A Peroxidase-Coupled Method for the Colorimetric Determination of Serum Triglycerides

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We describe an enzymatic method for rapid, precise measurement of serum triglycerides with use of sample/reagent ratios as large as 1:200. Hydrolysis of triglycerides is catalyzed by lipase to produce glycerol and free fatty acids. The glycerol generated is then phosphorylated by adenosine 5'-triphosphate in the presence of glycerol kinase. Oxidation of the resulting glycerol 3-phosphate to produce hydrogen peroxide is catalyzed by L-α-glycerophosphate oxidase. An intense red chromogen is produced by the peroxidase-catalyzed coupling of 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate with hydrogen peroxide. This sensitive chromogen system not only permits use of unusually small sample volumes, it also facilitates a linear response to serum triglyceride concentrations up to at least 10 g/L, while displaying good Ringbom (measure of accuracy) characteristics.

Additional Keyphrase: enzymatic methods

Determination of serum triglycerides has progressed from totally chemical methods (1, 2) through partly chemical–partly enzymatic methods in which alkaline saponification is used to hydrolyze the triglycerides followed by an enzymatic determination of the generated glycerol (3), to totally enzymatic methods in which lipases are used for the hydrolysis step (4, 5). The totally enzymatic methods currently are widely used because of their inherent simplicity, amenability to automation, and the excellent correlation of results with those by the chemical methods (4). However, many enzymatic methods will not measure serum triglycerides in high concentrations unless the sample is first diluted. Alternatively, when smaller sample volumes are used in some methods, the Ringbom characteristics (6) may affect the precision of the assay at low triglyceride concentrations, owing to the relatively low absorptivity of the measured analyte, nicotinamide adenine dinucleotide. In addition, from the evidence to be presented here, there is apparently a critical concentration of generated insoluble nonesterified fatty acid "soaps" that may cause light scattering, which in turn may cause an upward deviation in the calibration curve. This upward deviation is especially pronounced when measurement is made at 340 nm, where turbidity effects are quite noticeable. This nonlinear response compromises calculations, especially when automated devices are used that are limited to a reagent blank and one other point of a calibration curve.

We describe here the development and application of a totally enzymatic triglyceride procedure that allows the measurement of triglycerides in undiluted serum in concentrations up to at least 10 g/L. The reaction sequence is similar to others described previously (4, 5) up to the point where L-α-glycerophosphate oxidase catalyzes the generation of hydrogen peroxide from glycerol 3-phosphate. The hydrogen peroxide is then used in a peroxidase-catalyzed reaction to couple 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate to form a red quinonimine chromophoric compound with a maximum absorbance at 510 nm. This indicator reaction has previously been used in sensitive methods for determination of the other major lipid classes such as cholesterol (7) and choline-containing phospholipids (8) in the high-density lipoprotein fraction of serum, as well as lecithin in amniotic fluid (9), uric acid in serum and urine (10), and cholinesterase activity in serum (11).

The use of L-α-glycerophosphate oxidase in an enzyme-linkd system for the measurement of triglycerides was first reported for "dry-film" technologies (12). More recently,

CLIN. CHEM. 29/3, 538–542 (1983)
Wako Pure Chemical Industries, Ltd., has marketed a triglycerides kit (13) in which this enzyme is used. While this manuscript was in preparation, Fossati and Prencipe (14) published a procedure for an enzymic system that is very similar to the one presented here. The incorporation of an oxidase rather than a dehydrogenase in the reagent system facilitates the use of the above-described indicator reaction, which is approximately fourfold more sensitive in the visible region than is the reduction of NAD* in the ultraviolet region.

Materials and Methods

Apparatus. Spectrophotometers used throughout were a Beckman Model 25 (Beckman Instruments, Fullerton, CA 92634) and a Gilford Model 300N (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074), both equipped with flow-through thermostated microcuvettes.

Chemicals. Lipase (from Chromobacterium viscosum; glycerol-ester hydrolase; EC 3.1.1.3) and l-α-glycerophosphate oxidase (from Aerococcus viridans; glycerol-3-phosphate:O2 2-oxidoreductase; EC 1.1.3...) (GPO) were obtained from Ferment Biochem, Inc., Elk Grove Village, IL 60007.9 Glycerokinase (from Escherichia coli; glycerol-3-phosphotransferase; EC 2.7.1.30) (GK), peroxidase (from horseradish; hydrogen peroxide oxidoreductase; EC 1.11.1.7) (POD), adenosine 5′-triphosphate (ATP), Triton X-100, 4-аминоантипирин (AAP), α-cyclodextrin, and glycerol were from Sigma Chemical Co., St. Louis, MO 63178.

Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate was obtained from either Research Organics, Inc., Cleveland, OH 44125, or Bioi synth Chemicals, Skokie, IL 60077, or was synthesized by us as previously described (9). The bilirubin standard used was an above-normal bilirubin control serum obtained from Ortho Diagnostic Systems, Inc., Raritan, NJ 08869.

Unless stated otherwise, all chemicals were reagent grade.

Reagents. The triglyceride reagent was prepared in Tris HCl buffer (50 mmol/L, pH 7.6) to contain per liter, 0.1 g of Triton X-100, 1 mmol of AAP, 1.5 mmol of sodium 2-hydroxy-3,5-dichlorobenzensulfonate, 5 mmol of MgCl2, 0.5 mmol of ATP, 10 kU of POD, 4 kU of GPO, 0.25 kU of GK, and 100 kU of lipase. This reagent is stable for 24 h at 4°C. Standards were prepared from glycerol in de-ionized water.

Samples. All specimens used in this report were freshly (within 24 h) discarded samples obtained from the chemistry laboratories of Detroit Receiving Hospital/University Health Center.

Procedure. Pipet 1.0 mL of the enzymatic triglyceride reagent into a test tube. Add either 5 μL (lipemic serum) or 10 μL (clear serum) of serum or glycerol standard. Mix well and incubate at 37°C for at least 15 min. Measure the absorbances of samples and standard at 510 nm vs a reagent blank in which de-ionized water is substituted for sample.

Dilute over-range samples with a 9.0 g/L saline solution.

Results and Discussion

Initially, we optimized the reagent without lipase, using glycerol as the substrate. The reagent was then further optimized for the determination of triglycerides, which required that lipase and Triton X-100 be included.

Figure 1 presents the optimization of the reagent with respect to pH, Mg2+, ATP, and Triton X-100 concentrations.

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8 Nonstandard abbreviations: GPO, l-α-glycerophosphate oxidase; GK, glycerol kinase; POD, peroxidase; AAP, 4-аминоантипирин.

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Fig. 1. Optimization data for the change in absorbance at 510 nm with time (ΔA 510/min) as pH, ATP, MgCl2, and Triton X-100 concentrations were varied.

The optimum pH is 7.6, although the pH does not change the rate a great deal over the range shown. An ATP concentration of 0.75 mmol/L was chosen for the reagent. Although the rate increased to some extent when tested at higher concentrations, the final absorbance tended to decrease. The absolute requirement for Mg2+ in the reagent was 2.5 mmol/L or greater. To ensure a margin for error in reagent preparation, we chose to use 5 mmol/L. A Triton X-100 concentration of 0.1 g/L was chosen as optimum; its primary function is to increase the rate of hydrolysis of low-triglyceride sera, as is discussed below.

Using the reagent without lipase, we assayed 19 sera for free glycerol. The free glycerol concentration ranged between 8.0 and 174 mg/L, averaging 55 mg/L (as triglyceride). Although this glycerol concentration may be considered negligible, it may be corrected by using a serum blank with a reagent that lacks lipase. This type of blank has proven to be important in our experience in dealing with samples from patients who are undergoing some form of therapy with glycerol. None of the triglyceride concentrations reported here have been corrected for free glycerol, because the method with which they were compared does not include use of such a blank.

The complete triglycerides reagent was optimized to allow measurement over a wide range of concentrations without prior dilution of the sample. We found that the absorbance obtained for increasing volumes of serum sample increased linearly up to 50 μL without interference from other components of serum, such as protein. Therefore, low triglyceride concentrations—such as would be found in the serum of high-density lipoprotein fraction, for example—could be measured by simply increasing the sample volume. Conversely, high triglyceride concentrations, up to at least 10 g/L, could be measured by decreasing the sample volume. A 10-μL sample volume is optimal for concentrations up to 5 g/L. A 5-μL sample volume is appropriate for values between 5 and 10 g/L. Linear-regression analysis performed on the 5- and 10-μL standard curves gave the respective equations y = 0.00134x - 0.007 (r = 1.0000) and y = 0.00265x + 0.011 (r = 0.9997). Although we decided on a 5 and 10 g/L cutoff, it may be possible to extend these limits to 7 and 14 g/L if one is willing to accept slightly worse regression statistics: y = 0.00128x + 0.0256 (r = 0.9992), and y = 0.00259x + 0.024 (r = 0.9992).
suggested by the positive intercept, all of the points above the 5 and 10 g/L cutoffs tend to fall slightly below the standard curve. However, even at 14 g/L this error is only about 4.4%.

