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Preparation of a reusable enzyme strip for determination of serum cholesterol

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A reusable enzyme strip for the determination of cholesterol was prepared by co-immobilizing cholesterol esterase (CEase), cholesterol oxidase (COD) and peroxidase (POD) onto alkylamine glass beads affixed on a plastic strip by a non-reactive fixative. The co-immobilized enzymes retained 71.42% of initial activity of free enzymes with a conjugation yield of 11.0 mg g^{-1} beads and showed optimum activity at *p*H 6.6, when incubated at 32°C for 6 min. The strip was employed for direct determination of total cholesterol in serum. The minimum detection limit of the method was 2.14 mg dL^{-1} reaction mixture. The within batch and between batch coefficient of variation (CV) were <7.0% and <19.0%, respectively. The serum total cholesterol values as determined by the strip, were in good correlation (r=0.885) with those by commercial enzo-kit method employing free enzymes. The method is characterized by reuse of co-immobilized enzymes with great ease and unaffected by various serum substances.

Keywords: alkylamine glass, cholesterol, cholesterol esterase, cholesterol oxidase, enzyme strip, peroxidase, serum

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Introduction

The determination of cholesterol level in blood is essential in the diagnosis and medical management of heart disease such as atherosclerosis, nephrosis, diabetes mellitus, myxedema obstructive jaundice and cholelithiasis[1]. Among the various methods available for the determination of serum cholesterol, enzymic colorimetric method employing cholesterol esterase (CEase), cholesterol oxidase (COD) and peroxidase (POD) is comparatively simple, sensitive, specific and rapid, hence suitable for routine assay[2]. The method is so popular that its commercial kit is available. However, a bulk quantity of enzymes is required for a large number of clinical samples, which is expensive. The immobilization of enzyme onto insoluble support provides its reuse and thus renders it economical. The authors have developed methods for discrete analysis of serum total cholesterol employing CEase, COD and POD immobilized individually and co-immobilized onto free alkylamine glass beads, which reduced the cost of cholesterol determination[3-4] . However, the handling of free glass bead bound enzymes was tedious and time consuming and included the risk of losing them during the transfer of reaction mixture and their washing for reuse. The problem was overcome in the present study by affixing the glass beads on a plastic strip before co-immobilization of enzymes.

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Materials and Methods

Chemicals

Zirconia coated alkylamine glass beads (pore diameter 55 nm) (Corning Glass Works) Cholesterol esterase from bovine pancreas, cholesterol oxidase from *Brevibacterium* sp., peroxidase from horseradish, 4-aminophenazone and Triton X-100 (Sigma) Enzo kit for cholesterol determination (Bayer's Diagnostics, India), glutaraldehyde (BDH) "Lakme" nail enamel of silver white colour (No. 754, Dew drops) (Flora Cosmetics) were used. All other chemicals were of analytical reagent grade.

Preparation of Cholesteryl Acetate Solution

50 mg of cholesteryl acetate was dissolved in 1 mL Triton X-100 by slow heating and stirring until the solution was clear. Sodium phosphate buffer (0.05 *M*, *p*H 7) was added to make final volume to 100 mL (50 mg dL^{-1}) and stored at 4°C until use.

Combined Assay of Free CEase, COD and POD

An enzyme solution was prepared by dissolving 1 mg each of CEase, COD and POD in 3.0 mL 0.05 *M* sodium phosphate buffer (*p*H 7.0). A combined assay of free CEase, COD and POD was carried out as

described by Malik and Pundir[3] in a 15 mL test tube wrapped in a black paper and based upon H_2O_2 measurement generated from cholesterol by cholesterol oxidase, with a colour reagent consisting of 4-aminophenazone, phenol and peroxidase as chromogenic system. The reaction mixture consisting of 1.9 mL sodium phosphate buffer (0.05 *M*, *p*H 7.0), 0.1 mL enzyme solution and 0.01 mL cholesteryl acetate (50 mg dL^{-1}) was incubated at 37°C for 5 min, 1.0 mL colour reagent was added to the reaction mixture and kept at room temperature (30±2°C) for 15 min to develop the colour. A_{520} was read against blank in Spectronic–20 (Milton & Roy). The content of H_2O_2 generated in the reaction was calculated from standard curve between A_{520} vs H_2O_2 concentrations.

