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Amperometric Cholesterol Determination Using HRP Incorporated Carbon Paste Electrode

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Elevated level of serum cholesterol has become an utmost risk factor for several diseases. A method has been described for the construction of an HRP incorporated carbon paste based amperometric biosensor for cholesterol detection mounted on cellulose acetate (CA) membrane bounded with enzymes cholesterol esterase and cholesterol oxidase. Electrochemical measurements were implemented with Ag / AgCl as the reference electrode and silver wire as the auxiliary electrode under a capacity of -50mV. The optimum pH of the biosensor was pH 7.0. The incubation temperature and reaction time displayed in a wide temp range from 35 °C to 50 °C was 25 seconds. The linear relationship shown by the sensor was in range from 2.5 to 60 mg dL-1 of the substrate, cholesteryl acetate and amount of current (mA). The correlation coefficient, r was 0.9916 for n=20, being compared to standard colorimetric methods. The mean analytical recovery observed was 99.24±0.81 & 99.12±0.84 after addition of 100mg dL-1 and 200mg dL-1 cholesteryl acetate in serum samples respectively. Coefficient of variation (CV) calculated were <2% & <4 % within and between batch respectively. The present working electrode retained 50% of its initial activity when used continuously for 180 times over 35 days, stored in sodium phosphate buffer, (0.1 M, pH 7.0) at 4oC. There were no practical interferences observed in the present method.

Keywords: Cholesterol Esterase; Cholesterol Oxidase; Carbon Paste Electrode; Cellulose Acetate; Horseradish Peroxidase.

Measurement of serum cholesterol is required in the diagnosis and medical management of several diseases such as arthrosclerosis, nephritis, myxodema and obstructive jaundice, malabsorption, washing syndromes etc. (Hirany *et al*. 1997). Among the numerous methods available for cholesterol determination (Allian *et al*. 1974; Witte *et al*. 1974; Karube *et al*. 1982; Veldhoven *et al*. 1998; Shahnaz *et al*. 1998). Amperometric biosensor method is more rapid, sensitive and accurate. Several authors have been reported on the amperometric cholesterol biosensors since 1982, where the enzymes employed were cholesterol esterase (ChE), cholesterol oxidase (ChOx) and peroxidase got immobilized onto octyl-agarose gel and then placed on the electrode after activation with cyanogen bromide (Karube *et al*. 1982). Some authors have used nylon mesh and pyrrole membrane over a platinum electrode (Moody *et al*. 1988; Wolfgang *et al*. 1993). Carbon paste electrode was successfully used by Charpentier *et al*. 1995 after modified by hydroxymethyl

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ferrocene and hydrogen peroxide. Nuria *et al*, 2001 has employed graphite-70% Teflon matrix. Vidal *et al*, 2002 has used polypyrrole film co-entrapped with ferrocene monocarboxylic acid and incorporated onto platinized PT electrode. Pramanik *et al*. 2018 has formed a composite using polypyrrole, green reduced graphene oxide (gRGO) and cholesterol oxidase (ChOx) for the construction of biosensor for cholesterol diagnosis. There are many other matrices have been employed in various researches i.e. porous silicon (Song *et al*. 2006), electrochemically prepared polyaniline film (Singh *et al*. 2006), self-assembled monolayer of N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane (AEAPTS) (Prusty *et al*. 2007), ultra-thin dialysis membrane with photosensitive polymer (Endo *et al*. 2003), glassy carbon electrode modified with silica sol-gel matrix and prussian Blue (Jianping *et al*. 2003), pencil graphite electrode immobilized with apo-cholesterol oxidase (Dervisevic *et al*. 2016), conducing polypryrrile (PPY) membranes using entrapment method (Singh *et al*. 2004), sol-gel chitosan/silica hybrid composite material (Tan *et al*. 2005), electrophoretically deposited conducting polymer film derived from nano-structured polyaniline colloidal suspension (Dhand *et al*. 2007), amino-undecanethiol self-assembled monolayer (Solanki *et al*. 2007), modified graphene oxide based molecular imprinted polymer (Alexander *et al*. 2017). The choice of the matrix & membrane/film plays an important role in influencing the enzyme behavior as all the transformational dynamics related to substrate occurs at membrane-solution interface. Among the said matrices, Carbon is always observed to be the best support for the enzyme electrode fabrication (Stefan *et al*. 1999).

