Automated Measurement of Total Cholesterol and Triglycerides, in “Tandem,” on the Discrete Sample Analyzer, Gilford System 3500

Lynn Smith, Diane Lucas, and Gary Lehnus

We have developed an automated procedure on a discrete sample analyzer, Gilford® System 3500, which alternately measures both total serum cholesterol and triglyceride concentrations as a “tandem” procedure. We used Dow Diagnostic’s fully enzymatic, colorimetric reagents and aqueous standards to calculate unknowns ratiometrically. Cholesterol and then triglyceride reagent are dispensed into alternate cups; the produced color at 500 nm is measured after an ambient temperature incubation of 20 min. Reagent and sample carryover is less than 1.6%. Correlation coefficients of 0.997 for comparison of both automated tests with the manual methods at 30 ºC and a typical CV of less than 2.0% show this “tandem” procedure to be reliable and accurate.

Cholesterol and triglyceride determinations have become exceedingly important because they have been associated with various lipid disorders such as hyperlipidemia, diabetes mellitus, hypothyroidism, liver disease, atherosclerosis, and other metabolic derangements (1, 2). The quantitation of both lipid constituents is helpful in classifying familial hyperlipoproteinemias and, under appropriate circumstances, may be sufficient for diagnosis (3). Early clinical procedures required strong saponification chemicals and mineral acids in multistep processes (4-8). Modern procedures use microbial and animal enzymes to rapidly hydrolyze cholesterol and triglycerides, making them more adaptable to modern instrumentation (9-13).

Although the Gilford System 3500 (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074) is primarily a discrete sample analyzer, its programmable versatility allows it to be used as a dual-channel instrument. Prerequisites in the development of this tandem procedure include identical wavelengths, timing, temperature, and sample volumes; reagent compatibility; and the ability to under-reconstitute reagents for both tests.

The tandem procedure uses test parameters similar to the reagent manufacturer’s manual methods. The differences between the two methods are the reduced sample and reagent volumes, incubation at ambient temperatures, and elimination of the addition of acid in the triglyceride test immediately after sample hydrolysis to stabilize the final color. The addition of the acid reagent is unnecessary in the tandem procedure because the tests are processed in an exact time sequence.

Method and Materials

Serum samples are loaded on the instrument in duplicate. Serum and either cholesterol or triglyceride reagent are separately dispensed into alternate cups. After a 20-min incubation at ambient temperature, the reaction mixtures are sequentially aspirated and the amount of color formed is measured at 500 nm. The concentrations of total cholesterol or triglycerides in the samples are calculated ratiometrically, according to the respective standard’s absorbance.

In the cholesterol reaction, samples are incubated with cholesterol esterase and cholesterol oxidase, which convert cholesterol esters to cholest-4-en-3-one and hydrogen peroxide. Peroxidase converts 4-aminoantipyrine, in the presence of hydrogen peroxide and phenol, to a quinone imine chromogen.

\[
\text{Cholesterol esterase} \rightarrow \text{cholesterol} + \text{fatty acids}
\]

\[
\text{Cholesterol + O}_2 \rightarrow \text{cholesterol-4-en-3-one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} \rightarrow \text{quinone imine chromogen}
\]

The triglyceride reaction involves two microbial lipases that completely hydrolyze triglycerides to glycerol. Glycerol is converted to glycerol-1-phosphate by glycerol kinase, which then reacts with NAD in the presence of glycerol-1-phosphate dehydrogenase to yield dihydroxyacetone phosphate and NADH. The reduced NADH in the presence of diaphorase reacts with INT [2-(p-iophenolyl)-3-p-nitrophényl-5-phenyltetrazolium chloride] to yield a colored formazan.

\[
\text{Triglycerides} \rightarrow \text{glycerol} + \text{fatty acids}
\]

\[
\text{Glycerol + ATP} \rightarrow \text{glycerol-1-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-1-phosphate} + \text{NAD} \rightarrow \text{dihydroxyacetone phosphate} + \text{NADH}
\]

\[
\text{NADH} + \text{INT} \rightarrow \text{formazan} + \text{NAD}
\]
Table 1. Correlation of Tandem Cholesterol Determination with Two Other Methods