The colour reagent consisted of: 4-amino-phenazone, 50 mg; phenol 100 mg per 100 mL; sodium phosphate buffer, 0.05 *M* (*p*H 7.0). It was stored in amber colored bottle at 4°C until use and prepared freshly every week.

Preparation of Reusable Strip of CEase, COD & POD

Affixation of Alkylamine Glass Beads onto Plastic Strip

A plastic strip (15×1 cm) was cut from a plastic sheet. One end of this strip was made round with scissor and then scratched with a sand paper. A thin layer (0.1mm thickness) of 'Lakme' light cream nail enamel was applied onto it both sides of round end up to a height of 3.0 cm. Alkylamine glass beads (75 mg) were sprinkled uniformly on the wet layer with the help of aluminium foil and the strip was dried at room temperature (30±5°C) for 24 h.

Co-immobilization of CEase, COD & POD onto Affixed Alkylamine Glass Beads

The mixture of CEase, COD and POD was co-immobilized onto affixed alkylamine glass beads through glutaraldehyde coupling as described by Suman and Pundir[4] with modification: Alkylamine glass beads affixed on plastic strip were activated by dipping the strip into 3.0 mL 2.5% glutaraldehyde in sodium phosphate buffer (0.1 *M*, *p*H 7.0) in a 15 mL test tube for 2 h at room temperature (30±2°C). The strip was removed and washed with 0.1 *M* sodium phosphate buffer (*p*H 7.0) many times until the *p*H of washing discard was 7.0. The end of plastic strip containing glutaraldehyde activated glass beads was dipped into 3.0 mL of enzyme solution containing CEase, COD and POD in a 15 mL test tube so as to submerge all the beads and kept at 4°C for 48 h with occasional stirring. The strip was taken off from enzyme solution and dipped into 3 mL 0.05 *M* sodium phosphate buffer (*p*H 7.0) 3-4 times, until no activity was detected in the consequent washings. The unbound enzyme solution was tested for activity and protein. The protein bound to glass beads affixed on plastic strip was estimated by determining the loss of protein from the enzyme solution during im-mobilization. The strip was also tested for activity (Fig. 1).

Assay of strip bound CEase, COD and POD

The assay of strip bound CEase, COD and POD was carried out in the same manner as described for mixture of free enzymes except that mixture of free enzymes was replaced by strip bound enzymes and reaction mixture was increased by 110 μL and stirred continuously during incubation. The enzyme strip was taken out from the reaction mixture before addition of colour reagent. The control was run in the similar manner except that strip had only activated glass beads.

Reusability and storage of CEase, COD and POD strip

To reuse co-immobilized CEase, COD and POD, the 'enzyme strip' was washed by dipping it in a test tube containing 3.0 mL 0.05 *M* sodium phosphate buffer, *p*H 7.0 and shaking it gently for 10-15 sec. The process was repeated until A_{520} of washing discard was zero. The end of strip containing glass bead bound enzymes was kept dipped in reaction

