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Construction of an amperometric triglyceride biosensor using PVA membrane bound enzymes

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ABSTRACT

Objectives: A method is described for construction of an amperometric triglyceride (TG) biosensor using PVA membrane bound enzymes.

Design and methods: A mixture of commercial lipase, glycerol kinase, glycerol-3-phosphate oxidase and horseradish peroxidase was co-immobilized onto polyvinyl alcohol (PVA) membrane through glutaraldehyde coupling. The biosensor measures current when polarized at 0.4 V.

Results: The sensor showed optimum response within 2 s at pH 7.0 and 25 °C. The current (mA) was in proportion to concentration of TG in the range 0.56–2.25 mM. The minimum detection limit of the method was 0.21 mM. The analytic recovery of added TG was 94.3%. Within batch and between batch coefficients of variations (CV) were <5.85% and <4.13%, respectively. A good correlation ($r = 0.99$) was found. Among various serum substances tested, only cholesterol caused slight stimulation (20%).

Conclusions: An amperometric method was developed for determination of TG employing this enzyme electrode.

Introduction

Triglycerides (TG) along with cholesterol are the plasma lipids of most interest in the diagnosis and management of lipoprotein disorder [1]. It is a major indicator of nephritic syndrome, chronic hepatitis, pancreatitis, hyperlipidemia, liver disease, hypothyroidism, diabetes mellitus and alcoholism. Among the various methods available for determination of TG [2-6], biosensors are comparatively more simple, sensitive, rapid and possible at bedside of patients. Three types of TG biosensors have been reported: (i) DO metric TG biosensors which measure dissolved oxygen consumed in enzyme reaction [7], (ii) electrochemical biosensors which measure electrons generated from splitting of H_2O_2 under high potential [8] and (iii) potentiometric TG biosensor which measures voltage [9]. However electrochemical biosensors are more common in use as these are unaffected by environmental factors and easy to operate. In these amperometric biosensors, enzymes have been co-immobilized on collagen membrane [10], cellulose acetate (CA) [11], polyvinyl chloride (PVC) [12] and Prussian blue (PB) modified screen printed electrodes [13]. The collagen membrane had a problem of time (3 days) consuming preparation, long response time, low storage stability and limited electron communications. CA and PVC membranes are better than collagen membrane as these are more elastic, non-fragile and chemical resistant. However all these membranes had low permeability and conductivity and a major problem of desorption or leaching of enzymes during washing. Nevertheless, polyvinyl alcohol (PVA) membrane is elastic, non-fragile, had high mechanical strength, easy to prepare, resistant to microbial attack and have good electron communication. Further PVA membrane is the best polymeric candidate due to its excellent hydrophilic properties and the film formation ability in aqueous solution system [14] and thus an ideal material for making an enzyme electrode. The present report describes the construction of a triglyceride biosensor based on PVA membrane bound enzymes.

Materials and methods

Reagents and materials

Triton X-100 was from Sigma Aldrich; 3,5-dichloro-2-hydroxy benzene sulphonic acid (DHBS) was from E-Merk Germany; triolein and ATP were from SRL, Mumbai; and kit for enzymic colorimetric method (Enzo kit) for TG manufactured by M/s Erba Transasia

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Biomedical Ltd. (Daman, India) was purchased from M/s Haryana Scientific and Engineering Corporation, Rohtak. All other chemicals were of AR grade.

Preparation of mixture of lipase, GK, GPO and peroxidase

To prepare mixture of lipase, glycerol kinase (GK), glycerol-3-phosphate oxidase(GPO) and horseradish peroxidase (HRP), 3.0 mg of Reagent 1 of Enzo kit for TG consisting of lipase (110 U), GK (80 U), GPO (500 U),HRP (35 U) and 4-aminoantipyrine was dissolved in 1 ml of 0.02 M sodium phosphate buffer pH 7.0 and loaded on a Sephadex G-100 gel column (24×1 cm) preequilibrated with 0.02 M sodium phosphate buffer pH 7.0.The column was run in the same buffer at a flow rate of 0.5 ml/min. The fractions (2 ml each) were collected and tested for protein by Lowry method. The fractions showing presence of protein were pooled and treated as mixture of lipase, GK, GPO and HRP. The mixture was tested for combined activity of lipase, GK, GPO and HRP as given below.