The ability to measure high triglyceride concentrations without prior dilution of the sample is in part due to the high sensitivity of the indicator reaction and the wavelength of measurement. The sensitive chromogen system allows small sample volumes to be used, which in combination with measurement at 510 nm largely avoids the problem of turbidity that may result when the triglycerides are hydrolyzed. Methods in which triglycerides are measured by monitoring the change in absorbance at 340 nm may present serious problems from the formation of turbidity, because the effects of light-scattering are more pronounced in the ultraviolet than in the visible region. In addition, more turbidity is produced in these systems because relatively large sample volumes must be used.

To demonstrate this effect, we prepared a reagent to contain only Triton X-100, lipase, GK, ATP, and MgCl₂. To 1 mL of this reagent we added either 5 μL (present method) or 16.6 μL [ultraviolet method (4)] of a lipemic serum. The change in absorbance at 510 and 340 nm is illustrated in Figure 2. At 510 nm, only a slight decrease in absorbance is seen initially, which probably is caused by the hydrolysis of triglycerides by the lipase. However, at 340 nm, this initial clearing is followed by a large increase in absorbance, presumably ascribable to the formation of turbidity as fatty acids are released. This substantial increase in turbidity was seen only in the presence of the Mg²⁺, which is necessary for GK activation. We postulate that insoluble "soaps" are formed with the magnesium and the released fatty acids. When we added a fatty acid complexing agent, α-cyclodextrin, to the above reagent, in a final concentration of 1.0 g/L, the increase in absorbance at 340 nm was much less pronounced. The right side of Figure 2 shows the spectra of the three reaction mixtures, demonstrating that light-scattering is much more severe at 340 nm than at 510 nm. Bucolo and David (4) incorporated bovine serum albumin into their reagent, to bind the released fatty acids, but we find α-cyclodextrin to be much superior for this purpose. The small sample volumes and wavelength of measurement make it unnecessary to incorporate α-cyclodextrin into the reagent described here, although it can be included if desired.

Bilirubin interacts in the peroxidase-catalyzed reaction by competing for hydrogen peroxide and by contributing color to the final reaction mixture, which results in lower values for the analyte in question (15, 16). Table 1 shows the effects of bilirubin on results for this triglyceride procedure. Bilirubin substantially diminishes the apparent triglyceride value by virtue of its interaction in the peroxidase-catalyzed step. Attempts to prevent this interaction by including potassium ferrocyanide (10, 17, 18) or amidoxyprine (19) in the reagent were unsuccessful. Fossati and Prencipe (14) report that ferrocyanide prevents bilirubin interference in their triglyceride procedure, but we found that over the range of 1 to 80 μmol/L it actually worsened the bilirubin interference in our procedure. Although at moderately high bilirubin concentrations the degree of interference is slight, results for serum from a severely jaundiced patient could be less than the actual triglyceride value.

Hemoglobin not only acts as a pseudoperoxidase but also adds its visible spectrum to samples in which it is present (15, 16). However, at sample concentrations as great as 175 mg/L we observed no deleterious effects on our procedure.

Initially, we noticed that sera with a triglyceride concentration exceeding 5 g/L were hydrolyzed rapidly, while those with triglyceride concentrations less than 2 g/L were hydrolyzed much more slowly. Including 0.1 g of Triton X-100 per liter in the reagent increased the rate of hydrolysis of low-triglyceride sera to match that of the high-triglyceride sera and ensured uniform lipolytic activity across the entire range tested, presumably by forming mixed micelles with the triglycerides. Recently it was shown (20) that phospholipids promote hydrolysis in enzymatic triglyceride kits. This effect may be due to the detergent-like properties of the phospholipids. We did not find it necessary to use Triton X-100 to prevent cloudiness as others have (14).

Table 2 shows our precision data for serum containing low, normal, and high concentrations of triglyceride.

A major concern in an enzymatic triglyceride procedure is that hydrolysis of the triglycerides by the lipase be complete. If it is not, or is inconsistent, the triglyceride concentrations will be either underestimated or variably estimated. To study the hydrolysis of the triglycerides, we assayed serial dilutions of sera having low, normal, and high concentration of triglycerides, using both 9.0 g/L saline and a very low triglyceride serum as the diluent. The resulting curves

![Diagram](image)

**Figure 2. Effects of turbidity**

Left: change in absorbance with time for reaction mixtures at 510 nm (510), 340 nm (340), and 340 nm in the presence of 1.0 g of α-cyclodextrin per liter (340 + A-CD). Right: spectra of the corresponding final reaction mixtures.