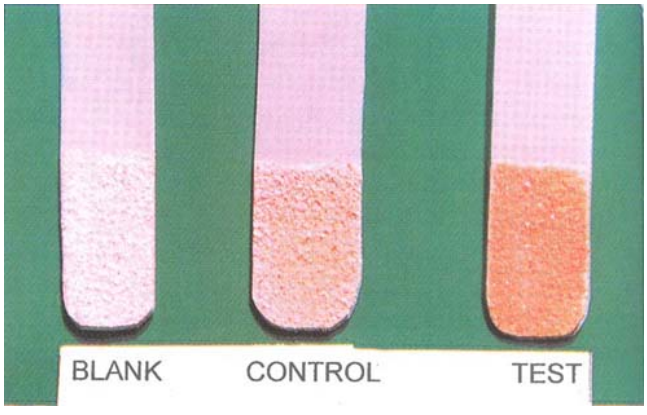

Fig. 1—BLANK–Blank, Plastic strip containing alkylamine glass beads affixed with nail paint; CONTROL–Plastic strip containing glutaraldehyde activated affixed alkyl-amine glass beads; TEST–Test, Plastic strip containing affixed alkylamine glass beads coupled to cholesterol esterase, cholesterol oxidase and peroxidase

buffer (0.05 *M*, *p*H 6.6) in a test tube at 4°C when not in use.

Determination of serum total cholesterol by CEase, COD and POD strip

Fresh serum samples of apparently healthy male and female adults were collected from local Medical Institute Hospital. To 0.1 mL serum was added 0.1 mL Triton X-100, 0.1 mL isopropanol and 0.2 mL sodium phosphate buffer (0.05 *M*, *p*H 7.0) and centrifuged at 4000 rpm for 5 min. The supernatant (0.4 mL) was collected of which 0.2 mL was used in assay. The assay of total cholesterol in serum was carried out under optimal assay conditions in the same manner as described for assay of strip bound CEase, COD and POD except that reaction buffer was decreased by 0.2 mL and cholesteryl acetate solution was replaced by 0.2 mL pretreated serum. The amount of cholesterol in serum was calculated from standard curve between A_{520} and cholesteryl acetate concentration ranging from 0.05 to 0.5 m*M*.

Results and Discussion

Commercial CEase (100 U mg^{-1}) from bovine pancreas, COD (150 U mg^{-1}) from *Brevibacterium* sp. (recombinant type) and POD from horseradish (500 U mg^{-1}) in mixture were co-immobilized through glutaraldehyde coupling onto alkylamine glass beads affixed on one end of a plastic strip by a non reactive fixative. The strip retained 71.42% retention of initial activity of free enzymes with a conjugation yield of 11.0 mg protein g^{-1} glassbeads, which is higher than that on free alkylamine glass beads (3.2 mg protein g^{-1})[5] (2.3 mg protein g^{-1})[4]. The strip not only provided the reuse of CEase, COD and POD but also overcome the tedious and time consuming handling of free glass beads bound to these enzymes[3]. The strip bound enzymes showed optimum activity at *p*H 6.6 when incubated at 32°C for 6 min. A comparison of some kinetic properties of CEase, COD and POD co-immobilized onto free and affixed alkylamine glass beads as given in Table 1, indicated that affixed glass bead bound enzymes would be better support than free glass beads for immobilization/co-immobilization of enzymes for cholesterol determination.

A simple, sensitive and specific method for discrete analysis of total cholesterol in serum was developed employing plastic strip bound CEase, COD and POD. The method is based on quantification of H_2O_2 generated from cholesterol by co-immobilized COD, which forms a coloured complex after coupling with 4-aminophenazone and phenol catalyzed, by co-immobilized POD. The method has the advantage that it provides the reuse of co-immobilized enzymes with enormous ease. The following analytic parameters were measured to evaluate the method.

Linearity

There was a linear relationship between A_{520} and cholesteryl acetate concentration ranging from 4.28 to 514.44 mg dL^{-1} reaction mixture, which is better than those employing individually immobilized and co-immobilized enzymes on glass beads (4.28 to 171.48 mg/dL and 5 to 50 mg/dL)[3,4].

Minimum Detection Limit

The minimum detection limit of the method was 2.14 mg dL^{-1} reaction mixture, which is lower than that by nylon mesh bound enzymes (8.57 mg dL^{-1})[6] and free arylamine glass bead bound enzymes (4.28 mg dL^{-1})[3] but higher than that of polarographic method (1.25 mg dL^{-1})[7].