<table>
<thead>
<tr>
<th></th>
<th>Manual method</th>
<th>3500 BMC procedure</th>
</tr>
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<tbody>
<tr>
<td>Correlation coefficient</td>
<td>0.997</td>
<td>0.993</td>
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<tr>
<td>Slope</td>
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<td>1.014</td>
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<td>Intercept</td>
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<td>-0.10</td>
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<td>No. samples</td>
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<td>79</td>
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Table 2. Correlation of Tandem Triglyceride Determination with Two Other Methods

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<td>Correlation coefficient</td>
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<td>0.983</td>
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<tr>
<td>Slope</td>
<td>0.958</td>
<td>1.035</td>
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<tr>
<td>Intercept</td>
<td>0.03</td>
<td>-0.19</td>
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<tr>
<td>No. samples</td>
<td>46</td>
<td>67</td>
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</table>

Instrumentation

The Gilford System 3500 discrete sample analyzer was used for the tandem automated procedure with program card and procedure (part no. 17831×82, Gilford). The reference instrument for the manual methods was a Model 250 Spectrophotometer (Gilford) with a temperature-controlled cuvet maintained at 25 ± 0.1 °C. A water bath was used at 37 ± 0.5 °C for incubation, and a Pipetter/Diluter (Gilford) was used to dispense samples.

Reagents

Reagents supplied by Dow Diagnostics (Dow Chemical Co., P.O. Box 68511, Indianapolis, IN 46287) were used throughout. Each reagent was reconstituted to two-thirds regular volume to allow for the addition of water to dispense samples and to maintain final concentrations similar to those in the manual methods. Each vial of cholesterol reagent (cat. no. 46650) was reconstituted with 10.0 mL of the supplied buffer containing phenol, and the triglyceride reagent (cat. no. 46676) was reconstituted with 7.0 mL of the supplied tri(hydroxymethyl)methylamine buffer. The following reconstituted reagent concentrations (per liter) give concentrations similar to those used by the manufacturer when additional water is added:

- **Cholesterol analysis.** 15.5 mmol sodium cholate, 3.4 mmol 4-aminooantipyrine, 32 mmol phenol, 102 U cholesterol esterase, 186 U cholesterol oxidase, and 71 300 U peroxidase; buffer, pH 6.7.

- **Triglycerides analysis.** 79 000 U lipases, 236 mg magnesium chloride, 1.4 g ATP, 5.7 g NAD, 190 mg INT, 44 000 U diaphorase, 720 U glyceral kinase, and 10 800 U glyceral-1-phosphate dehydrogenase; buffer, pH 7.4.

Reagents for the manual methods were prepared according to the package inserts.

Standards

Aqueous cholesterol and triglyceride standards were provided by the manufacturer in the reagent kits. The cholesterol standard contained 2 g of cholesterol per liter in ethylene glycol monomethyl ether, and the triglyceride standard contained 2.08 g of glycerol per liter, equivalent to a triolein concentration of 2 g/L.

Procedures

**Manual methods:** Reconstitute the cholesterol reagent with 15.5 mL of buffer as described in the package insert and preincubate at 37 °C for 7–45 min. Pipet 10 μL of blank, standard, and samples into disposable glass test tubes containing 1.5 mL of pre-warmed reagent, mix by inversion, and incubate at 37 °C for 10 min. Read absorbances at 500 nm within 15 min after hydrolysis, to avoid color deterioration.

Reconstitute the triglyceride manual method reagent as described with 5.5 mL of buffer solution and preincubate at 37 °C for 5–10 min. Pipet 20 μL of blank, standard, and samples into disposable glass test tubes containing 1.0 mL of pre-warmed reagent, mix by inversion, and incubate exactly 10 min at 37 °C; then immediately pipet 2.0 mL of acid reagent into each tube. Mix by inversion, then record absorbances at 500 nm.

Make calculations as described in the package inserts for both tests to obtain results in grams per liter.

**Automated procedure:** Reconstitute cholesterol and triglyceride reagents with 10.0 mL and 7.0 mL of reconstituting buffer, respectively, and allow to equilibrate to ambient temperature for 10–15 min. Program the instrument with the special program card. Dispenser A delivers 5 μL of sample and 0.25 mL of distilled water into each reaction cup. Dispenser B delivers 0.5 mL of cholesterol reagent into the first cup of each sample pair, and Dispenser C delivers 0.5 mL of triglyceride reagent into the second cup of each sample pair.

