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Determination of Serum Triglycerides by an Accurate Enzymatic Method Not Affected by Free Glycerol

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In this automated single-run enzymatic procedure for specific determination of triglycerides in serum, free glycerol is removed from the reaction mixture by pre-incubation with glycerol phosphate oxidase and peroxidase. The subsequent addition of lipase and the chromogen, 4-aminoantipyrine, results in the formation of color proportional to the amount of triglycerides in serum. Standards containing triolein in aqueous detergent are used to calibrate the method. For serum pools from the Centers for Disease Control with target values of 0.74, 1.41, and 2.63 mmol/L, the method produced biases of +0.01, −0.05, and 0.00 mmol/L, respectively (mean: −0.01 mmol/L or −0.4%). The mean coefficient of variation was 1.4% within and 2.5% between days; the combined CV, 2.9%. Ninety 6-μL serum samples can be analyzed per hour. The method is more accurate and precise than one based on an NADH-coupled enzyme system with separate addition of lipase.

Free glycerol concentrations in serum vary more than has been realized earlier (*1, 2*). Failure to allow for this could result in large errors in estimating the concentration of triglycerides in serum (*1, 2*). This is particularly relevant when enzymatic methods are used, because such methods usually compensate for the absence of an extraction step by assuming a constant concentration of free glycerol. Such an assumption may have led to some of the variability reported when triglycerides in serum have been measured enzymatically (*3*).

So far, attempts to overcome this problem have required separate analysis for free glycerol, with the attendant risk of between-run variation. Most analyzers do not allow analysis for free glycerol and for free-plus-triglyceride glycerol in the same run. More recently, a method in which free glycerol is measured after the pre-incubation phase of a single-run procedure has been described (*4*) involving an NADH-coupled reagent system. We found that by use of a new glycerol-3-phosphate oxidase–peroxidase kit we could remove free glycerol during a pre-incubation step. The system, referred to as GPO-PAP,[5] has several theoretical advantages over other methods: stability of reagents, photometric reading in the visible spectrum, more favorable equilibrium, and less product inhibition of the constituent enzymes (*5, 6*). We found that the GPO-PAP method is accurate and precise by single-run measurement of serum triglycerides in pools provided by the CDC.

Materials and Methods

The reagents used for estimating triglycerides by the GPO-PAP method were provided in the form of a kit supplied by Boehringer Mannheim GmbH, D-6800 Mannheim 31, F.R.G. The kit comprised two reagents. Reagent 1 contained glycerol kinase (EC 2.7.1.30), >0.4 U/mL; glycerol-3-phosphate oxidase (EC 1.1.3.21), >5 U/mL; peroxidase (EC 1.11.1.7), 0.3 U/mL; and ATP, >1 mmol/L. Reagent 2 contained esterase (lipase, EC 3.1.1.3), >6 U/mL; and 4-aminoantipyrine (*p*-aminophenazone), 0.7 mmol/L. Both reagents contained, per liter, 0.15 mol of Tris HCl buffer (pH 7.6), 17.5 mmol of $MgSO_4$, 10 mmol of EDTA-Na_2, 3.5 mmol of 4-chlorophenol, 1.5 g of sodium cholate, 6 μmol of potassium hexaferro(II)cyanate, and 1.2 g of detergent.

We used a discrete bichromatic analyzer (ABA-200; Abbott Inc., Irving, TX 75062). The difference between the absorbance reading on the second revolution (5.45 min after mixing the sample with Reagent 1 and immediately before addition of Reagent 2) and the fourth revolution (9.25 min after the addition of Reagent 2) was calculated. The following microprocessor program was used: filters, 500/600 nm; sample volume, 6 μL; volume of primary reagent (Reagent 1), 250 μL; volume of auxiliary reagent (Reagent 2), 250 μL; analysis time, 5 min; temperature, 37 °C; units, user; reaction direction, up; revolutions, 4; auxiliary position, 26; and auxiliary revolution, 2.

The GPO-PAP method was calibrated with a set of standards made by preparing a stable aqueous emulsion of triolein (cat. no. T7502; Sigma Chemical Co., St. Louis, MO 63178) by use of the non-ionic detergent Triton X-100 (no. 8603; Merck, D-6100 Darmstadt, F.R.G.) as described previously (*7*). Each ABA-200 tray of 32 cups was provided with five calibration standards. The standards, which contained 0.53, 1.05, 1.58, 2.10, and 3.16 mmol/L, were stored at 4 °C and new ones were prepared monthly, although they may well be stable longer.

An NADH-coupled reagent system, which also provides for the separate addition of lipase (esterase), was provided by Technicon Corp., Tarrytown, NY 10591. Their reagents T11-0941 and T11-0942 were used as instructed by the company except that we increased the concentration of the NADH-containing reagent threefold to take into account the different volumes used by the ABA-200 analyzer (sample volume, 10 μL; Reagent 1, 250 μL; and Reagent 2, 250 μL). Glycerol was obtained from Merck (cat. no. 4093).

