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Electrochemical Biosensor for Monitoring Insulin in Normal Individuals and Diabetic Mellitus Patients

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ABSTRACT

A biosensor is an analytical tool that comprises two essential components – an immobilized biocomponent, in intimate contact with a transducer that converts a biological signal into a measurable electrical signal. In the present study, electrochemical insulin biosensor is described. It has been developed by immobilizing red blood cells (RBC) in carbon paste electrode (CPE) which contain enzyme hexokinase and glucose-6-phosphate dehydrogenase ,adeniosine triphosphate(ATP),and insulin receptor with concomitant generation of NADPH and its oxidation is measured amperiometrically at 0.32 V. The response is linear, covers the calibration range 0.006-0.09 nM with stability of two weeks and its detection limit is 0.006 nM which is significantly lower than the existing methods. The results were statistically significant with p value less than 0.0001. The conventional analytical techniques used although precise, are time consuming where as developed insulin biosensor have the advantage of ease of use, eco-friendly, sensitive, low cost and lower detection limit. The developed insulin biosensor has been applied for detection of insulin in normal subjects, non-insulin dependent and insulin dependent diabetes mellitus patients.

Key words: Insulin biosensor, CPE, NADPH oxidation, Red Blood Cell.

INTRODUCTION

Insulin is the principal hormone responsible for glucose metabolism. It is synthesized in the cells of the islets of langerhans as the precursor, proinsulin, which is processed to form C- peptide and insulin and both are secreted in equimolar amounts into the portal circulation. It plays an important role in metabolism causing increased carbohydrates metabolism, glycogenesis and glycogen storage, fatty acid synthesis/triglyceride storage and amino acid intake, protein synthesis, increases transmembrane K^+ transport and decreases cyclic AMP level in adipose tissue and liver [1]. It serves as the predictor of diabetes and insulinoma (insulin –secreting tumors). Insulin concentrations are greatly reduced in insulin-dependent diabetes (IDDM) and other conditions such as hypopituiatarism. Insulin concentrations may be raised in non- insulin dependent diabetes (NIDDM), obesity, insulinoma and some endocrine dysfunctions, such as Cushing's syndrome and Acromegaly [2, 3]. Hence monitoring of insulin is important for diagnosis of IDDM patients and NIDDM.

As according to latest reports, rates of diabetes have increased markedly over the last 50 years. As of 2010 there are approximately 285 million people with the disease compared to around 30 million in 1985[4]. Insulin monitoring is

very important for clinical diagnosis. Presently, conventional methods of insulin analysis include(ELISA) enzyme linked immunosorbant assay and(IRMA) immunoradiometric assay[5, 6 and 7] .These methods are undoubtedly precise but suffer from the drawbacks of more response time, high cost and are laboratory bound. The conventional methods are hazardous as radioactive isotopes such as I^{125} or I^{131} are used for assaying insulin.

In the last few years various scientists have developed new technology which uses insulin electrochemical, amperometric, microvoltammetric electrode as sensors for measuring levels of insulin. These have the advantages of specificity, fast response time, low cost, portability, ease of use and a continuous real time signal. In the present study, electrochemical insulin biosensor has been developed.

In 1989, electrochemical oxidation of insulin was monitored by flow injection methods at nano molar level [8]. The carbon fibre microvoltammetric electrode modified with polynuclear ruthenium oxide/cyanoruthenate film had been developed for insulin with the detection limit at 0.5μM in 1993 [9]. Insulin was measured by amperometry based on oxidation-reduction couple (Ru-O/CN-Ru) using carbon fibre microelectrodes modified with a composite of ruthenium oxide and cyanoruthenate (Ru-O/CN-Ru) [10]. RuO was exploited on carbon fibre microelectrode by cycling the electrode potential. The film catalyzed the oxidation of insulin. The analytical performance of the modified electrode for insulin was characterized using flow injection analysis and gave a linear range of 0.10-1.0μM [11]. Then in 2000, two electrochemical catalytic systems based on the oxidation of insulin by chloro-complexes of iridium (lV) were developed. The lrOx film electrode was used as an amperometric detector for flow injection analysis of insulin with the range between 0.05-0.50μM and detection limit of 20nM [12].