In amperometric biosensors, hydrogen peroxide (H2O2), a by-product of enzymatic reactions, is the main analyte to be detected electrochemically at the electrode due to its direct oxidation at high potential (Guibault *et al*. 1984).

$$H_2O_2 \xrightarrow{E} O_2 + 2H^+ + 2e^-$$

When working at high potential, other metabolites present in various serum samples may cause interference due to their extra oxidational current (Cardosi *et al*. 1991). Hence, horseradish peroxidase (HRP) based amperometric biosensor is an attractive alternative to detect hydrogen peroxide. In HRP based biosensor, the analytical signal depends upon the analysis of the reduction of an oxidized redox mediator formed during the amperometric reaction. Many authors have reported the direct uptake of HRP on the electrode surface. This direct regeneration is acquired through direct transfer of electron from the enzyme's redox centre (associated to the ferroheme/ ferriheme pair) and the conduction sites present on the electrode surface as given in the following reaction.

$$HRP_{ox.} + 2e^- \rightarrow HRP_{red.}$$

Electron transfer between the HRP and the electrode is relatively faster as compared to other reductases. This mechanism is due to the tridimensional protein configuration, where the electron transfer is facilitated because of the presence of heme group in the outer region (Gorton *et al*. 1992). This direct transfer is mainly possible as the electrode surface provides efficient enzyme adsorption due to presence of carbon black or spectroscopic graphite. This HRP collaboration with the carbon paste has been well reported by Ghindilis *et al*. 1997. The enzyme location within the matrix maintains a sharp electrical contact of enzyme to the conducing site and hence on the electrode surface.

In view of this, the present enzyme electrode was fabricated with HRP incorporated in the carbon paste for direct and efficient transfer of electron at low working potential. ChE and ChOx were co-immobilized on to cellulose acetate (CA) membrane, so as to retain the stability & sensitivity of the principle enzymes in cholesterol determination. CA membrane has the advantage of large pore size due to which substrate easily diffuses and is available to the adsorbed enzyme in the CA membrane laminate. CA/ChE/ChOx membrane was mounting on carbon/HRP electrode for total cholesterol determination.

MATERIALS AND METHODS

Cholesteryl esterase from Pseudomonas species (165.8 Units/g), cellulose acetate, glutaraldehyde (25% solution), cholesteryl acetate,

Triton X-100, silver chloride (AgCl) and platinum powder were purchased from Sigma Aldrich Chemical Co., USA. Cholesterol oxidase from Streptomycin sp (500 units/ 10mg), Horseradish peroxidase (HRP) (80 U/mg), F.C. reagent were from SISCO Research Lab. Pvt. Ltd. Mumbai. All Chemicals and reagents employed were of analytical grade and purest quality.

Preparation of Cholesteryl Acetate Solution

Substrate used for cholesterol esterase was Cholesteryl acetate. Substrate (50 mg) was dissolved in Triton X-100 (1.0 ml) by slowly heating on magnetic stirrer until it becomes clear solution. Then, Sodium phosphate buffer (PB), (0.005M, pH 7.0) was added and final value was made upto 500mg dL-1. Solutions with varying concentrations ranging from 2.5 mg dL-1 to 750 mg dL-1 were formed by same method and preserved at 4°C for further use.

Preparation of Carbon Paste & HRP Incorporation

Carbon powder (1.0 gm) and ammonium chloride (0.1 mg) were mixed in given ratio with paraffin to obtain the paste consistency. A plastic hollow tube (2cm length and 2mm diameter) with one closed end was taken and the prepared paste was filled into the tube from the top/open end leaving 5mm empty space. 100 µl of HRP (0.5mg) and 25µl of 15% BSA were mixed with 2.5% glutaraldehyde and allowed to react for 20 minutes. Carbon powder (0.5g) and platinum powder (3µg) were mixed well and added to HRP and BSA suspension. Paraffin oil was added to attain the paste. Then the modified paste was inserted into hollow plastic tube from its open end. This developed into the main body of electrode. Silver wire was inserted into the carbon paste to obtain electrical contact. The tip of electrode tip was gently rubbed with the fine abrasive sheet to get an even surface. The electrode surface was washed with buffer and the open end was polished with an aluminium paper before use. The electrode was stored at 4°C for further use as shown in Fig. 1.

Cross-Linking and Adsorption of ChE and ChOx onto CA Membrane

Cholesterol esterase & cholesterol oxidase (0.5 mg each) were mixed with BSA (15mg) and dissolved in distilled water (25µl) containing 2.5% glutaraldehyde and left overnight. The mixture was uniformly spread onto 4cm2 CA membrane and left for half an hour for adsorption. Another CA membrane of its size was gently placed onto it to form membrane laminate.

