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Determination of serum triglycerides using lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase co-immobilized onto alkylamine glass beads

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A method for determination of serum triglycerides (Tgs) using lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase co-immobilized onto alkylamine glass beads (pore diameter 55 nm) through glutaraldehyde coupling was developed and evaluated. The minimum detection limit of the method was 0.54 m*M*. The analytical recovery of added triolein in the serum was 97.55±1.5% (mean ± S.D.). The mean value of serum Tgs, determined by the present method showed a good correlation (r=0.984) with the Bayer's kit method, employing free enzymes. The within and between batch coefficients of variation (CV) were <2.25% and <1.35% respectively. No significant loss of activity was observed, when co-immobilized enzymes were reused for about 200 times and stored at 4°C in distilled water. The cost of Tg determination for 200 serum samples was less, as compared with Bayer's kit method.

Keywords: Triglyceride, lipase, glycerol kinase, glycerol-3-phosphate oxidase, peroxidase, co-immobilization, alkylamine glass, serum.

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Triglyceride (Tg) level in serum is considered as a risk factor for coronary artery disease and is a valuable indicator of hypertension and atherosclerosis[1]. Although a number of methods are available for Tg determination in serum[2-7], they suffer from disadvantages, such as poor precision, expensive instrumentation, pretreatment and derivatization of the sample. However, enzymic colourimetric method offers ease of operation, good accuracy and specificity and its kit is available. In this method, Tgs are hydrolyzed by lipase to free fatty acids and glycerol, which is phosphorylated and oxidized by glycerol kinase and glycerol-3-phosphate oxidase, respectively to generate H_2O_2, which is determined by a colour reaction consisting of 4-aminophenazone, 3, 5-dichloro-2-hydroxybenzene sulphonate and horseradish peroxidase as chromogenic systems[8]. But, the method requires bulk quantities of free enzymes for a large number of clinical samples, thus making it expensive for routine assay. Earlier, we co-immobilized lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase onto alkylamine glass beads through glutaraldehyde coupling and demonstrated the better suitability of co-immobilized enzymes, as compared to individually immobilized enzymes in determination of serum Tg[9]. Here, we describe a method of determination of serum Tgs using co-immobilized lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase on to alkylamine glass beads. The method is also compared with currently used Bayer's kit method[10].

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Materials and Methods

Lipase (EC 3.1.1.3) from porcine pancreas (40-70 U/mg), glycerol kinase (EC 2.7.1.30) from *Cellulomonas* species, glycerol-3-phosphate oxidase (EC 1.1.3.2.1) from *Aerococcus viridans* (113 Units/mg), horseradish peroxidase (EC 1.11.1.7) (RZ=1.3), 3, 5-dicholro-2-hydroxy benzene sulphonate (DHBS), 4-aminophenazone, L-α-glycerol phosphate and Triton X-100 were purchased from Sigma Chemical Co. St. Louis, U.S.A. Glutaraldehyde (25%) was from BDH, Poole, England. All other chemicals were of analytical grade. The Bayer's kit for enzymic colourimetric determination of Tg was purchased from Bayer's Diagnostics India Ltd, Baroda. Zirconia-coated alkylamine glass beads (pore diameter 55 nm) were gift from Prof. H H Weetall, Corning Glass Works, New York.

Co-immobilization of enzymes

A mixture of lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase in 0.1 *M* sodium phosphate buffer (*p*H 7.0) in the ratio 100:50:10:1 of unit activity was co-immobilized on to alkylamine glass beads through glutaraldehyde coupling as described[9]. The protein bound to the glass beads was estimated using Bradford[11] method, by determining the loss of protein from the enzyme mixture during immobilization.

Standard curve for triglyceride with co-immobilized enzymes

Triolein was used as standard triglyceride. The emulsion of triolein of different concentrations in the range of 0.54-13.6 m*M* was prepared and used in the assay of co-immobilized enzymes[9]. In a conical flask (15 ml) wrapped in black paper, 0.95 ml of 0.1 *M* glycine-NaOH buffer (*p*H 8.5 consisting of 0.63 µmol ATP, 0.27 µmol 4-aminophenozone, 0.54 µmol $MgCl_2$, 1.53 µmol DHBS, and 0.95 µmol tritonX-100 and 250 mg alkylamine glass beads bound to all four enzymes were preincubated at 37°C for 5 min with continuous stirring. The reaction was started by adding 50 µl of triolein emulsion of varying concentrations. After incubation at 37°C for 15 min in dark, the reaction mixture was withdrawn with the help of Eppendorff pipette and transferred to a cuvette, A_{510} was read using a UV and visible spectrophotometer and a standard curve between various triolein concentrations and A_{510} was plotted.

