Automated Colorimetric Method for Estimating Serum Triglycerides

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A colorimetric adaptation of the fluorometric method of Noble and Campbell is described. Decreased sensitivity, caused by substituting colorimetry for fluorometry, has been improved by increasing the proportion of serum to isopropanol extraction fluid from 1 in 21 to 1 in 11, and by placing an adjustable range expander between colorimeter and recorder. This accurate and reliable adaptation has obviated the need for an expensive fluorometer.

Additional Keyphrases atherosclerotic heart disease • fluorometry • normal values

The estimation of serum triglycerides has become an increasingly popular request, generally in combination with a serum cholesterol and (or) a serum uric acid, as a result of continued interest in atherosclerotic heart disease. In suspected cases of familial hyperlipoproteinemia, clinical laboratory determinations include—besides the appearance of a fasting serum specimen—serum cholesterol, serum triglycerides, and in certain cases lipoprotein electrophoresis to assist in clinical diagnosis of the various Frederickson et al. types (2) of familial hyperlipoproteinemia. Several accurate manual and semiautomated techniques are available for measurement of serum triglycerides (3-5), but these are rather complex and depend on accurate timing of the steps involved in the determination.

In an attempt to find a relatively simple and inexpensive method to cope with small batches of triglycerides, I tried a method (6) based on a modification of the phenylhydrazine and potassium ferricyanide method, in which a red dye is produced, but without success, owing to the instability and doubtful purity of the phenylhydrazine reagent. Fletcher's (7) manual method was found to be relatively simple and reliable for small batches, after several minor modifications. This method is based on the Hantzsch condensation reaction between an amine, a β-diketone, and an aldehyde (8). Isopropanol extracts are prepared, phospholipids removed by silicic acid, and the glycerides are saponified to free glycerol. The glycerol is oxidized to formaldehyde, which is then condensed with diacetylacetone and ammonia to form a yellow compound, 3,5-diacetyl-1,4-dihydro-lutidine. The final color is measured colorimetrically.

As the work load increased, possible use of an automated procedure was investigated, but it was soon evident either the methods were still relatively time consuming or required an expensive fluorometer. An attempt to automate Fletcher's (7) method was unsuccessful, because the KOH in isopropanol (50 g/liter) produced a fine precipitate in the system.

I describe here the colorimetric adaptation of a modified fluorometric AutoAnalyzer (Technicon Corp., Tarrytown, N.Y. 10591) system (1, 9).

Materials and Methods

The manifold was assembled as described by Noble and Campbell (1), except D1, K0, and G0 glass connections were replaced by A6, K3, and H1 connections, respectively. These changes improved the bubble pattern and mixing of reagents. If blanks are included regularly, the KOH line is placed in isopropanol:water (85:15, by vol), and it is then advisable to use a Solvaflex pump tubing for this line.

Unknowns and standards were run at 30 per hour, blanks at 40 per hour.

Reagents were as described by Noble and Campbell (1), except silicic acid (analytical grade, 100
mesh; Mallinckrodt Chemical Works, St. Louis, Mo. 63160) was used instead of zeolite. Sufficient periodate reagent is prepared to last about seven days.

Standards were prepared from triolein (99% pure, Sigma Chemical Co.). From a stock solution containing 10 mg of triolein per milliliter of isopropanol, working standards of 50 to 300 mg of triolein were prepared. Standards were stored at 4°C in amber-colored bottles. These standards are treated the same way as the unknowns except that 0.5 ml of standard is added to 4.5 ml of isopropanol and 0.5 ml of water.

Modification of colorimeter and recorder. An adjustable range expander was inserted between colorimeter and recorder, and set to give a 2× expansion of the peaks; 408-nm filters were used in the colorimeter with a no. 2 aperture; the recorder was set in the usual manner.

Procedure

Extractions. Serum or plasma, 0.5 ml, was added with an Oxford pipet to 5 ml of isopropanol in a disposable plastic tube with cap and immediately mixed thoroughly on a Vortex mixer. Then about 0.5 g of silicic acid mixture was added to each tube, which was then capped and vigorously shaken for 10 min in a horizontal shaker. The tubes were centrifuged for 5 min at 3,500 rpm without their caps (if the extracts are to be used immediately, otherwise with the caps). After removing the caps, the tubes were allowed to stand 5 min to allow settling of any silicic acid that may have fallen off the cap into the supernatant fluid.

Blanks, if desired, were determined as described by Kessler and Lederer (9).