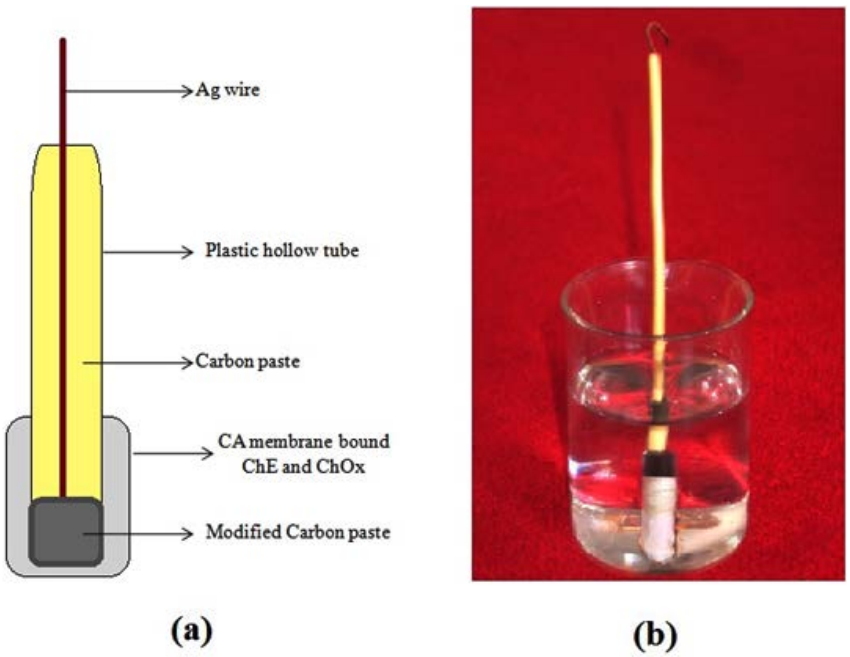

Fig. 1. Working electrode with CA membrane bound cholesterol esterase & cholesterol oxidase mounted on carbon paste electrode with incorporated HRP. (a) Schematic image (b) Real image

Fabrication of Carbon Paste Enzyme Electrode

CA membrane with adsorbed enzymes was wrapped around carbon paste electrode through paraffin film that formed a working electrode. This working electrode along with auxiliary and reference electrode was connected through Potentiostat.

Electrochemical Measurements

All the electrochemical measurements were taken by using a potentiostat. A three-electrode electrochemical cell system was used consisting of working, reference and auxiliary electrode. The amperometric testing of the working electrode was done at a potential of +0.7V. The substrate cholesteryl acetate (500mg dL-1) was transferred to the electrochemical cell which contains sodium phosphate buffer (0.02 M, pH 7.0). In the electrochemical cell, biochemical reactions occurred are given as:

$$Esterified\ cholesterol + H_2O \xrightarrow{Cholesterol\ esterase} cholesterol + fatty\ acid$$

$$Cholesterol + O_2 \xrightarrow{Cholesterol\ oxidase} choletenone + H_2O_2$$

$$H_2O_2 \xrightarrow{Peroxidase} O_2 + 2H^+ + 2e^-$$

Collection of Serum Samples

The left over serum samples were collected from apparently healthy persons and individuals diseased from hypertension, hyperthyroidism, myxodema, arteriosclerosis, certain anaemia, nephritis, obstructive jaundice, malabsorption from different age groups from clinical lab, Department of Biochemistry, Pt. B.D. Sharma PGIMS, Rohtak. The consent of the patients for collection of blood was already taken by the Institute. Four individuals were included per age group.

Preparation of Standard Curve for Total Cholesterol

A standard curve was processed using various concentration of cholesteryl acetate in the final range 2.5 mg/dl to 100 mg/dl as mentioned in above segment by observing the current response of electrode in milliampere under optimal working conditions. A constant potential of -50mV versus Ag/AgCl was applied in a reaction beaker containing 3 ml of sodium phosphate buffer (0.02 M, pH 7.0) and 0.2ml of cholesteryl acetate. A curve was plotted and evaluated between cholesteryl acetate amount and current (mA).

Detection of Total Cholesterol Level in Healthy & Diseased Individuals by Electrode

The total cholesterol concentration in several serum samples was measured by using electrodes of both enzymes. The assays for total cholesterol amount in serum samples were evaluated by the similar method as mentioned above in the section of preparing the standard curve

