Sensors & Transducers Magazine (S&T e-Digest), Vol.67, Issue 5, May **2006,** *pp.561-567*

Sensors & Transducers

ISSN 1726-5479

© 2006 by IFSA

http://www.sensorsportal.com

Preparation of Oxygen Meter Based Biosensor for Determination of Triglyceride in Serum

M. BHAMBI, MINAKSHI and C.S. PUNDIR*

Biochemistry Research Laboratory, Deptt of Bio-Science, M D University,
Rohtak 124001 Haryana, India

* Author for correspondence Fax: 91 1262 274076; E-mail: pundircs@yahoo.com

Received: 11 March 2006 Accepted: 24 May 2006 Published: 29 May 2006

Abstract

A method is described for preparation of a dissolved oxygen meter (make Aqualytic, Germany) based triglyceride biosensor employing a polyvinyl chloride (PVC) membrane bound lipase, glycerol kinase (GK) and glycerol-3-phosphate oxidase The biosensor measures dissolved O_2 utilized in the oxidation of triglyceride (TG) by membrane bound lipase, glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO), which is directly proportional to (TG) concentration. The biosensor showed optimum response within 10-15 sec at pH 7.5 and 39.5 ºC. A linear relationship was obtained between the (TG) concentration from 5mM to 20mM and oxygen consumed (mg/L). The biosensor was employed for determination of triglyceride in serum. The within and between batch coefficient of variation (CV) were < 2.18 % and < 1.7% respectively. The minimum detection limit of the biosensor was 0.35 mM. A study of interference revealed that ascorbic acid, cholesterol and bilirubin caused 13%, 15%, and 12% interference, respectively .The biosensor is portable and can be used outside the laboratory.

Keywords: Triglycerides, Lipase, GK, GPO, co-immobilization, enzyme electrode , dissolved oxygen meter, Polyvinyl chloride membrane, serum

1. Introduction

Increased triglyceride level may be a risk factor for coronary artery disease (CAD) [1]. Total triglyceride level in serum is valuable indicator of abnormalities in lipid metabolism, atherosclerosis and hypertension [2]. A number of methods available for triglycerides determination in serum such as chemical [3], enzymic[4] fluorimetric[5] and bioluminescent[6], chromatographic[7] and HPLC [8] methods have not become popular, because of their poor precision, expensive instrumentation, pretreatment and derivatization of the analyte. Enzymic colorimetric method employing lipase, glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO) and peroxidase, is comparatively more simple sensitive and specific hence suitable for routine. However, analysis of large number of samples requires the enzymes in bulk quantity, which is expensive. The co-immobilization of enzymes on to some insoluble support not only permits their reuse but also reduces the cost of procedure . Further, an immobilized system due to the shorter diffusional distance and restricted diffusion out to the surroundings, is more sensitive towards the low concentration of the substrate for the enzyme than a corresponding system with the enzymes separately immobilized. Lipase, GK, GPO and peroxidase have been co-immobilized onto free and affixed alkylamine glass beads and used for determination of serum triglycerides [9]. Recently amperometric determination of triglyceride has been reported based on immobilized enzyme electrode. A TG enzyme sensor for amperometric determination of triglyceride based on O_2 meter has a draw back that it requires expensive chemicals such as NADH and peroxidase and also not much sensitive [10]. Another triglyceride biosensor employing lipase glycerol dehydrogenase and diaphorase immobilized onto collagen membrane could measure serum triglyceride but not instantaneously. Collagen membrane is not stable and has to be changed weekly [11]. A porous silicon based biosensor employing immobilized liapse has also been prepared for triglyceride measurement as fatty acid generated in this method caused change in pH which was measured as shift in capacitance voltage and thus not a sensitive method [12] The present report describes the preparation of TG biosensor based on a highly sensitive dissolved O_2 meter employing commercial lipase, GK, and GPO co-immobilized onto polyvinyl chloride (PVC) membrane. Further, the PVC membrane has the advantage of high chemical resistance, low cost, non toxic, high affinity and response to simple ligand chemistry.

