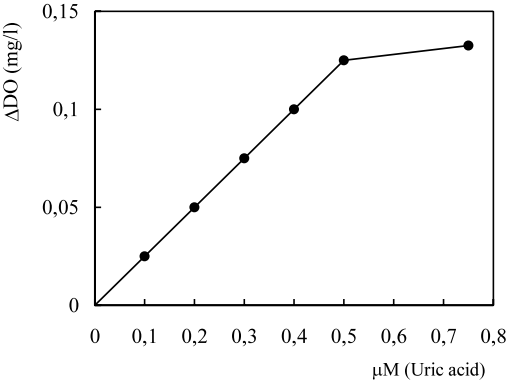

Fig. 4. Standard curve of the biosensor. (Phosphate buffer; pH 7.5, 50 mM; T 35 °C. The activity of urate oxidase, peroxidase, the percentage of glutaraldehyde and gelatine amount were kept constant at 64.9×10^{-3} U cm^{-2}, 512.7 U cm^{-2}, 1.25% and 11.8 mg cm^{-2}, respectively.)

pounds such as uric acid, L-aspartic acid, citric acid, L-glutamic acid, glycolic acid, oxalic acid, and glucose were examined. The biosensor response obtained for uric acid was accepted as 100% and compared to the biosensor responses obtained for the other substances. There were no responses for the other substances which were used for the substrate specificity of the biosensor.

3.2.4. Storage stability

Storage stability experiments were made for a long storage period to detect decreases in the biosensor response. During this period enzymes used in the biosensor construction lost their activities as related to time. In order to determine the storage stability of the biosensor, measurements were carried out periodically every week for a month. The biosensor prepared was used for only this purpose and during the period it was stored at 4 °C. After the storage period, it was determined that the remain activity of the biosensor was more than 83% of its initial activity (Fig. 5).

3.3. Uric acid determination in urine

Fig. 4 shows that the biosensor have a linear range between 0.1 and 0.5 μM uric acid concentrations. Normal urinary excretion of uric acid is within the range 1.5–4.4 mmol 24 h^{-1} [18] so the biosensor can be used for the measurement of uric acid levels in 7500-fold diluted urine sample because of the linear range of the biosensor is between 0.1 and 0.5 μM. Dilution procedure also minimized the interference effects of substances which would come from urine. The analytical results obtained for two methods are given in Table 1. Table 1 shows the results obtained from standard and standard addition methods by using the biosensor and the kit methods for urine samples. From the standard method uric acid level in urine sample was found as 2180 μM l^{-1} by the biosensor and 1978 μM l^{-1} by the kit. Uric acid concentration in urine was also determined by using standard addition method for the biosensor and the kit. According to the results if we compare two methods it can be said that results related to uric acid concentration in urine sample obtained by using the biosensor based on urate oxidase–peroxidase coupled enzyme system were found to be more sensitive, accurate and specific in comparison to the results of uric acid kit method.

Fig. 6 shows the curves obtained for the biosensor by using standard and standard addition methods.

From this figure it was obvious that the biosensor developed by the urate oxidase–peroxidase coupled enzyme system was very sensitive and it was not affected from the interference effects of the substances that would be in urine.

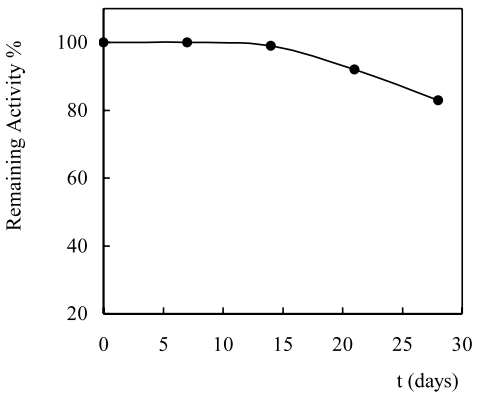

Fig. 5. Storage stability of the biosensor. (Phosphate buffer; pH 7.5, 50 mM; T 35 °C. The activity of urate oxidase, peroxidase, the percentage of glutaraldehyde and gelatine amount were kept constant at 64.9×10^{-3} U cm^{-2}, 512.7 U cm^{-2}, 1.25% and 11.8 mg cm^{-2}, respectively.)