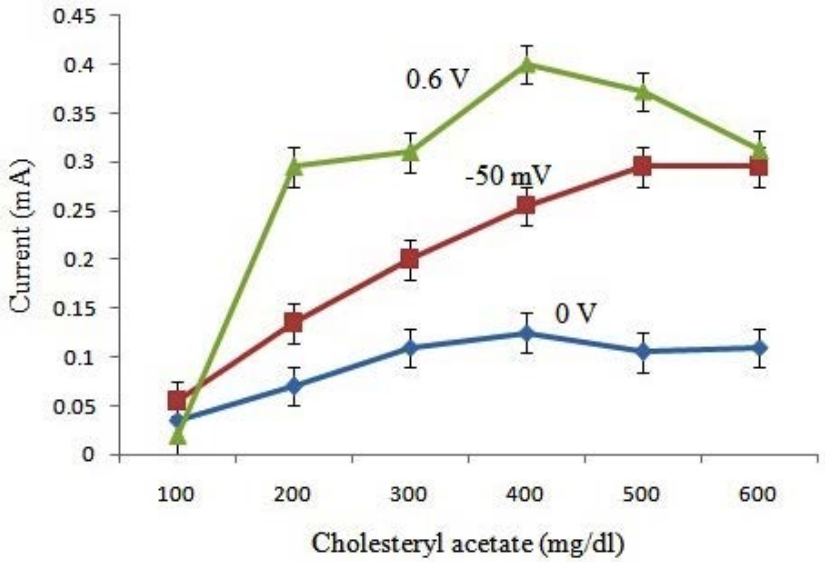

Fig: 2. Amperometric response curve for Carbon paste electrode as a function of cholesteryl acetate concentration at -50mV, 0V and 0 .6V, pH 7.0. Error bars representing standard deviation of three values

but here, the cholesteryl acetate was replaced by 0.2 ml serum. The total cholesterol concentration was extrapolated from the standard curve plotted between cholesteryl acetate concentration and electrical responses in milliampere (mA).

Detection Limit

Minimum and maximum limit of detection (LOD) were measured by amperometric detection of enzyme electrode responses of enzyme at different concentration of cholesteryl acetate ranging from 2.5 mg/dl to 100 mg/dl and the measurement of current was done by potentiostat. The minimum LOD for cholesteryl acetate was observed from their standard curve.

Percentage Recovery

Reliability of the methods was determined by measuring the cholesteryl ester content into the serum samples before and after addition of two different concentration of cholesteryl acetate i.e. 100 mg/dl and 200 mg/dl. The Percentage recovery of added cholesteryl acetate was measured.

Precision

Reproducibility of the present method was studied by determining the cholesteryl acetate content in six serum samples continuously within batch i.e. for same day and between batches i.e. for one week after their storage at -20oC. Coefficient of variation (CV) was calculated in both batches using the following formula.

$$CV = \sigma \times 100 / \alpha$$

Where,

σ = SD

α = mean of series

Accuracy

Accuracy of fabricated biosensing method was determined by measuring the concentrations of total cholesterol in serum samples (n=20). Measurement was done by Bayers enzo kit method in which free cholesterol esterase, cholesterol oxidase and peroxidase enzymes were employed by enzymic colorimetric method (x) and by the present method (y). The obtained values from both the different methods were related to each other through regression equation, and value of correlation coefficient (r) was calculated using the equation below:

$$r = \frac{n\Sigma xy - \Sigma x \Sigma y}{\{n\Sigma x^2 - (\Sigma x^2)\}\ \{n\Sigma x^2 - (\Sigma y^2)\}}$$

Where,

'x = value obtained by reference method.

y = values obtained by present method'.

Effect of Interfering Substances

Influence of several compounds present in blood i.e. potassium chloride, copper sulphate, zinc sulphate, sodium chloride, pyruvate, glucose,

Table 1. Total cholesterol concentrations measured from serum samples of apparently healthy and diseased persons employing working electrode.

Age group (n=08)	Sex	Total serum cholesterol mg/dl(mean±S.D.) Healthy persons	Total serum cholesterol mg/dl(mean±S.D.) Diseased persons
< 10	M	153.16±4.08	228.19±7.38
	F	144.92±8.06	231.51±13.34
11-20	M	169.94±17.78	241.96±19.46
	F	164.82±18.28	244.18±14.78
21-30	M	188.18±8.82	251.44±18.18
	F	186.98±18.04	258.96±19.58
31-40	M	198.02±3.28	273.62±27.13
	F	189.82±17.44	272.19±18.96
41-50	M	208.56±12.22	289.92±35.82
	F	196.44±8.18	203.14±41.91
51-60	M	222.38±18.46	271.68±36.11
	F	218.78±14.64	276.22±27.88
61& Above	M	225.43±18.82	280.11±38.78
	F	225.12±11.28	281.59±42.45

magnesium sulphate, citrate, Ca2+, EDTA, uric acid, ascorbic acid, acetone, urea and billirubin were detected by their addition into the reaction mixture particularly at their specific concentration (0.1 mM) before adding the cholesteryl acetate.