2. Materials and Methods

2.1 Chemicals and instruments

Lipase from Porcine pancreas, GK from *Cellulomonas spp,* and GPO from *Aerococcus viridans and* peroxidase from horseradish, 4-aminophenazone, L-α-glycerophosphate and Triton X-100 (Sigma Chemical Co., USA), Glutaraldehyde (25%), polyvinyl chloride (PVC) 3,5-dichloro-2-hydroxybenzene sulphonate (E. Merk Germany) , olive oil and ATP and isopropylmeristate from SISCO Research Laboratories Ltd., tetrahydrofuran (THF) from Qualigens fine chemicals, Mumbai were used. All other chemicals were of analytical reagent grade. Thermo Spectronic-20 (Milton & Roy Co. USA.) and dissolved oxygen meter (Make-Aqualytic Germany, Model No. OX-24) were used. The kit for enzymic colorimetric determination of triglycerides was from Miles India Pvt. Ltd., Baroda.

2.2 Assay of mixture of free lipase, GK, GPO and peroxidase

The combined assay of free lipase, GK, GPO and peroxidase was carried out in a test tube[4]. The reaction mixture consisted of $MgCl_2$ (0.54μmol), ATP (0.63 μmol), potassium ferricyanide(1.0 μmol), 4-aminophenazone(0.27 μmol), 3-5-dichloro-2-hydroxy benzene sulphonic acid (DHBS)(1.53 μmol), Triton

X-100(1.0 µmol) per litre of 0.1 M sodium phosophate buffer, pH 7.0 All the four enzymes were dissolved in 1.0 mL 0.1M sodium phosphate buffer pH 7.0 in a ratio 100:50:20:1. To 900 µL reaction mixture, 50µL enzyme solution was added and pre-incubated at 37°C for 5 min. The reaction was started by adding 50µL of 20 % olive oil emulsion. After incubation at 37°C for 15 min, A_{510} of reaction mixture was read and H_2O_2 generated in reaction mixture was extrapolated from standard curve between H_2O_2 and A_{510}.

2.3 Preparation of PVC membrane and activation

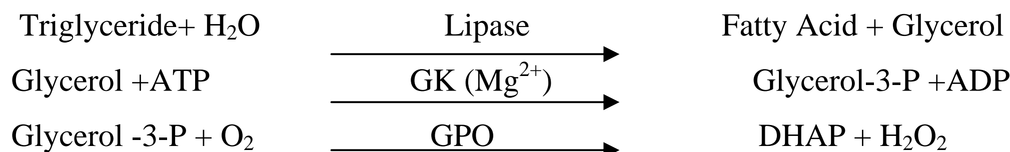
PVC 0.06g and 150µl isopropylmeristate were dissolved in 5ml of tetrahydrofuran (THF). The polymer solution was poured into petridish (Diameter: 9cm). Both the polymer solution was rotated on a horizontal flat surface to get an even distribution of polymer over the glass. The polymer solution was covered with a glass lid to allow slow and controlled evaporation of the solvent and creation of a membrane of even thickness.

2.4 Co-immobilization of enzyme on PVC membrane

PVC membrane was washed in double distilled water and small piece of (2×1) was cut and activated with 2.5% glutaraldehyde. After activation of membrane, excess of glutaraldehyde was decanted and membrane was washed with 0.1 M sodium phosphate buffer (pH-7.0) until the pH of washing was 7.0. One ml enzyme mixture of lipase*, GK and GPO* was added to activated membrane and kept at 4°C for varying time with occasional stirring. The unbound enzyme was decanted and tested for activity and protein [5]. The membrane was washed 3-4 times with reaction buffer (sodium phosphate , pH-7.0, 0.1M) until no activity was detected in the washing.

2.5 Construction and testing of biosensor

The membrane laminate of lipase, GK and GPO was mounted over the sensing part of combined electrode of dissolved O_2 meter with paraffin film. The enzyme electrode was then connected to the main apparatus of the dissolved O_2 meter. The electrode sensor was dipped into 1.8 ml of sodium phosphate buffer (0.1 M. pH 7.0) in a 10 ml glass beaker. The meter was put on and the reading displayed on the screen was read in terms (mg/l). The meter reading was kept on hold and then 0.1 ml olive oil emulsion was added into the reaction buffer. Dissolved O_2 of the reaction buffer was measured again. When the reading became constant, the difference of oxygen content between the two readings was noted, which provided the amount of O_2 consumed in the enzyme reactions, as follows.