Preparation of triolein solution

Triolein was used as a substrate for lipase, GK, GPO and HRP. Triolein emulsion was prepared as described [15]. Solutions of different concentrations of triolein ranging from 0.1 to 20 mM were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and stored at 4 °C until use.

Assay of mixture of free lipase, GK, GPO and HRP

The assay of mixture of free lipase, GK, GPO and HRP was carried out in a 15 ml conical flask wrapped in a black paper [2]. The reaction mixture consisted of 0.54 μmol $MgCl_2$, 0.63 μmol ATP, 1.0 μmol potassium ferrocyanide, 0.27 μmol 4-aminophenazone, and 1.53 μmol DHBS, 1.0 μmol of Triton X-100/liter of 0.1 M sodium phosphate buffer, pH 7.0. To 900 μl reaction mixture, 50 μl of mixture of enzymes was added and pre-incubated at 37 °C for 5 min. The reaction was started by adding 50 μl of 22.6 mM triolein. After incubation at 37 °C for 15 min, A_{510} was read. H_2O_2 generated in reaction was calculated from standard curve between A_{510} versus H_2O_2 concentration. The unit activity of mixture of enzyme was expressed as 1 nmol H_2O_2/min/ml.

Preparation of PVA membrane

A polyvinyl alcohol (PVA) membrane was prepared as described by Singh et al. [16]. PVA powder (500 mg) was dissolved in 5 ml sodium phosphate buffer (0.05 M, pH 7.0) and heated gently to boil.

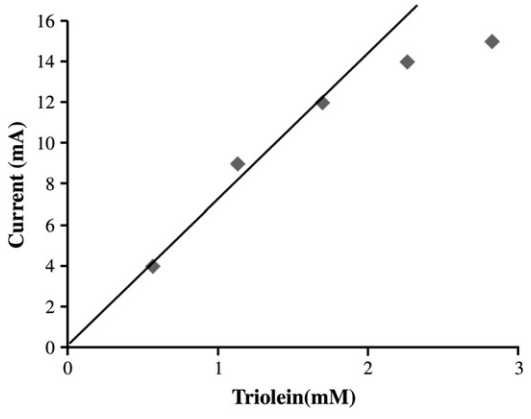

Fig. 1. Standard curve of triolein using TG biosensor based on PVA membrane bound enzymes.

The solution was spread in a glass petriplate of 10 cm diameter with the help of a glass rod and left overnight for drying. Membrane was removed gently and stored at 4 °C in 0.05 M sodium phosphate buffer, pH 7.0, until used.

Co-immobilization of enzymes on PVA membrane

The mixture of enzymes (0.3 ml) and glutaraldehyde (0.15 ml of 2.5% in 0.02 M sodium phosphate buffer, pH 7.0) was spread evenly onto PVA membrane and kept overnight at room temperature for co-immobilization. The membrane was washed in reaction buffer and tested for activity.

Construction of amperometric TG biosensor and response measurement

An amperometric biosensor for measurement of TG was constructed by mounting PVA membrane containing immobilized lipase, GK, GPO and HRP, onto Pt electrode with a parafilm to construct a working electrode. This working electrode along with Ag/AgCl as reference and Cu wire as auxiliary electrode were connected through electrometer (Make: Keithley Japan, Model 6517A/E). The electrode was dipped into 4 ml reaction mixture [3.0 ml 0.05 M sodium phosphate buffer pH 7.0 and 1.0 ml triolein solution] and the electrodes were polarized at different potential (in volts) and current (mA) generated was measured in electrometer. The current was also measured at varying concentration of triolein. The principle of working of the biosensor is as follows.

$$\text{Triglyceride} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[MgCl_2]{GK} \alpha-\text{Glycerol} - 3 - \text{phosphate} + \text{ADP}$$

$$\text{Glycerol} - 3 - \text{phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 \xrightarrow{0.4V} 2H^+ + O_2 + 2_e^-$$

$$2_e^- \xrightarrow{\text{PVA membrane}} \text{Pt electrode}$$

$$GK = \text{Glycerol kinase} \qquad GPO = \text{Glycerol} - 3 - \text{phosphate oxidase}$$

For control, PVA membrane (without immobilized enzymes) was mounted on Pt electrode. The rest of the procedure was same as for the working electrode.