RESULTS AND DISCUSSION

Procedure of co-Immobilization of ChE & ChOx onto CA Membrane

Glutaraldehyde, a bifunctional compound, has two aldehyde groups that covalently links to amine groups of protein molecules (lysine or hydroxylysine) that provides more physical stable complex as compared to prepared by the physical aggregation. Additionally, BSA reinforced the compact ester structure of the enzyme that helps in enhancing stability. The stable tri-complex (BSA+ChE+ChOx) due to its large molecular size could not leach out through the large pores of CA membrane. CA membrane has the advantage of large pore size through which substrate diffuses in

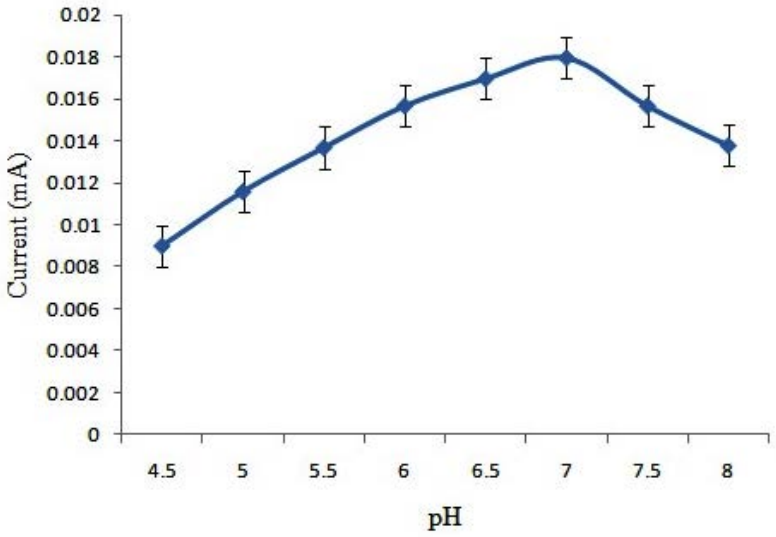

Fig. 3. Effect of pH on the working electrode at -50mV. Error bars representing standard deviation of three values

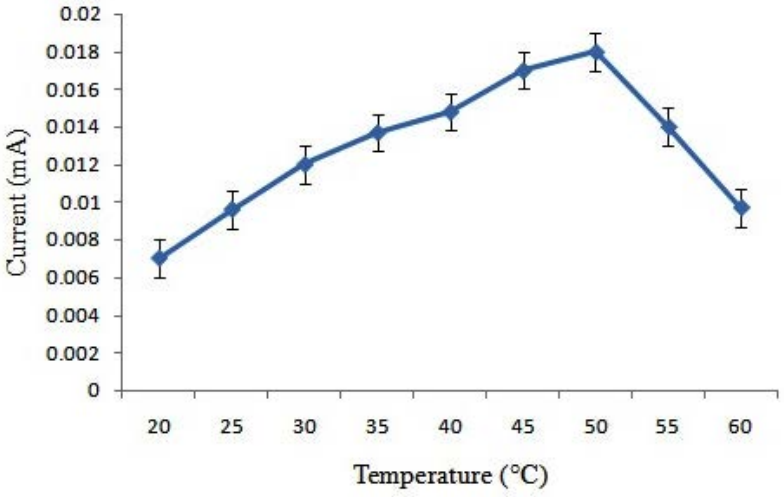

Fig. 4. Effect of temperature on the response of biosensor based on carbon paste working electrode at -50mV. Error bars representing standard deviation of three values

easily and is available to the adsorbed enzyme in the CA membrane laminate. BSA is used to increase the size of the multi-enzyme complex and prevents its leakage from the CA membrane pores.

Effect of Applied Potential

The response was detected by applying three potentials of – 50mV, 0V and +0.6V and fluctuations occurred in current has been shown in Fig. 2. A cathodic potential of -50 mV has shown the optimum response in reduction of the hydrogen peroxide for detection of total cholesterol.

Effect of pH

The dependence of the biosensor response on pH was investigated. The working electrode has been subjected to a pH ranging from 4.5 to 8.0 at an interval of 0.5. The optimum working pH was observed between pH range 6.5-7.5 at which the biosensor showed highest activity. With further increase in pH, the activity of biosensor decreased as shown in Fig. 3. This concluded that biosensor has shown an efficient usage for cholesterol quantification in intracellular fluid pH