$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Fatty Acid} + \text{Glycerol}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK }(Mg^{2+})} \text{Glycerol-3-P} + \text{ADP}$$

$$\text{Glycerol-3-P} + O_2 \xrightarrow{\text{GPO}} \text{DHAP} + H_2O_2$$

2.6 Storage of electrode

The enzyme electrode was stored in reaction buffer, 0.1 M sodium phosphate (pH 7.0) at 4°C when not in use. To reuse the electrode, it was washed with distilled water at room temperature followed by reaction buffer and then dried with a tissue paper.

2.7 Determination of triglyceride in serum

It was carried out as described above for testing of enzyme electrode under its optimal assay conditions except that olive oil emulsion was replaced by fresh serum sample. The triglyceride in serum was extrapolated from standard curve between olive oil concentration vs. O_2 consumed (mg/L) (Fig 1).

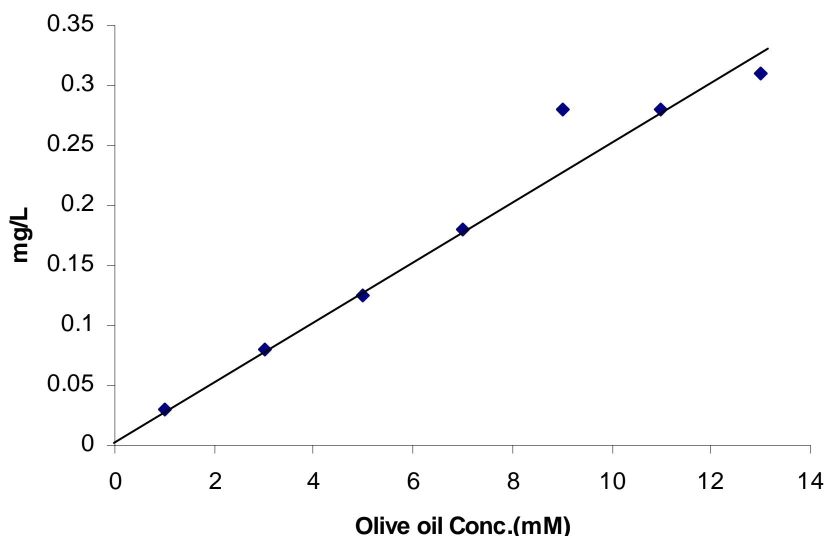


Fig.1. Standard curve of triglyceride obtained with PVC membrane bound lipase, GK and GPO employed in dissolved oxygen based biosensor.

3. Results and Discussion

Commercial lipase, GK and GPO were co-immobilized onto PVC membrane with a conjugation yield of 15 mg / 4cm^2 and 65.7 % retention of initial activity of mixture of free enzymes (Table 1). A procedure is described for construction of a dissolved O_2 meter based triglyceride biosensor (enzyme sensor) based on a highly sensitive dissolved O_2 meter employing PVC membrane bound enzymes. The triglyceride biosensor is based on measurement of dissolved O_2 consumed in GPO catalyzed oxidation of glycerol-3-phosphate obtained from TG by membrane bound lipase and GK which is directly proportional to TG concentration. The pH of co-immobilized enzyme was increased from pH 7.0 to 7.5 with incubation temperature from 37^{0}C to 39.5 ^{0}C after co immobilization. The response time of consumption of O_2 was 10-15 seconds. The K_m for olive oil employing the PVC membrane bound enzymes was decreased from 21.1 to 1.16mM and V_{max} from 19.6mg/L was to 0.58 mg/L after co-immobilization (Table 2). The changes in kinetic parameter of enzymes after co-immobilization were due to change in enzyme configuration, steric effects, micro-environmental effects ,bulk and diffusion effects. The biosensor has the advantage over other biosensors that PVC membrane restricts the diffusion of high molecular weight biological materials through it and thus prevents the interference in biosensor functioning. The following criteria were studied to evaluate the method.

Table 1. Co-immobilization properties of Porcine pancreas lipase, Cellulomonas spp, glycerol kinase (GK) and Aerococcus viridans glycerol-3-phosphate oxidase(GPO) onto PVC membrane (2×1cm) through glutaraldehyde coupling.