In determining the cholesterol and triglyceride factors, the instrument divides the concentration of the standard by the difference between absorbance of the standard and of the blank [standard conc/(A \_standard - A \_blank) = factor]. Sample absorbance values are converted to grams per liter by the following formula: (A \_sample - A \_blank) X factor = sample value in g/L. The blank and standard absorbances and the factors are printed as a check on the instrument/reagent system.

Load the reaction strips over the sample cups and set the instrument controls as instructed by the tape printout; start the program by depressing the RUN push button. The samples are automatically processed in sequence, alternating cholesterol and triglyceride reagent addition. The instrument times the 20-min hydrolysis period and aspirates the mixtures into the thermal cuvet, which is regulated at 25 ± 0.1 °C. After a 5-s equilibration time, the instrument reads the absorbances and prints the results.

Results

**Correlation**

Patients' samples, less than one week old and stored tightly capped at 4 °C until use, and commercially available lyophilized controls were used to evaluate the tandem cholesterol/triglyceride procedure. Comparisons with manual methods and BMC's System 3500 procedures (Tables 1 and 2) were made. The BMC triglyceride procedure uses a serum blank for each sample and calculates results from the difference in absorbance between blank and sample. The tandem cholesterol procedure correlates to the Liebermann–Burchard method (4, 5) with a slope of 1.010 and a correlation coefficient of 0.981. The tandem triglyceride procedure correlates to the
Table 4. Day-to-Day Precision of Tandem Determination

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol, g/L</th>
<th>Triglycerides, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>assays</td>
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<tr>
<td>Mean</td>
<td>1.453</td>
<td>2.080</td>
</tr>
<tr>
<td>SD</td>
<td>0.020</td>
<td>0.032</td>
</tr>
<tr>
<td>CV, %</td>
<td>2.53</td>
<td>1.58</td>
</tr>
</tbody>
</table>

Worthington Diagnostic saponification method with a slope of 1.046 and a correlation coefficient of 0.994.

Precision

Precision data are listed in Tables 3 and 4 for the tandem cholesterol/triglyceride procedure. Within-run precision was evaluated on replicate assays of four pooled sera having cholesterol values ranging from 1.02 to 3.23 g/L and triglyceride values ranging from 0.76 to 3.86 g/L. Results showed coefficients of variation of less than 1.7% for cholesterol and 1.5% for triglycerides (Table 3). Day-to-day precision was accumulated over a three-day period on four pooled sera with concentrations ranging from 1.45 to 3.00 g/L for cholesterol and 0.83 to 3.08 g/L for triglycerides. Freshly reconstituted reagent and standards were used for each assay. Results showed coefficients of variation of less than 2.6% for cholesterol and 3.5% for triglycerides (Table 4).

Carryover

Carryover studies were performed to determine the cross-contamination effects between the alternately aspirated cholesterol and triglyceride sample/reagent mixtures. Human samples were selected that contained abnormally high and low cholesterol and triglyceride concentrations. Each high or low sample was separately assayed in quadruplicate, then reassayed with the tandem procedure, alternating various concentration combinations of cholesterol and triglyceride samples. Each set of four results was averaged to establish mean values for separate and tandem runs and used in a formula proposed by Broughton (14) in a scheme for instrument evaluation. Overall carryover was calculated to be less than 1.6% (Table 5).

Linearity

Linearities of the automated tandem procedure were determined by assaying dilutions of pools of human sera with abnormally high concentrations of cholesterol, triglyceride, or both. Each pool was diluted with human sera with low amounts of analyte to give appropriate concentrations. Observed results, y, are plotted vs. expected results, x (Figures 1 and 2).

Acceptable linearities were obtained up to 6 g/L for cholesterol and to 5 g/L for triglycerides. Values greater than these should be diluted with low-concentration sera and reassayed.

Discussion

Most discrete sample analyzers cannot be used as a dual-channel system to measure two serum constituents within the course of one analysis; the Gilford System 3500, however, can be used to routinely assay both total cholesterol and triglyceride serum concentrations as a tandem procedure.

Assays for total cholesterol and triglyceride concentrations are frequent tests on patients exhibiting various lipid disor-
We have shown that this procedure is comparable to the manual methods, System 3500 BMC procedures, and to Liebermann–Burchard and saponification methods. It offers excellent precision and repeatability with economical use of reagents, minimum use of personnel, and readily available commercial reagents. Other reagents cannot be interchanged for this procedure.

We thank Dr. Andris Indriksons and Robert Mack (Dow Diagnostics) for their help with this procedure.

References