The immobilization of Ru {ll} was done on a carbon surface. The reversible voltammetry for the Ru ll/lll redox couple was observed. Flow injection amperometric determination of insulin at pH 7.0 with the detection limit of 2nM was observed [13]. In 2001, a simultaneous amperometric measurement of glucose and insulin was developed. An insulin sensitive RuOx-modified electrode was developed. The amperometric sensor has linearity up to 1000nM insulin and 14mM glucose [14]. The multiwalled carbon nanotubes and chitosan were coated on a glass carbon electrode and the redox chemistry of insulin was studied with the detection limit of 30nM and range of 0.1-3.0 μM [15].

To improve the sensitivity of the method for the detection of insulin, carbon ceramic electrodes were fabricated by sol-gel technique. The modified electrode was used for insulin detection chronoamperometrically. This developed electrode had sensitivity 0.45nM with detection range of 0.5-500nM [16]. In 2008, scientists of University of Pittsburgh developed a novel biosensor for live cell imaging of insulin which is highly sensitive .This invention is related to the field of imaging intracellular proteins, which uses fluorescent probes capable of changing color over time to identify and track the synthesis, trafficking, and secretion of intracellular insulin[17].

In the present study, detection limit of the newly developed electrochemical biosensor is 0.006 nM which is lower than the existing methods. The automated immunoassay method has the detection limit of 2μIU/ml and ELISA has the detection limit of 0.5MμIU/ml. Real time sample analysis has not been carried in different insulin sensors. The developed electrochemical biosensor is novel with respect to biocomponent (RBC) which is immobilized in carbon paste and response is linear. The developed biosensor has been applied for monitoring insulin in normal individual, diabetic patients – non-insulin dependent and insulin dependent diabetes mellitus patients. The characteristics of the developed insulin biosensor are reported in the following section.

MATERIALS AND METHODS

2.1 Subjects:

The present study comprised of 90 patients from government and private hospital. The consent from the patient was taken who were enrolled for study and approval from ethics committee of the institute. The insulin levels were assayed by the developed biosensor as well as by the photometry and ELISA technique.

2.2 Blood Sample: The serum sample was used for insulin determination.

2.3 Chemicals and Reagents:

Various reagents of analytical grade used were NaCl solution , phosphate buffer saline (K_2HPO_4/KH_2PO_4), NaOH ,

insulin(Bovine Insulin 40U/L) , glucose , NADP solution, KCl , were procured from SRL Chemical Ltd., Torrent Pharmaceuticals Ltd., S.D fine Chemicals. Carbon powder was obtained Sigma chemicals and mineral oil from Fluka-Germany. A glass tube 5cm long and 2-3 mm in diameter was used for the fabrication of carbon paste RBC-electrode (procured from CSIO-Sec-30, Chd).

2.4 Fabrication of Insulin Biosensor

Procedure:

The carbon paste insulin electrode was prepared by hand mixing carbon paste (4:1, carbon powder: mineral oil) for 20 mins.Then the paste was mixed with biocomponent (red blood cells) isolated from 2ml of human blood plasma. A portion of the resulting paste was packed into the end of a 5cm long glass tube (2-3mm diameter).The paste was filled to the tip to a height of 1cm; electrical contact to its inner end was made with a 0.2mm diameter copper wire.

