Ratiometric Triglyceride Analysis by Use of a Centrifugal Analyzer

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We describe a simple, reliable, enzymatic method for triglyceride analysis, in which triolein is used as a standard. Use of a centrifugal analyzer greatly decreases required time and reagent cost. Results correlate well with those obtained by a standard automated continuous-flow method.

Additional Keyphrases: AutoAnalyzer • GEMSAEC • three procedures compared • normal values

It is important to have reliable, reproducible, rapid, and inexpensive methods for triglycerides and cholesterol, because both analyses are required for hyperlipoproteinemia screening. Colorimetric and fluorometric methods all share the common problem of separating triglycerides and excluding interfering substances such as glucose and phospholipids. Non-polar solvents such as nonane (1) and isopropyl ether (2) satisfy this requirement at the expense of poor bubble patterns and frequent manifold tubing changes on “AutoAnalyzers” (Technicon Instruments Corp., Tarrytown, N. Y. 10591).

Enzymatic analysis of triglycerides is expensive but precise and highly specific (3). Centrifugal analyzers circumvent these problems; they require small sample and substrate volumes, are rapid, and the results are extremely reproducible. The proposed method differs from current methods by use of a pure triolein standard and by evaluation of the linear portion of the reaction slope.

Materials and Methods

Apparatus

Triglycerides were analyzed with the “GEM- SAEC” analytical system (Electro-Nucleonics Inc., Fairfield, N. J. 07006) (Figure 1).

Reagents

A “Triglyceride Kit, Biochimica Test Combination No. 15989, Neutral Fat and Glycerol” was used (Boehringer Mannheim Corp., New York, N. Y. 10007). The instructions were followed as indicated in the kit for the dilution of vials 1-3. Vial 4 was further diluted with 3.0 ml of 2 molar NH₄SO₄. A working substrate was prepared by adding 25.0 ml of vial 1, 1.0 ml of vial 2, and 0.2 ml of vial 3 to a 50-ml plastic bottle labeled “working reagent I.”

A stock standard was made by dissolving 5.00 g of triolein (Calbiochem No. 6459, grade A) in 100 ml of ether-ethanol (1:1 by vol). The working standard was prepared by placing 500 μl of the stock solution in a 250-ml Erlenmeyer flask containing 50 ml of 0.5 molar alcoholic potassium hydroxide. The flask was covered with Parafilm and incubated for 30 min in a 70 °C water bath. (The contents should be swirled twice during incubation.) After incubation, the flask was removed, and cooled. Then 20 ml of de-ionized water and 100 ml of 0.15 molar magnesium sulfate were added to the flask, and the contents mixed thoroughly and centrifuged in 50-ml centrifuge tubes. The supernate was decanted into two 50-ml polyethylene bottles containing “poly seal” caps and stored at 4 °C. This standard is stable for at least three weeks when stored at 4 °C.

Procedure

The sample is prepared by placing 200 μl of serum in a 12 × 75 mm Falcon Plastics tube No. 2053, and

Fig. 1. The GEMSAEC
adding 500 μl of 0.5 molar alcoholic KOH. The contents of the tubes are mixed well, capped, and incubated at 70 °C for 30 min. When hydrolysis is complete, the tubes are cooled, and 1.0 ml of 0.15 molar magnesium sulfate is added. The contents of the tubes should be mixed well, centrifuged, and the clear supernate transferred to sample cups for analysis with the GEMSAEC.

Glycerol Analysis

The distribution disc is loaded with the Rotolader (III) as follows: sample volume, 50 μl; flush volume (water), 60 μl. The sample is placed in cup position C, and the reagent arm in position C. The 16 positions of the distribution disc are loaded according to the following arrangement: a water blank in position 1, a standard in position 2, samples from patients in positions 3 thru 15, and a control in position 16. The Rotolader deposits the patient sample and 400 μl of substrate (minus glycerokinase) into the C well. The 25 μl of glycerokinase are added manually to well B.

The following GEMSAEC settings are used in the analysis:

- Reaction temperature: 30 °C
- Wavelength: 340 nm
- Filter position: 335–385 nm
- Reaction mode: Rate
- Running mode: Auto
- Initial reading: (IR) 10 s
- Reading interval: (IR) 90 s
- No. of readings: (NR) 2

The triglyceride program, entered into the computer before the samples are run, contains the following information: S = IR 10; S = RI 90; S = NR 2; S = SC 125; S = Kt 1; S = TF 1; S = TC 1; S = AD 4; S = HI 175; S = LO 35; S = SA 0.4; and S = XX 1.