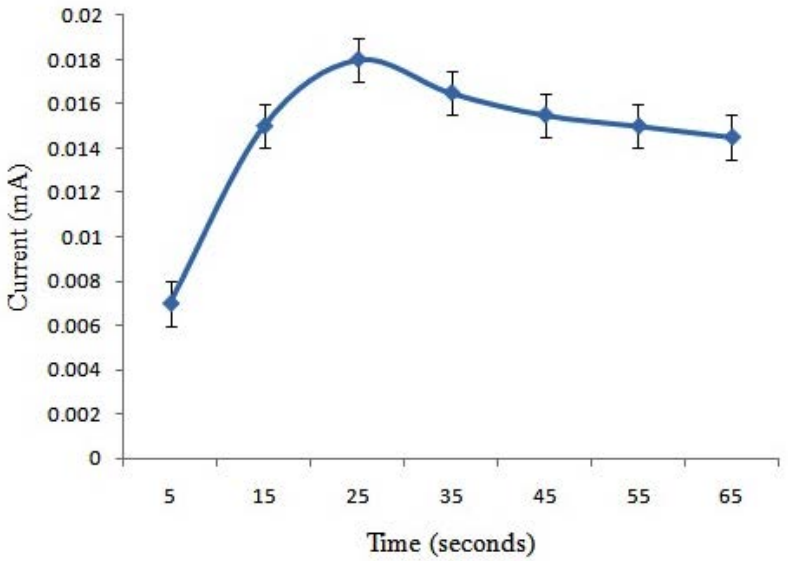

Fig. 5. Effect of incubation time on the response of working electrode at -50mV. Error bars representing standard deviation of three values

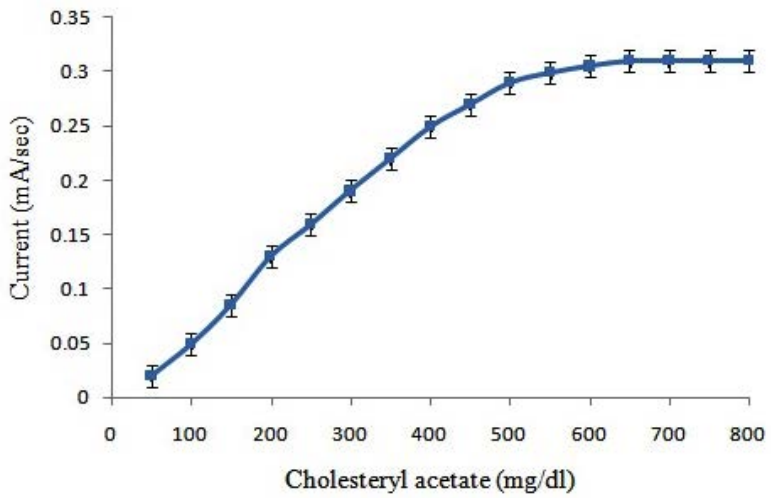

Fig. 6. Effect of concentration of cholesteryl acetate varying from 50 mg/dl to 750 mg/dl on biosensor employing carbon paste working electrode. Error bars representing standard deviation of three values

range i.e. pH 7.4, which is similar to that of mixture of free enzymes & other report (pH 7.0) [Singh *et al*. 2004; Charpentier *et al*. 1995; Singh *et al*. 2006), but higher than that of pH 6.5 (Singh *et al*. 2006). A possible reason for the change in activity with pH could be the changes in acidic and basic amino acid side chain around the active site in the microenvironment. These changes are caused by the interactions between basic residues of enzyme and glutaraldehyde during cross linking (Singh *et al*. 2006).

Effect of Incubation Temperature

The optimum temperature of the biosensor was measured in different temperature values between 20oC to 60oC at a regular interval of 5oC. The electric response was increased as the temperature was increased in the range 35°C to 50°C after it showed a decline in the activity (Fig. 4). Hence, the temperature of reaction mixture in all the subsequent experiments was kept at 50oC, which is much higher than the mixture of free enzymes (40°C) (Suman *et al*. 2003) but comparable to that prepared by Singh *et al*. 2006 (48°C) and higher than that reported earlier in temp range 30 to 45°C (Singh *et al*. 2004). The reason for the rise in the optimum temperature could be the conformational changes of enzyme after co-immobilization. Another reason might be the steric hindrance or diffusion effect that protected the enzyme against heat denaturation. The real temperature of bulk solution was generally less than that of microenvironment (Kennedy *et al*. 1975).

Response Time

To determine the suitable incubation period of the working electrode, response time was evaluated by measurement of amperometric responses between time period from 5 sec to 65 sec with an interval of 10 sec. During the period of 5 sec to 25 sec, an increase in the response was observed and after this, the response became stable (Fig. 5). Thus, the potentiostat observations were recorded after 25 sec in all subsequent assays. This response time is higher as compared to other biosensors as the site of generation of H2O2 (CA membrane) and its reduction (carbon paste) are differentiated. Rate of diffusion is the limiting factor in response generation.