2.5 Experimental Part

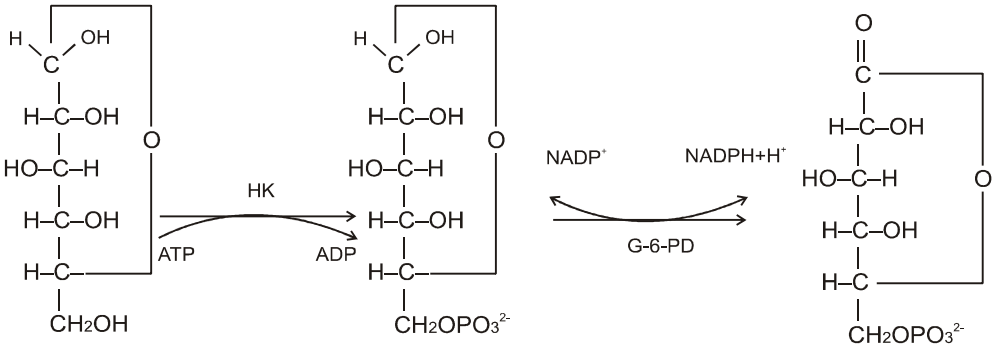
The experiments were run with the blank, standard and unknown samples. The oxidation curve was observed at 0.32 V which is the oxidation potential of NADPH [18] and for the blank, the oxidation potential curve was observed at 0.27V. Optimization of various parameters for production of NADPH was studied which includes temperature, reaction time, glucose concentration, NADP+ and RBC (for immobilization).

Instrumentation and measuring procedure

Electrochemical measurement was performed using a polarograph (Systronic model 1632) modified as an amperometer. The three-electrode system consisted of a CPE- RBC electrode as the working electrode, an Ag/ AgCl as the reference electrode and a platinum wire as the auxiliary electrode. The experiments were carried out in an electrochemical cell holding 40 ml of reaction mixture, cocktail in a sequence ABCDA. Solution A (NaOH), solution B (serum of normal individual, IDDM and NIDDM patients) which serves as a source of insulin, solution C (glucose) , D($NADP^+$) and phosphate buffer saline(PBS) of pH 7.4 and KCl at temperature of 27 ± 2˚C. The current- voltage curves were taken with the voltage range of 0-0.75 V with a scan rate of 5 mv/sec. The oxidation of NADPH was studied at + 0.32V amperometrically and the anodic current was measured. Reliability of the developed electrochemical insulin biosensor was checked by quantitative analysis of NADPH generation by photofluorometer. The reproducibility of the ten fabricated CPE-RBC electrodes was checked which gave the same peak height with same insulin concentration with Mean±S.D- 1.7±0.005.

RESULT AND DISCUSSION

Conversion of Glucose inside red bood cells :



HK – Hexokinase Enzyme

G6PD – Glucose- 6- Phosphate Dehydrogenase

Fig.1 Diagrammatic representation of NADPH production

3.1. Method of Development

The developed electrochemical insulin biosensor contains CPE-RBC electrode. The red blood cells contain enzymes hexokinase, glucose-6- phosphate dehydrodegenase, ATP and insulin receptor. This enzymes machinery is used for insulin detection. The insulin facilitates the transport of glucose across rbc membrane by glucose permease transporter [18, 19] which is converted to glucose-6- phosphate by hexokinase and further to 6-phosphogluconate by glucose-6- phosphate dehydrodegenase with the concomitant generation of NADPH. The reactions are shown in Fig.1 .The NADP and glucose were added exogenously to drive the reaction in forward direction. The amount of NADPH produced is proportional to insulin concentration and signal was observed at oxidation potential of + 0.32 V.

3.2 Insulin standard curve

The insulin standard curve was constructed as shown in Fig. 2 with the linear range of 0.006-0.09 nM and the signal was obsereved at potential of 0.32V and for the blank, signal was observed at 0.27V.