Results and Discussion

The GEMSAEC was programmed to read absorbance at 60-s intervals for a total of 10 readings. The change in absorbance for this period was observed on hydrolyses of 12 patient samples and four triolein standards. Sixty-four to seventy percent of the total 9-min change in absorbance was reached at 90 s for the patients, except for one patient who had markedly elevated triglycerides. The triolein standard had undergone 85–91% of the total absorbance change by 90 s. We concluded that more than 80% of the glycerol had been transformed enzymatically, and the slow continual increase in absorbance noted in the samples from patients was attributable to some other competing reaction. Figure 2 illustrates data for representative samples.

Free glycerol was determined in sera from 31 patients having triglyceride values ranging from 64 to 359 mg/100 ml. The mean free glycerol in these sera, calculated as triglyceride, was 10.5 mg/100 ml, a concentration of free glycerol similar to that found by other investigators (4). We saw no correlation between free glycerol and triglyceride concentration.

Six triolein standards ranging in concentration from 150 mg to 1500 mg/100 ml were processed in the same manner as a serum sample. Two sera that gave very high results were diluted as follows: a two-fold dilution for patient No. 26 and three- and five-fold dilutions for patient No. 43. The 125-mg standard was used for the ratiometric rate calculation in these elevated samples in the same manner as described in the method. The data are presented in Figure 3. Analytical linearity extends to a triglyceride content of at least 1275 mg/100 ml.

Sera from 39 patients were analyzed both enzymatically with GEMSAEC, and colorimetrically with the AutoAnalyzer. The sample was prepared for the AutoAnalyzer by the extraction and purification procedure first described by Laurell (2) and later used by Kraml and Coeys (5) in a semi-automated method. Laurell's technique of using isopropyl ether in

![Fig. 2. Representative absorbance-time curves for triglycerides](image)

Triolein standards (Interrupted lines) of 125, 500, 1000 mg/100 ml and patients' sera ranging in concentration from 224 to 1598 mg/100 ml (solid lines) are plotted

![Fig. 3. Curve for standards and sera from two patients: calculated vs. found](image)

Patient No. 43, undiluted and 3- and 5-fold dilutions (●); Patient No. 26, undiluted and a 2-fold dilution (△); triolein standards, 150–1500 mg/100 ml (○). Ordinate: calculated; abscissa, found
the presence of silicic acid yields an extract free of phospholipids, glucose, and free glycerol. The plotted data (Figure 4) have a slope of 0.949, a Y intercept of 6.244, and a correlation coefficient of 0.985. The difference demonstrated by these two methods is that the enzymatic method measures free glycerol in the serum. From the slope and Y-intercept we calculate the results to be 6.5 mg/100 ml greater for total triglyceride by the enzymatic (GEMSAEC) method.

The enzymatic and colorimetric methods described in this paper were compared with a popular enzymatic method (6), in which lipase is used to hydrolyze the triglyceride. Determinations for 42 patients’ sera by these methods showed good agreement between the enzymatic (GEMSAEC) and the colorimetric (AA) procedures, but agreement with the enzymatic (lipase) method was not as good—three patients’ sera had only a third of the activity while one patient had four times the activity found by the other two methods. In addition, the analytical concentration range without sample dilution is about half of the range of the enzymatic method described in this paper. The time saved by hydrolyzing the triglyceride with lipase is derived at the expense of increasing GEMSAEC running time, which is more crucial.

A pooled serum sample was included with each group of patients’ sera being analyzed for triglycerides. During a month, 49 determinations were done on this serum pool by three different technologists. The mean result was 114.4 mg/100 ml (CV, 4.7%).

A range of 40 to 150 mg of triglycerides per 100 ml was obtained for sera from 100 normal individuals by the AutoAnalyzer method. Because the enzymatic method gave triglyceride values averaging 6.5 mg/100 ml greater than results obtained by the AutoAnalyzer, we adjusted the normal range to 47–157 mg/100 ml. A further review of the normal range with this method will be undertaken when a larger normal population can be evaluated.

The GEMSAEC system can analyze 150 hydrolyses per hour for triglycerides. At least 100 sera may be analyzed with the kit (for 50 determinations), thus reducing the per sample cost by half.

References