Optimization of working conditions of TG biosensor

Various kinetic properties of PVA membrane bound enzymes mounted on Pt electrode such as optimum pH, incubation temperature, time for maximum response and effect of substrate (triolein) concentration were studied amperometrically to optimize the biosensor.

Amperometric determination of TG in serum

Blood samples (1 ml each) were drawn from apparently healthy male and female (25 each) and persons having a diagnosis of hypertriglyceridemia of various categories such as atherosclerosis, diabetes mellitus and pancreatitis after 12 h fast at Pt BDS PGIMS, Rohtak hospital and centrifuged at 1500 g for 10–15 min and their supernatant (serum) was collected. TG content in serum was determined by the present biosensor in same manner as described above for its response measurement, under its optimal working conditions except that triolein was replaced by serum. The current

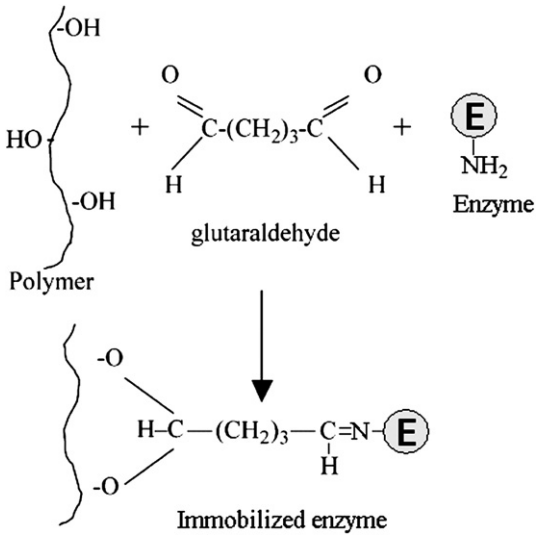

Fig. 2. Chemical reactions involved in co-immobilization of enzymes onto PVA membrane through glutaraldehyde.

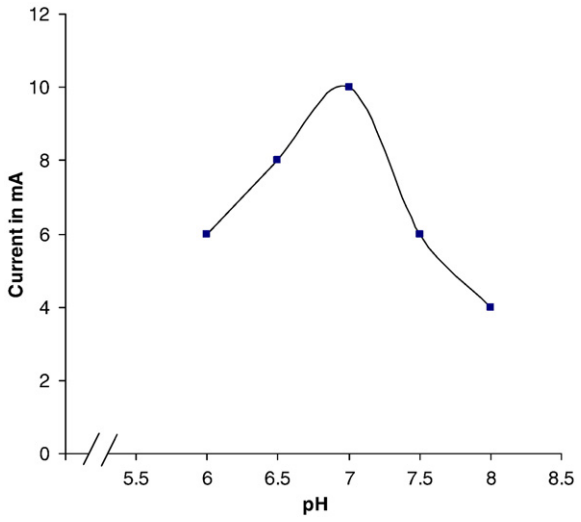

Fig. 4. Effect of pH on response of TG biosensor based on PVA bound enzymes.

(mA) was measured and the amount of TG in serum was extrapolated from standard curve between triolein concentrations and current (in mA) prepared under optimal working conditions (Fig. 1).

Evaluation of TG biosensor

The biosensor was evaluated by studying analytic recovery, precision and accuracy/correlation. In order to determine accuracy of the present method, the TG values in 10 serum sample were determined by standard enzymic colorimetric kit method as well as by the present method. The kit method involves preparation of working solution by mixing reagent 2 and reagent 3 in equal ratio and incubation of 0.9 ml working solution with 0.1 ml serum sample at room temperature for 15 min and reading A_{510} in UV and visible spectrophotometer (Shimadzu-1700, Japan) and calculating TG by running standard triolein solution. The effect of various possible interfering substances found in blood such as uric acid, cholesterol, ascorbic acid, bilirubin, glucose, pyruvate and glutathione was also studied at their physiological concentrations.