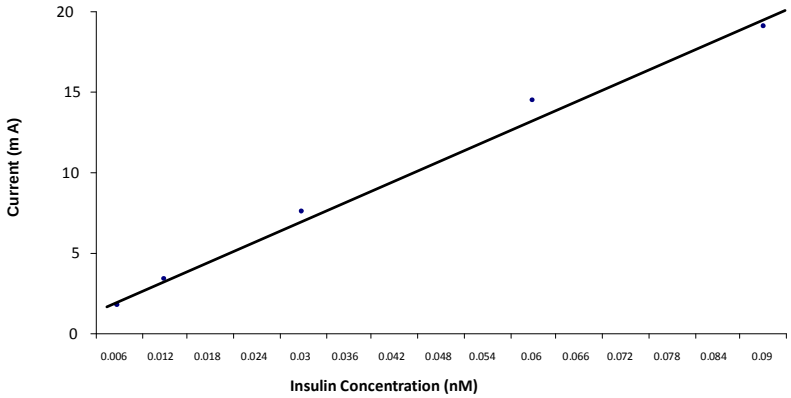


Fig. 2. Standard Curve for Insulin

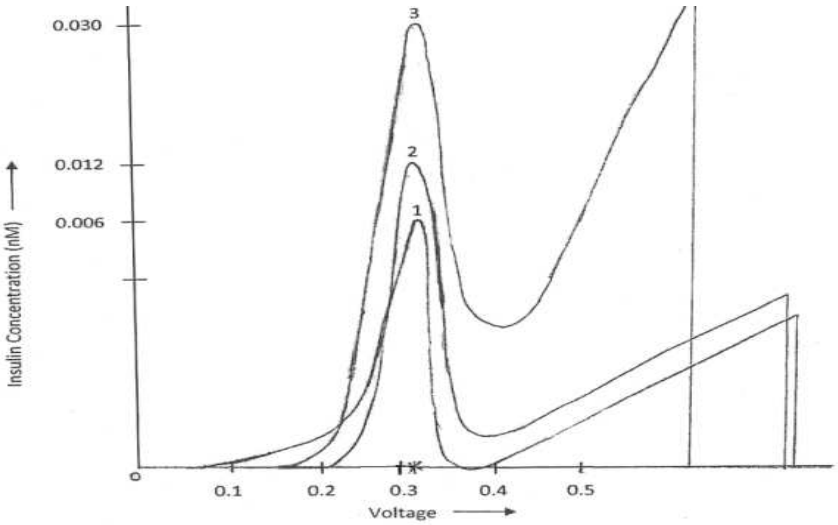


Fig. 3 A voltagram of insulin (1) 0.006 nM (2) 0.012 nM insulin (3) 0.030 nM insulin, PBS pH = 7.4, current = 5mA, *Voltage= 0.32V, CPE- RBC electrode.

3.3 Surface characterization of the RBC electrode

The blood volume of biocomponent (RBC) to be immobilized in the carbon paste was optimized and the 1.5ml, 2.0 and 3.0ml was taken under study. These three fabricated electrodes obeyed the bioassay principle characterization, but the current value came to be highest with 2.0ml of biocomponent as shown in Table 1 and Fig. 4. Though the number of cells in 3ml volume of blood was more, yet peak height was comparatively less as overcrowding of RBC cells hampered the facilitated transporter. The current-voltages curve for 1.5, 2.0, 3.0ml of biocomponent is shown in Fig. 3. A sharp peak at 0.32V oxidation potential is observed for 2.0ml of biocomponent.