4. Conclusion

As a result of this work the biosensor developed based on urate oxidase–peroxidase coupled enzyme system was found to be more advantageous in comparison to other methods known, such as chemical [19], Radiochemical-HPLC [20], voltammetric-coulometric [21] and enzymatic-spectrophotometric methods [22]. The novel biosensor is more suitable especially for routine uric acid analysis, it is simple to construct and it is sensitive, specific and doesn't require any expensive apparatus and by using the biosensor more than 50 measurements were done. Moreover, the high cost of urate oxidase and peroxidase used in the kit is a factor that limits widespread use of the enzymatic

Table 1
Determination of uric acid levels in urine by the biosensor and kit methods by using standard addition[a]

Sample	Method	Found uric acid content of sample ($\mu M\,l^{-1}$)	Sample+adding uric acid ($\mu M\,l^{-1}$)	Found total uric acid ($\mu M\,l^{-1}$)	Recovery (%)
Urine	biosensor	2180	4182	4162	99.5
	kit	1978	4182	3960	94.7

[a] The average of 5 analysis.

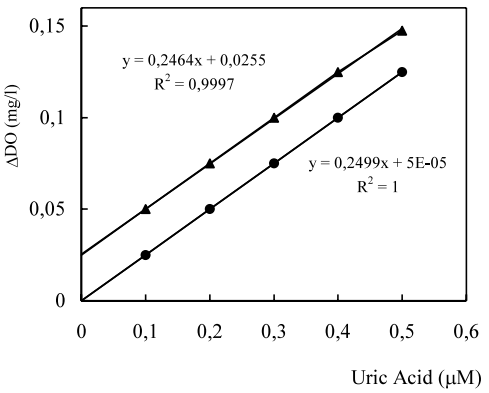

Fig. 6. Calibration curves obtained for standard and standard addition methods. (●-●) standard curve, (▲-▲) standard addition curve. The activity of urate oxidase, peroxidase, the percentage of glutaraldehyde and gelatine amount were kept constant at 64.9×10^{-3} U cm^{-2}, 512.7 U cm^{-2}, 1.25% and 11.8 mg cm^{-2}, respectively.)

colorimetric method for routine purpose. However, the immobilization of the enzymes on the teflon membrane surface with gelatine and glutaraldehyde reduces the cost of the procedure, considerebly. If the biosensor was compare to other methods, we can say that the lowest detection limit was obtained by the biosensor developed based on the urate oxidase–peroxidase coupled enzyme system. While the lowest detection limit of uric acid in our method was 0.1 μM, the detection limits of uric acid for the other methods were given below. Such as urate oxidase–peroxidase system immobilized onto alkylamine glass and arylamine glass (420.8 μM) [22], automated analysis using immobilized urate oxidase (105.2 μM) [23], a chemiluminescence method using immobilized urate oxidase (88.4 μM) [24], an enzymatic colorimetric method using soluble urate oxidase (131.5 μM) [25], automated analysis using urate oxidase bound to protamine (10.5 μM) [26], radiochemical method using soluble urate oxidase (8.8 μM) [20], chemiluminescence method using soluble urate oxidase (5.2 μM) [27], and amperometric flow-injection determination (1.78 μM) [28]. By using a reagentless amperometric urate biosensor based on uricase and peroxidase enzymes immobilized in carbon paste, uric acid was detected between 3 and 100 μM concentration range [15].

In our previous study we developed an amperometric urate oxidase enzyme electrode and by using the electrode we could sensitively detect uric acid until 5.0 μM [16]. In the present study we have developed a new biosensor based on urate oxidase–peroxidase coupled enzyme system that has 0.1 μM uric acid detection limit and is not reported in the literatures until now. By using the biosensor we can easily detect uric acid more sensitive, specific and accurate than the other methods. Furthermore if we consider to substrate specificity, S.D. and variation coefficient results of the biosensor it is obvious that the biosensor can be used easily and reliable for the selective clinical determination of urinary uric acid.

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