Results

Figure 1 shows a chart record of a set of standards, preceded by two prolonged periods of aspirating single extract from a fresh serum pool and followed by 22 separate extracts from the same pool. Values for the pool range from 97 to 113 mg of triolein per 100 ml (mean, 105.7; standard deviation, ±3.8). During this run there was about 1–2 small divisions of drift on the chart paper. Results for 20 extracts from a second fresh serum pool ranged from 185 to 207 mg of triolein per 100 ml (mean, 198.8; standard deviation, ±5.6). Values for 20 extracts from a further serum pool ranged from 280–300 mg of triolein per 100 ml (mean, 295.3; sn, ±4.6). Double sets of standards (150 mg of triolein/100 ml) are regularly spaced throughout runs, and the maximum variation between values obtained is no greater than ±8. The standard curve is linear up to 300 mg triolein per 100 ml.

Table 1 shows the day-to-day reproducibility for assay of two separate pools on 10 successive runs. Recovery was measured in each run by mixing two known sera and subjecting the mixture to the above procedure. Recoveries within runs and between runs varied between 94% and 104%.

Normal values. Determinations were performed for 81 healthy individuals after an overnight fast. The values obtained ranged from 40 to 160 mg of triolein per 100 ml (mean, 104.3; standard deviation, ±28.1). Serum uric acid and cholesterol concentrations were normal for all these individuals. The normal range and mean compare well with those obtained from other published methods.

Table 1. Triglyceride Concentration of Two Serum Pools as Determined on 10 Occasions

<table>
<thead>
<tr>
<th>Date (1970)</th>
<th>Pool 1 mg of triolein/100 ml</th>
<th>Pool 2 mg of triolein/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/10</td>
<td>95</td>
<td>142</td>
</tr>
<tr>
<td>14/10</td>
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<td>150</td>
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<tr>
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<td>160</td>
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<td>155</td>
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<tr>
<td>13/11</td>
<td>102</td>
<td>150</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>94.6 ± 1.8</td>
<td>150.7 ± 3.9</td>
</tr>
</tbody>
</table>
Comparison with Kessler and Lederer (9) method. Thirty samples covering a range 60 to 2,300 mg of triolein per 100 ml were subjected to both procedures. Close correlation was observed \( r, 0.981 \).

Discussion

In the Kessler and Lederer (9) method a 1 in 21 dilution of the serum was used, but I used a 1 in 11 dilution, to increase the sensitivity of the reaction. Under similar extraction conditions they found that triglycerides are completely recovered if the serum to solvent ratio is in the range 1:10 to 1:25. Recovery was complete, with a 1 in 11 dilution, in a range of 50 to 500 mg of triolein per 100 ml. An attempt to perform the estimation using the original serum to isopropanol dilution of 1:21 and a 3 1/4 expansion on the adjustable range expander yielded results 20 to 30 mg of triolein/100 ml greater than found with the present system and a 11-fold dilution. There was more drift and a lack of consistency in the results within runs. Blanks were run on standards and unknowns by by-passing the first coil in the 50°C water bath and acidifying the unsaponified extracts directly by adding the periodate and acetyl acetone reagents. The blank values for the standards and unknowns were consistently small, and for ordinary routine purposes these blank determinations can be omitted. However, for batches of three commercially available control sera, the results obtained were almost double the stated figure in two, while those for the third were 10-15% greater. Blanks for these sera showed that one had a blank value equivalent to approximately 50 mg of triolein per 100 ml (stated triglyceride value for product is 68), the second had a negative blank, and the third yielded a blank equivalent to about 10-15% of the stated figure. It is now routine to run a blank on the quality control sera before each run. Aliquots of ordinary laboratory pooled sera, kept frozen, appear to be the most consistent quality control.

It is not necessary to subject the dilute standards to the silicic acid mixture: identical peaks were produced when silicic acid treated standards were compared with nontreated standards.

When the Solvaflex tubing has deteriorated and is to be replaced, it is advisable to change all the other pump tubes at the same time, even though after 30-40 hours use they still appear to be in good condition; they nevertheless have been stretched fully along with the Solvaflex tubes, and if they are not replaced drift increases, because decreased amounts of KOH are available for saponification as the run progresses.

Lately, I have used a double twenty-foot glass coil in place of the double forty-foot coil. By increasing the temperature of the bath to 55-60°C, identical results have been obtained, with a considerable saving in time and reagents, particularly isopropanol. Wash between samples is much improved, enabling the samples to be run at 40 per hour.

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References