Enzymes added (mg protein)	3.5
Enzymes coupled (mg protein)	2.3
Conjugation yield (mg/cm^2)	3.75
Retention of specific activity (%)	65.7

Table 2. A comparison of kinetic properties of mixture of free and co-immobilized lipase, glycerol kinase, glycerol-3-phosphate oxidase onto PVC membrane employed in dissolved oxygen meter based biosensor.

Parameters	Mixture of free enzyme	Enzymes co-immobilized onto affixed glass beads	Enzymes co-immobilized onto PVC membrane employed in sensor
Optimum pH	7.2	8.0	7.5
Optimum Temp (°C)	37	40	39.5
Response time	15 min	20	10 ÷ 15 s
K_m (mM)	21.1	18.6	1.16
V_{max} ((nM/H_2O_2/min)	19.6	12.5	0,58 mg/L

Linearity

As the concentration of olive oil emulsion was increased, the consumption of O_2 was also increased. A linear relationship was obtained between the olive oil concentration ranging from 0.25mM to 14 mM and oxygen consumed (mg/L) (Fig2).

Detection limit

The minimum detection limit of the method was 0.25 mM/l of serum, which is lower than method employing alkylamine glass bead bound enzymes [9].

Determination of triglyceride in serum

The present biosensor was employed for determination of triglyceride in serum. The mean triglyceride value in sera obtained from apparently healthy adults (age 20-40 yrs) was 85.2 -175 mg/dl in females, while in males the mean triglyceride value was 92-190 mg/dl in male.

Precision

The content of triglyceride was determined six times in one run (within batch) in six serum samples on first day and after one week storage at –20 ^{o} C (between batch). The results presented in Table 3 showed that triglycerides values of these determinations agreed with each other and within batch and between batch coefficient of variation (CV) were <2.18 & 1.7 % respectively, showing the high reproducibility & reliability of the method.

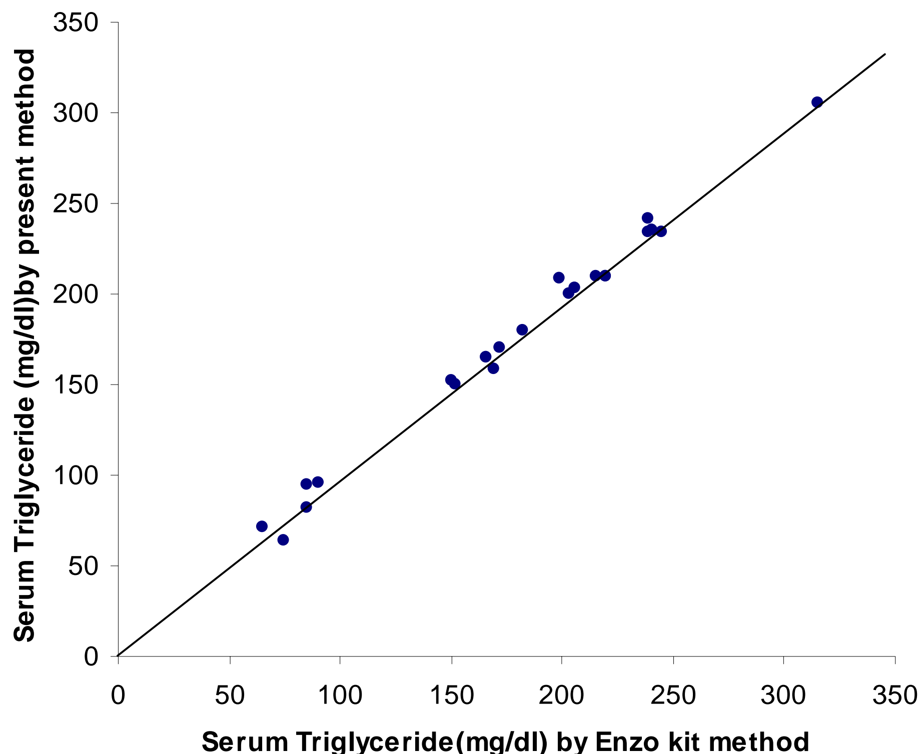


Fig. 2. Correlation between serum tryglyceride (TG) value determined by commercial enzo-kit method employing soluble enzyme (x-axis) and the present method (y-axis) using PVC membrane bound lipase, e, GK and GPO employed in dissolved oxygen based biosensor.