Results and discussion

Co-immobilization of lipase, GK, GPO and HRP onto PVA membrane

A mixture of commercial lipase, glycerol kinase, glycerol-3-phosphate oxidase and horseradish peroxidase was co-immobilized covalently onto PVA membrane through glutaraldehyde cross linking, with 70% retention of initial activity of free enzyme. The following reactions might have occurred in immobilization (Fig. 2).

Scanning electron microscopy

Scanning electron microscopy (SEM) of the PVA membrane and enzymes immobilized in PVA membranes are shown in Fig. 3A and B,

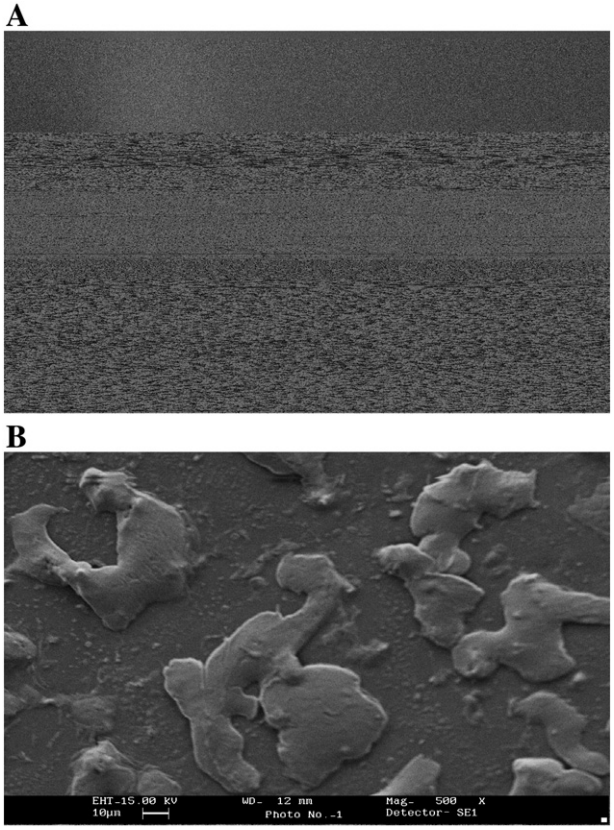

Fig. 3. Scaning electron microscopy pictures of (A) a PVA membrane and (B) enzymes immobilized on PVA membrane.

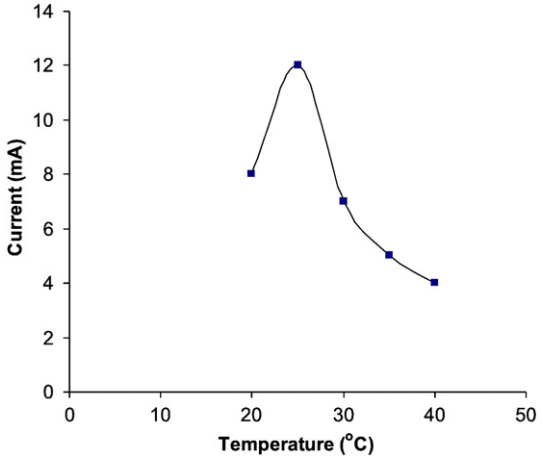

Fig. 5. Effect of incubation temperature on biosensor based on PVA bound enzymes.