Serum pools with known triglyceride target values measured by the reference method of Carlson et al. (*8*) were provided by the Clinical Chemistry Standardization Section of the CDC. They were received in Wageningen in the frozen state and were stored at −20 °C until analysis. Serum pools for internal control were prepared from sera of healthy normolipemic subjects and stored similarly; the concentration of triglycerides in these pools was measured by the chemical method of Soloni (*9*).

Results

We assessed the GPO-PAP method from our data on more than 100 estimations of each of three CDC pools with target values in the range from 0.74 to 2.63 mmol/L (0.66 to 2.33 g/L). The results obtained, summarized in Table 1, show that the GPO-PAP method has a mean bias with respect to the

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[5] Nonstandard abbreviations: GPO-PAP, glycerol-3-phosphate oxidase–*p*-aminophenazone; CDC, Centers for Disease Control, Atlanta, GA 30333.

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Table 1. Concentration of Triglycerides in Serum Pools Provided by the Clinical Chemistry Standardization Section of the CDC As Measured by the Present Method

CDC pool code	CDC target value, mmol/L[a]	Results of GPO-PAP method: Days	n	Mean concn, mmol/L	Coefficient of variation, % Within day	Between days	Combined	Bias mmol/L	Bias %
50	0.74	9	108	0.75	2.1	5.1	5.5	+0.01	+1.8
MQC	1.41	9	109	1.36	1.4	2.9	3.2	−0.05	−3.3
40	2.63	9	108	2.63	1.1	1.6	1.9	0.00	+0.2
Mean	1.59	9	325	1.58	1.4[b]	2.5[b]	2.9[b]	−0.01	−0.4

[a] Triglycerides: 1 mmol/L = 0.89 g/L. [b] Calculated by taking the average of the squares of the SDs for the three pools.

CDC target values of only 0.01 mmol/L (0.4%), and a mean CV of 2.9%. Only 6 μL of serum was required to obtain such results, and 90 samples could be analyzed per hour.

The GPO-PAP method was also applied to two internal-control pools that we had previously analyzed extensively by a chemical method involving extraction (*9*). The enzymatic values were about 4% higher than the chemical values (0.84 vs 0.81 mmol/L for one pool and 1.72 vs 1.67 mmol/L for another). The CV for 108 analyses done during nine days was 4.8% for the first pool and 2.5% for the second.

We tested the efficiency with which free glycerol was eliminated during the pre-incubation by adding glycerol to serum to give a final concentration of 5.6 mmol/L (about 50 times physiological concentrations). The value obtained for serum triglycerides both before and after the addition was 1.67 mmol/L.

Our attempts to adapt the NADH-coupled enzyme system to a single-run procedure on the ABA-200 met with failure. Reproducibility was poor, both with triolein and with glycerol as calibrators, and the mean values obtained for CDC pools differed widely from target values (0.66 vs 0.74, 1.47 vs 1.41, and 2.96 vs 2.63 mmol/L when we used triolein calibrators with nine analyses per pool). We investigated the source of error by following the time course of the absorbance at 340 nm of the NADH-containing reagent when incubated at 37 °C. In the absence of serum and lipase the absorbance of the NADH reagent declined by 12% in 14.5 min, which is the time required for the test. Various rates of decline were found when the NADH reagent was combined with different sera or with glycerol-containing solutions that simulated sera of different glycerol content.

Discussion

Enzymatic methods for determination of triglycerides offer theoretical advantages over chemical methods: increased specificity and (sometimes) greater ease of automation, both of which can lead to greater precision and accuracy. However, these expectations have not been fully realized, and this may explain why chemical methods are still preferred in many laboratories. Methods for the determination of triglycerides should take into account the wide variations in the concentration of free glycerol in sera obtained from patients and in commercial control sera. Failure to do so has probably contributed to the unsatisfactory performance of some enzymatic methods (*1, 2*).

Here we have reported on an enzymatic method based on the use of lipase, glycerokinase, glycerol-3-phosphate oxidase, and a dye, the absorbance of which can be measured in the visible range. The same chromogen and wavelength are used as in the cholesterol oxidase–peroxidase method for cholesterol, with which many laboratories performing lipid estimations are familiar. The present method is shown to be both precise and accurate for determination of triglyceride concentrations in the normal and mildly hypertriglyceridemic range. In addition, it is not influenced by free glycerol in serum and, being automated, it allows many samples to be analyzed with use of small amounts of both serum and reagents. Such performance is required for use in epidemiologic studies where small differences between mean values for different populations are studied (*10*).

So far, methods that correct for free glycerol have relied on NADH-coupled reagents (*4, 11*). In our hands, the NADH-containing reagent showed a drift in baseline absorbance, which precluded its use in a single-run procedure without parallel blanks. The advantages of a glycerol phosphate oxidase–peroxidase system over an NADH-coupled system have been described before (*5, 6*) and are confirmed by our results. Previous studies (*6*) have demonstrated a linear response for the GPO-PAP method up to 11.4 mmol/L, which is an encouraging prospect for the use of this method for hypertriglyceridemic sera.

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