Table3. Precision study of serum triglyceride by O_2 meter based TG biosensor employing PVC membrane bound lipase, glycerol kinase, glycerol-3-phosphate oxidase.

6 (Within assay)		
Mean (mM l^{-1})	**S.D**	**CV%**
5.5	0.121	<2.18
6 (Between assay)*		
2.52	0.043	< 1.706

*Sample were assayed after one week storage at -20ºC

Accuracy

To test accuracy of method, the serum triglycerides values was determined in 20 serum samples of healthy and diseased persons by both the present method (y) and standard enzymatic colorimetric kit method (x). The values obtained by both methods showed a fair correlation (r = 0.973) with regression equation being $y = 0.9543x + 6.1286$ (Fig.2).

Reusability and storage

The enzyme electrode did not show any noticeable change in its initial activity after its 100 uses over a period of 50 days, when stored in 0.1M sodium phosphate buffer pH 7.5 at 4°C.

Interference study

The effect of the following metabolites on the activity of co-immobilized enzyme system was studied at their physiological concentrations i.e. glucose, pyruvate, citrate, uric acid, cholesterol, EDTA, glutathione, acetone, urea and ascorbic acid. None of the metabolite studied had any affect except for ascorbic acid, cholesterol and bilirubin, which caused 13% 15% and 12% inhibition respectively.

References

[1]. S.G. Klotzsch, and J.R McNamara, *Triglyceride measurement*: A review of methods and interferences,. *Clin. Chem*. 36 (1990), pp. 1605-1613.

[2]. J. Wallach, *Interpretation of diagnostic tests,* in: Little brown (Ed.), Lippin cott. Raven Publishers, Philadelphia 1996., PA 19106.

[3]. E. Van Handel, and D.B. Zilversmit, Micromethod for the direct determination of serum triglycerides. *J. Lab. Clin*. Med.50 (1957), pp. 152-157.

[4]. P. Fossati, and L. Prencipe, Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin. Chem.* 28 (1982), pp. 2077-80.

[5]. J.E. Voysey, and D.C. Wilton, Rapid, sensitive fluorometric determination of serum triglyceride by measuring lipase liberated fatty acids, *Clin. Chem.,* 40 (1994), pp. 14-17.

[6]. O.H. Lowry, N.J. Rosenbrough, A.L Farr,. and R.J. Randall, Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* 193 (1951), pp. 265-275.

[7]. I.Bjorkhem,., K Sandelin,. and T Anders,. A simple, fully enzymatic bioluminine – Scent assay for triglycerides in serum. *Clin. Chem.,* 28 (1989), pp. 1742-1744.

[8]. M.Okazaki, N Komoriya,., H Tomoike, N Inowe, S. Itoh, and S. Hoosaki, Quantitative detection method of triglycerides in serum tlipoproteins and serum free glycerol by high performance liquid chromatography. *J. Chromatogr. B. Biomed. Sci. Appl.,* 709 (1998), pp. 179-187.

[9]. Minakshi and CS Pundir ,Preparation of reusable enzyme strip for determination of serum triglyceride. *Indian J. Chem. Technol*. 12 (2005), pp.539-542 (Published by NISCAIR New Delhi).

[10]. A.K. Tim, and D.C. Gray, Amperometric determination of glycerol and triglycerides using an oxygen electrode. *Analyst,* 109 (1984). pp. 453-456.

[11]. W. Handani, S.K. Shia, and G.G. George, Amperometric enzymic determination of serum of triglycerides in serum. *Anal. Chem.* 54 (1982), pp. 1987-1990.

[12]. R.R., Reddy, Anju Chadha, Enakshi Bhattacharya, Porous silicon based potentiometric triglyceride biosensor. *Biosens. Bioelectronics,* 16 (2001), pp. 313-317.

2006 Copyright ©, International Frequency Sensor Association (IFSA). All rights reserved.
(http://www.sensorsportal.com)