Effect of Substrate (Cholesteryl Acetate) Concentration

The optimum substrate concentration for the working electrode was determined by employing different concentration of cholesteryl acetate from 50 mg dL-1 to 750 mg dL-1 in the reaction mixture as shown in Fig.6. A hyperbolic curve was observed between electrode current measurements and substrate concentration upto 550 mg dL-1 of final concentration. These results was lower than amperometric detection done by employing octyl agarose gel bound enzyme i.e.

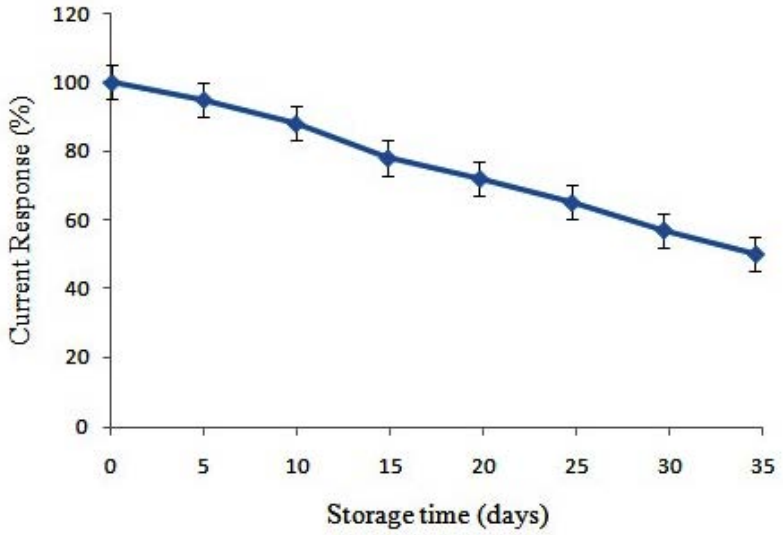

Fig. 7. Storage stability of the working biosensor based on carbon paste electrode at 4°C. Error bars representing standard deviation of three values

1000 mg dL-1 (Karube *et al*. 1982) and have shown similar responses as biosensor showing linearity form 50-500 mg dl-1 of cholesterol oleate (Singh *et al*. 2006), 1 to 8mM of cholesterol ester (Singh *et al*. 2004), 500mg dL-1 cholesterol (Singh *et al*. 2006) and 8µM cholesterol (Vidal *et al*. 2004).

Effect of Serum Metabolites

Several metabolites were added to study the effect of interferences i.e. glucose, lactate, ascorbic acid, uric acid, acetone, EDTA, glucose, and bilirubin. All the metabolites were added to solution before adding cholesteryl acetate. There were no practical interference observations in the present method. Singh *et al*. 2006 (Singh *et al*. 2006) has observed earlier that uric acid and ascorbic acid (0.6V) enhanced the current measurements in his research. This was due to their high potentials that contributed to increment of oxidation current.

Effect of Metal Ions and Metal Salts

The influence of various metal salts such as magnesium chloride, sodium chloride, copper sulphate, potassium chloride, calcium sulphate, zinc sulphate each at a final concentration of 1.0 mM was detected by the action of both the working electrodes. Only Mg2+ stimulated the electrochemical response of the working electrodes slightly. No effects have been observed by rest of the metals.

Storage Stability and Reusability

The enzyme electrode suffers from a loss of about 50% activity when repeatedly for 180 times used up to a period of 35 days. The electrode was restored at 40C in PB solution after each use. The stability is enhanced by the membrane coating as it prevents the leaching of enzyme which enhances the reusability also (Fig-7).

Minimum Detection Limit

The L.O.D of the working electrode was 2.5 mg dL-1, which is ten times lower than that of cholesterol biosensor with minimum detection limit of 25 mg dL-1 cholesterol (Singh *et al*. 2006), and 3 times less than polarographic method employing soluble enzymes with detection limit 12.5 mg dL-1 (Noma *et al*. 1976), but more than the methods using silica gel bound enzyme with detection limit of 0.12 mg dL-1 (Yao *et al*. 1985).

Analytical Recovery

Analytical recovery was measured for added cholesteryl acetate in the samples to measure the accuracy of present method. The mean analytical recovery obtained for added cholesteryl acetate i.e. 100 mg dL-1 and 200 mg dL-1 in six serum sample was 99.24±0.81 & 99.12±0.84 respectively. These values were comparable to the electrochemical method that have used alkyl amine glass bound enzyme i.e. 95-102% recovery for the added 192 mg dL-1 and 145 mg dL-1 cholesterol concentration respectively (Reddy *et al*. 1994).