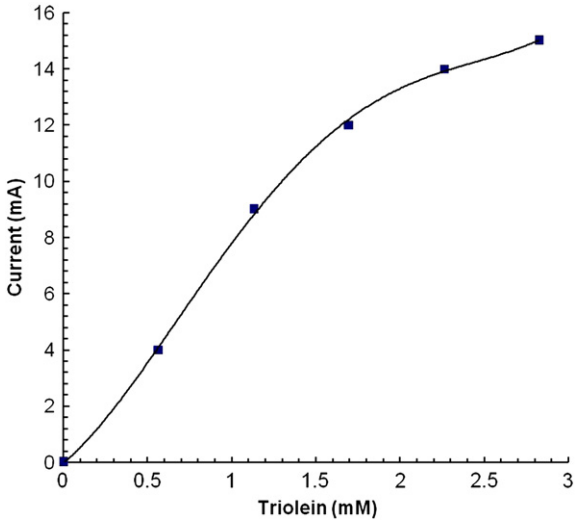

Fig. 6. Effect of triolein concentration on biosensor based on PVA bound enzymes.

respectively. The SEM of the PVA membrane without bound enzymes (Fig. 3A) indicates a uniform polymeric layer of PVA, whereas globular structures due to immobilization of enzymes in the PVA membrane are seen in Fig. 3B.This suggests that the enzyme molecules are uniformly packed in the PVA membrane.

Construction of TG biosensor

A method is described for preparation of an amperometric TG biosensor employing PVA membrane bound lipase, GK, GPO and HRP. TG biosensor showed good response in the form of current (mA). As the current generated was optimum at +0.4V, it was selected for standardization of working conditions of biosensor.

Optimization of TG biosensor

Optimum pH

To study effect of pH on biosensor response, the pH of reaction mixture was varied from pH 6.0 to 8.0 at an interval of 0.5, using 0.1 M sodium phosphate buffer. The sensor showed optimum response at pH 7.0 (Fig. 4). This optimum pH of biosensor was slightly lower than that for free enzyme (pH 7.2) (Fig. 4). However optimum pH of the present electrode (pH 7.0) was lower than amperometric sensor based on Prussian blue modified screen printed electrodes (pH 8.0) [13] and Pt electrode mounted with PVC membrane bound enzymes (pH 7.5) [12] but slightly higher than biosensor based on CA membrane bound enzymes (pH 6.5) [11].

Table 2
Analytical recovery of added triolein in the serum, as measured by biosensor having PVA membrane bound lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase.

Triglyceride added (mg/dl)	Triglyceride found (mg/dl)	% Recovery
–	156.9	–
10	157.4	94.3
20	161.5	91.3

Optimum temperature

The sensor performance was evaluated in the temperature range from 20 to 40 °C at an interval of 5 °C. The sensitivity was increased as the temperature was increased up to 25 °C after which it was declined (Fig. 5). Hence in all the subsequent experiments, the temperature of reaction mixture was kept at 25 °C. This optimum temperature is lower than that for PVC membrane [12], based on oxygen meter biosensor (39.5 °C) [7] and amperometric biosensor based on collagen membrane (37 °C) [10] but similar to CA membrane bound enzyme sensor (25 °C) [11].

Effect of substrate concentration

The effect of substrate concentration on the response of biosensor was measured by varying the triolein concentration from 0.56 to 2.25 mM. A hyperbolic relationship between triolein concentration up to a final concentration of 2.26 mM was obtained after which it became constant (Fig. 6). Lineweaver–Burk plot between the reciprocals of triolein concentration and activity of co-immobilized enzymes (Fig. 7) gave K_m for triolein and I_{max} as 3.33 mM and 35.7 mA, respectively, which is higher than that of CA membrane bound enzymes (10 mM) [11] and PVC membrane bound enzyme [12]. A comparison of various kinetic properties of polyvinyl alcohol (PVA) membrane bound enzymes with those of free and CA and PVC membrane bound lipase, GK GPO and HRP is summarized in Table 1.

Determination of TG with biosensor

An amperometric method for analysis of serum triglycerides was developed using the present biosensor. The following criteria were studied to evaluate the method.