Table 1 **Volume of Biocomponent Optimization for CPE**

S. No.	Volume (ml)	Current (cm.)
1	1.5	7.9
2	2	18.1
3	3	15.9

S.D± 5.36

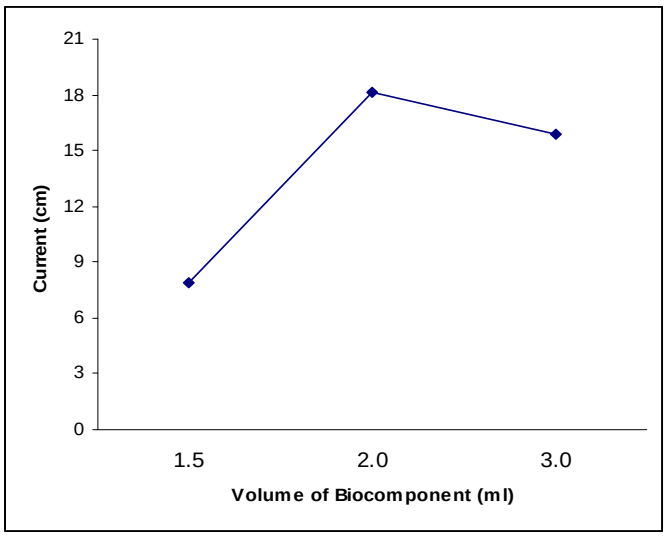


Fig. 4 Effect of Biocomponent volume on NADPH Production

3.4 Reliability

The reliability of the developed biosensor for insulin estimation has been checked with photofluorometer by detection of NADPH production and results are comparable as shown in Table 2. Insulin level of normal healthy subject, NIDDM, and IDDM determined by the developed biosensor were found to be comparable (data not shown) by the conventional method (ELISA). Hence, the developed biosensor is reliable.

Table 2: Comparison of electrochemical insulin biosensor and Photofluorometer methods for determination of insulin concentration

S. No.	CPE-rbc Insulin Conc. (nM)	Photofluorometer Insulin Conc. (nM)
1	106	106.9
2	131.1	126.6
3	75	87
4	96	90
5	95.4	99

3.5 Stability of biocomponent

The developed CPE-RBC electrode is stable for two weeks if kept at pH 7.4 and temperature 2-8ºC in phosphate buffer saline [20]. The regeneration of the biocomponent is not fast and hence CPE-RBC electrode is of single use.

4. Application of the developed insulin biosensor

The developed biosensor has been applied for determination of insulin in normal individual, IDDM and NIDDM patients. Normal range for healthy individuals
is 2-150 nM/ml [6, 7].

Table 3 Comparison of Insulin Concentration in Normal individuals, IDDM and NIDDM Patients using CPE-RBC electrode

	Normal Individual (n=30)	IDDM (Fasting) (n=30)	IDDM (After insulin injection) (n=30)	NIDDM (n=30)
Mean	72.78	0.03000	177	84.7
SD	16.9	0.00500	20	4.66
SEM	5.3	0.00204	10.0	1.47
p	p<0.0001	p<0.0001	p<0.0001	p<0.0001

Table 3 shows the results and statistical analysis of normal individual, IDDM and NIDDM patients. The insulin levels of NIDDM are close to the normal individuals whereas the IDDM patients have lower insulin level than normal range. The results were statistically significant by this developed method i.e. p value<0.0001.The history of the patients correlates with the results as they were taking antidiabetic drugs daily to maintain normal glucose level. Some IDDM patients had high normal insulin levels as those patients were taking insulin intravenously daily. This developed method has been patented with the support of Technology Information Forecasting and Assessment Council (TIFAC), Punjab State Council for Science &Technology (PSCST), and Department of Science and Technology (DST) and Indian Patent vide application no. is 1792/DEL/2006 under the title “Biosensor for Estimation of Insulin” [21].

CONCLUSION

In this paper, an electrochemical biosensor has been developed for detection of insulin concentration in normal individuals, IDDM and NIDDM patients. The developed biosensor exhibits excellent sensitivity. The linear range achieved for insulin detection is 0.006 – 0.09 nM and response time is 25min. The storage stability of the biocomponent achieved is two weeks if stored in PBS of pH 7.4 at 2-8ºC. The reliability of the developed biosensor has been checked with photofluorometer and the results are comparable. The conventional analytical technique used is no doubt precise but it is time consuming and mostly lab bound whereas the biosensor approach has the advantage of specificity, fast response time, simplicity of construction of CPE-RBC electrode, it exhibits great prospects in the field of biosensor. The analysis of insulin by biosensor approach is a remarkable step far ahead of the old conventional method of electrochemical detection with a lower detection range. Because of high sensitivity, the developed electrochemical biosensor is suitable for monitoring insulin level in IDDM patients whose serum insulin level is quite low.

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