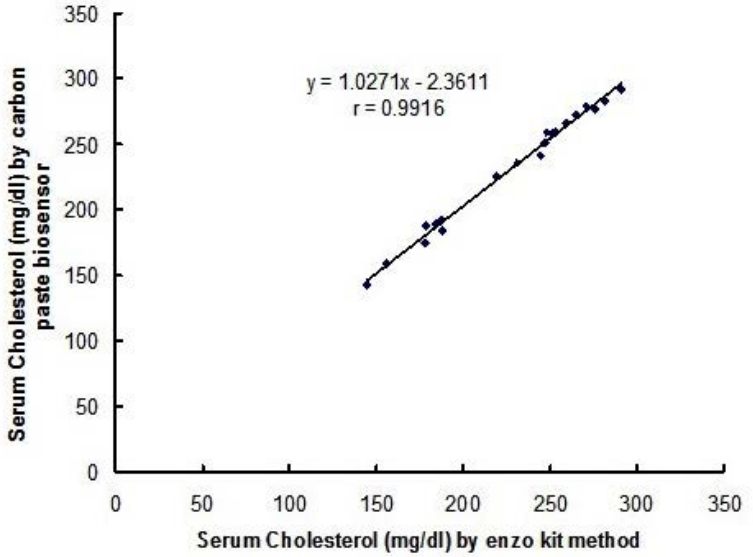

Fig. 8. Correlation between total cholesterol concentration observed by enzo kit process using soluble enzymes (x axis) and by biosensing device (y-axis) based on carbon paste electrode

Precision

Reproducibility and reliability of present device was studied by determing the cholesteryl acetate content in six serum samples continuously on the same day i.e. within batch and for one week after their storage at -20oC i.e. between batch. The results concluded that the values obtained were in close agreement to each other and coefficient of variation (CV) found was <2% & < 4 % for biosensor employing carbon paste within and between the batch, which is similar to the consequences reported by amperometric method using silica gel bound enzyme (<1.5% for all samples) (Tabata *et al*. 1981), cholesterol detection method using phosphotungstic acid/ MgCl2 (within batch 5.0 % and between batch 8.2% (Heuck *et al*. 1985) and flow injection method employing controlled pore glass immobilized ChE and ChOx (within batch < 1.0 and between batch < 2.5%) (Fernandez *et al*. 1987). The low per cent CV values shows the accuracy and reproducibility of the present method.

Accuracy

Accuracy was determined by comparing the cholesterol values from 20 samples by enzymatic colorimetric method (x) and the results obtained by current biosensing device (y). A good correlation (r = 0.9916) was shown by the results got by present working electrode with those by standard enzymatic colorimetric method by using regression equation of y1 = (01.0271x + 2.3611) as shown in Figure 8. The results observed by both the methods are also in correlation to the previously reported methods by Bachorik *et al*. 1988 and Zeng *et al*. 2019.

Concentration of Total Cholesterol in Various Serum Samples

Using the present method, the total cholesterol content in obtained serum samples was measured by present biosensing device employing carbon paste working electrode. The total cholesterol amount in serum samples from healthy persons ranged from 140 mg dL-1 to 220 mg dL-1 for males and 135mg dL-1 to 220 mg dL-1 for females. Total cholesterol content detected by carbon paste electrode in serum samples of apparently healthy individuals was observed to be from 153.16 to 225.43 mg dL-1 for male and from 144.92 to 225.12 mg dL-1 for female as shown in Table. 1. The total cholesterol amount measured from the diseased persons from various hyper cholesterolimic diseases was evaluated and found in the range 228.19 to 280.11 mg dL-1 for male and 231.51 to 281.59 mg dL-1 for female employing carbon paste electrode (Table.1). The results obtained were also compared to the results obtained by analytical methods i.e. Carr and Drekter's colorimetric method (211 mg dL-1) (Blomhoff *et al*. 1973), GLC method (194 mg dL-1), and HPLC method (range was 131-335 mg dL-1) (Duncan *et al*. 1979).

CONCLUSION

Fabrication of carbon paste working electrode for amperometric detection of total cholesterol has been presented in the current work. To provide direct electron transfer HRP was employed into carbon paste. However, to maintain and enhance the stability & sensitivity of the enzymes i.e. ChE & ChOx were co-immobilized on to CA membrane through glutaraldehyde coupling. To determine total cholesterol a reducing potential, CA/ChOx/ChE membrane was placed onto the carbon paste electrode with incorporated HRP. The biosensor worked efficiently at -50mV and exhibits linearity with concentration in the range 2.5-6.0 mg dL-1. The optimum temperature range is 35-50°C and the optimum pH range is 6.5-7.5. The effect of various serum metabolites was studied at their physiological concentrations. Added cholesteryl acetate (100mg dL-1 and 200mg dL-1) in six serum samples showed the mean analytical recovery of 99.24±0.81 & 99.12±0.84 respectively. Coefficient of variation (CV) for the cholesterol amount were <2% & < 4 % for within and between batch respectively.

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