Evaluation of TG biosensor

Linearity

There was a linear relationship between current (mA) and triolein concentration ranging from 0.56 to 2.25 mM in reaction mixture, which is similar to that based on PVC membrane [12] but lower than that for dissolved oxygen (DO) metric biosensor (5–20 mM) [7] and comparable to enzymic sensor (0.3–2.5 mM) [8] porous silicon

Table 1
A comparison of kinetic parameters of free and co-immobilized lipase, glycerol kinase, glycerol-3-phosphate oxidase and horseradish peroxidase on CA membrane and poly vinyl alcohol (PVA) membrane.

Parameter	Mixture of free enzymes	Co-immobilized on CA membrane (10)	Co-immobilized on PVC (Narang et al., 2008)	Co-immobilized on PVA membrane (present work)
Optimum pH	7.2	6.5	7.5	7.0
Optimum temperature	37 °C	25 °C	35 °C	25 °C
Thermal stability (at 70 °C for 15 min)	30%	43%	45%	70%
Time for linearity	15 min	40s	30sec	15sec
K_m (for triolein)	21.1 mM	8.76 mM	6.0 mM	3.33 mM
V_{max} (for triolein)	19.6 nmoles/min	0.83 nmoles/min	1.15 nmoles/min	35.7 mA/min (I_{max})
Stability at 4 °C during regular use for 6 months	42%	50% loss in 25 days	50% loss in 40 days	50% loss in 50 days

Table 3
Within and between assay coefficients of variation (CV) for determination of triglycerides in the serum samples, by biosensor based on PVA membrane bound lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase.

n	Triglycerides (mg/dl)	CV (%)
Within assay (5)	107.38 ± 6.28	5.85
107.4		
104.65		
98.7		
115.34		
110.8		
Between assay (5)	118.94 ± 4.93	4.14
116.7		
111.6		
121.7		
120.4		
124.3		

membrane based biosensor (0.2–2.1 mM) [9] and CA membrane bound enzyme sensor (0.2–3.5 mM) [11].

Detection limit

The minimum detection limit of amperometric biosensor was 0.21 mM, which is similar to CA membrane based enzyme electrode (0.2 mM) [11] but lower than DO metric biosensor (0.35 mM) [7], potentiometric biosensor (0.42 mM) [9], amperometric biosensor (0.32 mM) and higher than PVC membrane bound enzyme sensor (0.1 mM) [12].

Recovery

In order to check the accuracy of the present methods, the analytical recovery of added triolein in the serum was determined. The mean analytical recoveries of added triolein (10 and 20 mM) in serum sample were 94.3% and 91.3% (Table 2) which is higher than that of CA membrane based biosensor (89%) [11] and also than that of Prussian blue modified screen printed electrode (81%) [13] and lower than amperometric enzyme biosensor (98%) [10].

Precision

In order to check the reproducibility and reliability of the method, the triglyceride content of the sample in one turn (within batch) and after storage at −20 °C for 1 week (between batches) were determined. The results showed that the triglyceride value of these determinations agreed with each other and within batch and between

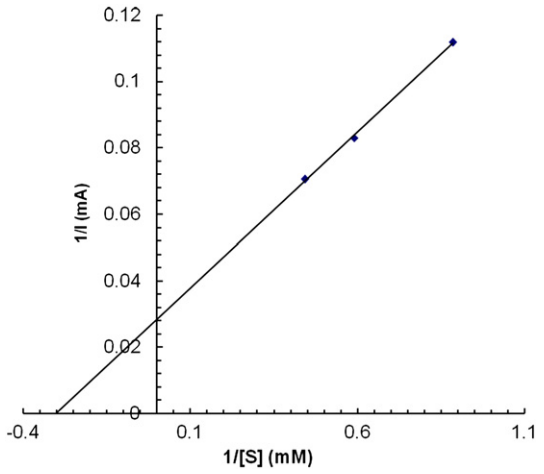

Fig. 7. Lineweaver–Burk plot for effect triolein concentration on biosensor based on PVA bound enzymes.