Determination of serum triglycerides

Blood (1 ml) was withdrawn intravenously from apparently healthy and diseased individuals suffering from hypertriglyceridemia and centrifuged at 2000 *g* for 10 min at 4°C, and the serum was collected and used for Tg determination. The assay of serum Tg was carried out as described above for preparation of its standard curve, except that triolein was replaced by 50 µl of serum. The amount of serum Tg was extrapolated from the standard curve between triolein concentrations in the range 0.5-14.0 m*M* *vs* A_{510}.

Reuse of alkylamine immobilized enzymes

To reuse the co-immobilized enzymes, the glass beads bound to enzymes were washed with distilled water 5-6 times and finally with 0.1 *M* glycine-NaOH buffer (*p*H 8.5) by adding these along the sidewalls of the reaction flask, followed by gentle shaking. The washing discard was removed each time with the help of an Eppendorff pipette carefully, avoiding the loss of glass beads. The beads were stored in 0.1 *M* glycine-NaOH buffer (*p*H 8.5) at 4°C, when not in use.

Results and Discussion

A method for determination of serum Tg using co-immobilized lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase on to alkylamine glass beads has been evaluated. The H_2O_2 generated from Tg by co-immobilized lipase, glycerol kinase and glycerol-3-phosphate oxidase is measured colourimetrically with co-imobilized peroxidase by additive coupling of 4-aminophenazone with DHBS and the resulting quinoneimine dye is measured at 510 nm. The following parameters were studied to evaluate the method.

Linearity

Linearity of the present method was up to 13.6 m*M* (1203.5 mg/dl), which is better than that of Bayer's kit method (up to 200 mg/dl)[10]. The lower detection limit of the method was 0.54 m*M* (47.7 mg/dl).

Recovery studies

The reliability of the method was evaluated by determining the recovery of triolein added in serum. The per cent recoveries of triolein in serum (1.13 m*M*/l and 2.26 m*M*/l) were 96.3±1.3 and 97.55±1.15 (mean ± S.D.).

Precision

The Tg level of the same serum samples in one run (within day) and after 1 week of storage at -20º (between days) were determined to check the reproducibility and reliability. The within and between batch coefficients of variation (CV) for serum Tgs were calculated and found to be <2.25% and <1.35%, respectively. The Tg values in the same serum were very close to each other. These values of within and between batches CV were comparable with those reported values using different methods such as mass fragmentography (<2.4% and 1.2%)[12], bioluminescent assay, where CV decreased with increasing signal (<8.6% to 4.8%)[13] and fluorimetric method (<2.5%)[14].

Triglyceride values in serum

The Tg content of sera samples collected from apparently healthy individuals of different age and sex using the present method was found in the range 78.65-200 mg/dl and 68-180.65 mg/dl for males and females, respectively, which were comparable with the reference range of 102-199 mg/dl and 76-162 mg/dl for males and females, respectively[15]. The Tg was also determined in the sera of patients of hypertriglyceridemia associated with atherosclerosis by the same method and found to be in the range 236-1250 mg/dl and 200-1200 mg/dl for males and females in adults, respectively.

Accuracy

To evaluate the accuracy, triglyceride values in serum samples were determined by the commercial

Bayer's kit method employing free enzymes (x) and the present method using co-immobilized enzymes (y) and compared with each other. The Tg values obtained by these methods showed a good correlation (r = 0.984) with the regression equation being y = 1.016x-2.50.

Interference study

The effect of the various metabolites, such as glucose, pyruvate, citrate, uric acid, cholesterol, EDTA, glutathione, acetone, urea and ascorbic acid on the combined activity of all four co-immobilized enzymes was studied at their physiological concentrations. Only ascorbic acid, cholesterol and bilirubin caused 13%, 25% and 14% inhibition of the co-immobilized enzymes, respectively.

Storage stability

Co-immobilized enzymes lost 20% of their initial activity during its regular use for about 200 times when stored in distilled water at 4°C for 6 months, while mixture of all four native enzymes lost 42% of its initial activity under similar storage conditions.

Cost-effectiveness

The present method is economical, as the cost of 200 serum Tg samples was found to be lower (Rs. 809.46), than the Bayer's enzo kit (Rs. 2170) available in India. Further, the method does not require any sophisticated equipment, except a colourimeter, which is not expensive and easy to operate.

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