**Table 1. Effects of Bilirubin on the Peroxidase-Catalyzed Triglyceride Determination**

<table>
<thead>
<tr>
<th>Bilirubin added* (mg/L)</th>
<th>Triglyceride found (g/L)</th>
<th>Change, %</th>
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<tr>
<td>0</td>
<td>2.500</td>
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<tr>
<td>150</td>
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<tr>
<td>175</td>
<td>2.150</td>
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</tbody>
</table>

*Added to glycerol standards.
Table 2. Precision for the Proposed Procedure

<table>
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<th>Between-run</th>
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<tbody>
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<td>$\bar{x}$</td>
<td>SD</td>
<td>CV, %</td>
<td>$\bar{x}$</td>
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<tr>
<td></td>
<td>mg/L</td>
<td></td>
<td></td>
<td>mg/L</td>
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</tr>
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<td>2.0</td>
<td></td>
<td>1 090</td>
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<td>10 930*</td>
<td>141</td>
<td>1.3</td>
<td></td>
<td>11 630*</td>
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</tbody>
</table>

n = 20 tests each.
* For the high-triglyceride serum, 5 $\mu$L of sample was used.

(Figure 3) were all linear, with coefficients of correlation ranging from 0.9995 to 0.9999. Moreover, all of the saline-dilution curves intersected at zero, and all of the low serum triglyceride dilution curves intersected at the value corresponding to the triglyceride concentration of the low serum. These curves do not prove complete hydrolysis of the triglycerides, but the degree of hydrolysis is at least demonstrably constant. Furthermore, the rise curves for several sera and similar concentrations of glycerol standards were found to reach an equilbria in color production within 13 min, suggesting again that if hydrolysis is not complete, it is at least constant. Moreover, color production—and therefore presumably lipolytic activity—does reach an equilibrium. Thus the timing of the measurement of the color is not critical.

We compared the present method with the method used in the Abbott VP system, which is essentially the original enzymatic method of Bucolo and David (4). We assayed by both methods 110 sera with triglyceride concentrations ranging from 0.5 to 7.5 g/L. As Figure 4 illustrates, the Abbott method gave consistently slightly higher values than did the proposed method. Perhaps this bias is ascribable, at least in part, to the formation of turbidity by the free fatty acids released by the lipase (as discussed above) in the Abbott reagent. In the Abbott method, absorbance is measured at 340 nm, where the effect of turbidity is very pronounced (cf. Figure 2). Although the values are slightly lower, results by the present method do correlate well with the Abbott version of the original enzymic procedure (4), as evidenced by the equation of the calculated line of regression: $y = 1.06x + 1.96$, $r = 0.995$. We selected these samples to obtain a wide range of results, not with the aim of determining a reference interval. However, the regression equation allows us to calculate that this range would be 6 to 7% lower than for the original enzymatic procedure as applied to the Abbott VP. This is a statistically significant difference ($p < 0.001$) by the paired Student's $t$-test, but probably is not clinically significant.

The proposed procedure has several inherent advantages over presently available techniques. In contrast to other systems, especially those in which measurements are made in the ultraviolet, hydrolysis of the triglycerides does not produce any significant optically interfering turbidity. The lipase used facilitates, if not complete, certain constant hydrolysis. The choice of a visible-range, sensitive-indicator reaction, by allowing the use of small sample volumes, also decreases aberrations caused by turbidity, whether present initially or generated by enzymatic hydrolysis. The colorimetric indicator reaction is about fourfold as sensitive as the ultraviolet systems, twice as sensitive as the commercially available one in which p-chlorophenol is used (13), and almost 4.5-fold as sensitive as the original phenol reaction (7). Moreover, p-chlorophenol is only slightly soluble in water, has a definite phenolic odor, and may be both a health and disposal problem (21). The sodium 2-hydroxy-3,5-dichlorobenzenesulphonate used in this procedure is quite water soluble, does not have a characteristic phenolic odor, and is not corrosive. Although no data are available, one would not expect it to be a health hazard, as is phenol, because of its inherent solubility characteristics.
Supported in part by a grant-in-aid to B.Z. from the Michigan Heart Association and to J.D.A. from Wayne State University.

References