Recovery Studies

The percentage recoveries of added cholesteryl acetate in serum (85.74 & 171.48 mg dL^{-1}) as measured by the present method were 88.8 and 86.7%, respectively, which are lower than that by enzymes co-immobilized on free alkylamine glass beads (98%)[5] and electrochemical method (98.5%)[8].

Table 1—A comparison of kinetic parameters of CEase, COD and POD co-immobilized onto free and affixed alkylamine glass beads

Kinetic parameters	Enzyme co-immobilized on free alkylamine glass beads[4]	Enzyme co-immobilized-immobilized on affixed alkylamine glass beads
Optimum *p*H	7.0	6.6
Temp for max activity (°C)	37	32
Time for linearity (min)	12	6
Substrate conc. for saturation	50 to 500 mg dL^{-1}	42.87 to 514.44 mg dL^{-1}
Stability after storage at 4°C for 45 d	62%	50%

Precision

To assess the reproducibility and reliability of the method, total cholesterol of the six serum samples in one run (within batch) and after storage at -20°C for two week (between batch) were determined. The results showed that within day and between day coefficient of variation (CV) were 6.62 and 18.56%, respectively (Table 2), which are comparable to that by other methods such as electrochemical method employing alkylamine glass bound enzyme (1.6% for intrabatch and 3.2% for interbatch)[8] and amperometric method using silica gel bound enzyme (<1.5%)[5], and enzymes co-immobilized onto free arylamine glass (<1.5 & <4.0%)[3] and chemical method (3.3%)[9].

Accuracy

To check the accuracy of the present method, the total cholesterol values in twelve serum samples as determined by present method (y) were correlated with those obtained by commercial enzo kit method employing free enzymes (x). The values obtained by both the methods agreed with each other and showed a good correlation with r = 0.885., which is better than that for method employing co-immobilized enzymes on to free alkylamine glass beads (r = 0.83)[4].

Determination of Total Cholesterol in Serum

The content of total cholesterol in sera of apparently healthy adults as measured by the present method was in the range of 160 to 257.22 mg dL^{-1} for male and 150 to 222.92 mg dL^{-1} for female, which is in the established normal range (150-250 mg dL^{-1}). Earlier serum total cholesterol as determined by co-immobilized enzymes on free arylamine glass was 164.68 to 192.96 mg dL^{-1} for male and 152.20 to 195.45 mg dL^{-1} for female [3].

Interference Study

To study the interference of various substances found in serum such as NaCl, KCl, sodium bicarbonate, creatinine, albumin, vitamin C, vitamin D, estrogen, progesterone and testosterone in the method, these substances were added individually in the reaction mixture at their physiological conc. None had practically any effect on the strip bound enzymes, similar to those for immobilized on free controlled pore glass beads[10] and zirconia coated alkylamine glass beads[3].

Reusability and Storage Stability

The immobilized enzyme strip did not show considerable loss in its activity upto 20 d, during its regular use, when stored in reaction buffer at 4°C. However, the strip lost 50% activity after its 120 times use during the span of 30 d.

Table 2—Within and between assay coefficient of variations (CV) for determination of total cholesterol in serum by co-immobilized CEase, COD & POD onto affixed alkylamine glass beads

Cholesterol (mg/dL)		Mean	% CV
Within assay			
*	**		
222.92	222.92		
197.20	180.05		
222.92	222.92	219.49	6.62
231.49	222.92	214.34	
222.92	222.92		
Between assay			
*	***		
145.75	128.61		
222.92	171.48		
222.92	188.60	215.00	18.56
222.92	188.60	195.74	
257.22	248.60		
222.92	248.60		

*Reading on starting day
**Reading within same day
***Reading after one week

Conclusion

A reusable enzyme strip of CEase, COD and POD has been prepared and employed for determination of serum cholesterol. The method has the advantage over other existing enzymic methods employing free enzyme[2], enzymes immobilized individually and co-immobilized onto alkylamine glass beads[3,4] is that it provides the reuse of the enzyme with enormous ease in handling.

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