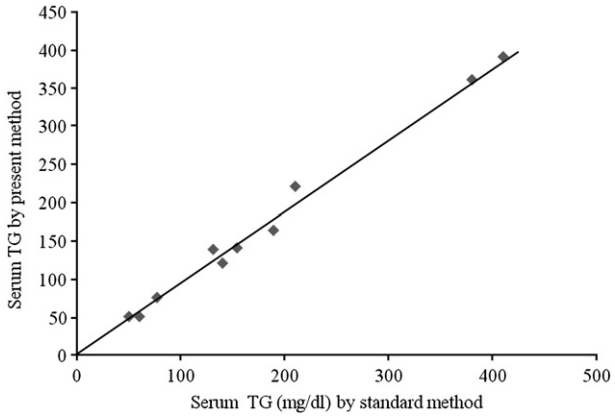

Fig. 8. Correlation between serum triglyceride (TG) level as measured by biosensor based on PVA membrane bound lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase (y) and standard enzymic colorimetric method employing free enzymes (x).

batch coefficients of variation (CV) were 5.85% and 4.14% (Table 3) which represents no change in the actual TG concentration as per the NCEP lipid guidelines 2001. Earlier within and between batch CV were <2.18% and <1.7% [7], <2.8% and <4.9% [13], <2.53% and <3.21% [12], 6.2 and 7.4% [10], and <8% and <4% [11] for biosensors and 0.5% and 4.5% for standard enzymic colorimetric method [17].

Accuracy

In order to know the accuracy of present method, the level of triglyceride in 10 serum samples was determined by standard enzymic colorimetric method (x) and compared with those obtained by present methods (y). The serum TG values obtained by both the method matched with each other and showed a good correlation $r = 0.992$ (Fig. 8). This 'r' is better than those by CA membrane bound enzyme sensor (0.91) [11] and PVC membrane bound enzyme sensor ($r = 0.91$) [12] but comparable to that by other amperometric biosensor ($r = 0.993$) [10] and DO metric biosensor ($r = 0.97$) [7].

Interference study

To study the effect of serum metabolites on the response of sensor glucose, uric acid, cholesterol, ascorbic acid, bilirubin, urea and pyruvate were added into reaction mixture at their physiological concentrations. Only cholesterol increased the sensor response by 20%, while others had no effect (Table 4).

Storage stability and reusability

The amperometric sensor lost 50% of its initial activity during its regular use for about 100 times over a period of 50 days (Fig. 9), when stored in 0.1 M sodium phosphate buffer, pH 7.0 at 4 °C. This

Table 4
Effect of interfering substances for determination of serum triglycerides by TG biosensor based on PVA membrane bound lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase.

Substances added	% Effect
None	100
Glucose	100
Uric acid	100
Cholesterol	120
Ascorbic acid	100
Bilirubin	100
Urea	100
Pyruvate	100

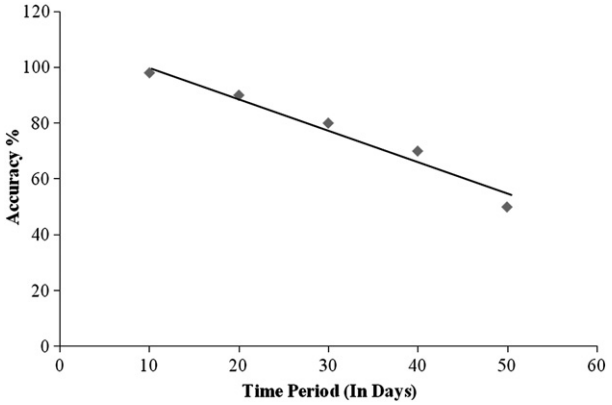

Fig. 9. Effect of storage (in cold) on the accuracy of TG biosensor based on PVA membrane. The accuracy was measured vs. reference method (enzymic colorimetric method)

reusability of this biosensor was better than DO metric enzyme sensor [7].

Conclusion

A mixture of lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase was immobilized covalently onto PVA membrane through glutaraldehyde coupling. This enzyme membrane conjugate was employed for construction of an amperometric biosensor for determination of TG in serum. This biosensor has advantage over previous membrane based enzyme sensor that the PVA membrane is non-fragile, had high mechanical strength and ionic conductivity, easy to prepare and provides better flow of electrons.

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