Direct Manual Determination of Serum Total Cholesterol with a Single Stable Reagent

D. R. Wybenga, V. J. Pileggi, P. H. Dirstine,1 and John Di Giorgio

We describe a simple, direct, and specific manual method for quantitative determination of total cholesterol in serum. In the method, which requires no extraction of the serum, a single stable reagent and 50 μl of sample is used. Serum cholesterol concentrations determined by this method did not differ statistically from those obtained by the reference method of Abell et al. Hemoglobin, bilirubin, and γ-globulin do not interfere unless present at markedly supranormal concentrations. Inter-run precision is about ±3% (95% confidence limits).

Additional Keyphrases ferric perchlorate-ethyl acetate • interference by bilirubin, hemoglobin, γ-globulin • intermethod comparison

Several different color reactions have been used in attempts to develop a satisfactory method for direct determination of total serum cholesterol. These include the Liebermann-Burchard (1, 2), p-toluenesulfonic acid (3), and ferric chloride-sulfuric acid reactions (4, 5). The latter has received the most attention because it is four or five times more sensitive than the Liebermann-Burchard reaction (6) and requires a smaller serum sample.

Zlatkis et al. (4) added a fixed volume of concentrated sulfuric acid, glacial acetic acid, and ferric chloride to serum previously diluted with glacial acetic acid. They claimed that positive bilirubin or protein interference (falsey high results) was negligible if the absorbance was measured at 560 nm. However, Furst and Lange (7) critically examined this method and found it unsuitable for routine use because a small variation in the volume of added concentrated sulfuric acid and the consequent variation in temperature change were responsible for differences in analytical results. Several investigators (8–15) later confirmed the critical nature of the sulfuric acid addition. In Tonks’ (8) review of cholesterol methodology, he recommends the Leffler (9) modification of the Zlatkis method, which includes an isopropanol extraction of cholesterol with concurrent precipitation of serum proteins.

There is an obvious need for a simple, direct method for determination of cholesterol, in which a single stable reagent is used and which has specificity approaching that of methods requiring extractions. The method we describe satisfies the above criteria and offers, for the first time, a direct method that is relatively free of interference by bilirubin or hemoglobin.

Materials and Methods

Reagents1

(a) Sulfuric acid. Concentrated, reagent grade.
(b) Ethyl acetate. “Spectroquality” solvent (Matheson, Coleman & Bell, E. Rutherford, N. J. 07073), used without redistillation.
(c) Ferric perchlorate. Nonyellow, containing excess HClO4 (catalog no. 40, G. Frederick Smith Chemical Co., Columbus, Ohio 43223). This reagent usually contains 58 to 75% ferric perchlorate.

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1 Available as a reagent set from the Dow Chemical Co., Diagnostic Products, P.O. Box 1656, Indianapolis, Ind. 46206.
Keep desiccated after reagent bottle has been opened.

(d) **Cholesterol reagent.** Dissolve 520 mg of reagent c in 600 ml of reagent b, contained in a 2-liter Erlenmeyer flask. Place the flask in an ice bath and cool the contents to 4°C. Add gradually, in small portions, 400 ml of cold concentrated sulfuric acid. Mix after each portion is added, but do not allow the temperature to exceed 45°C. The reagent is stable for at least a year when stored in an amber bottle at 25°C, or two years when refrigerated.

(e) **Cholesterol standard.** Two hundred milligrams of cholesterol (Pfanstiehl Labs, Inc., Waukegan, Ill. 60086) per 100 ml of glacial acetic acid.

**Procedure**

(a) Mark three vials\(^1\) “blank,” “standard,” and “unknown,” respectively. Since the final color is developed in these vials, they may be used as cuvets in reading absorbances.

(b) Transfer 5.0 ml of cholesterol reagent to each vial.

(c) Add 50 \(\mu\)l of cholesterol standard and 50 \(\mu\)l of serum or plasma (do not use oxalated plasma) to the vials marked “standard” and “unknown,” respectively, and mix the contents of each vial thoroughly for at least 10 s.

(d) Simultaneously insert all vials into a heating block (such as the Dow Diagnostest Heating Block, catalog no. 58770) set at 100°C.

(e) Simultaneously remove all vials exactly 1.5 min later and immerse them in tap water (20°C or cooler) for 5 min. Remove, dry the exterior of the vials, and mix their contents by inversion.

(f) Set the photometer on zero with the blank and read the absorbance of standard and unknown at 560 nm (Dow Diagnostest Photoelectric Colorimeter, or see Table 1 for wavelength setting or filter to be used with various instruments).

**Results and Discussion**

**Composition of Cholesterol Reagent**

We first tried to develop a single stable reagent by using the constituents used by Zlatkis et al. (4). Numerous reports (16–22) indicate that the reproducibility of methods for cholesterol based on the ferric chloride reaction depends on the purity of the reagents and the way sulfuric acid is mixed with the acetic acid–ferric chloride–sample mixture. We thought that premixing the reagents before adding the serum would eliminate the latter variable and also simplify the procedure. Therefore, in our early experiments, we combined sulfuric acid, acetic acid and ferric chloride in the same ratio reported by Zlatkis et al. (4). When 50 \(\mu\)l of serum or cholesterol standard was added to this combined reagent, maximum color (at 560 nm) developed in 30 min at room temperature. Results of this modified procedure were compared with those obtained with the semiautomated method of Kessler et al. (23). The values obtained with the combined reagent averaged 10% greater, and the discrepancies were even larger for specimens of icteric or hemolyzed serum. Furthermore, the combined reagent was stable for only two weeks at room temperature.

Some investigators (10, 13, 23) claim improved specificity for the ferric chloride–sulfuric acid methods if glacial acetic acid is used that has been distilled from chromium trioxide. Such distillation removes traces of aldehydes, which react with tryptophan-containing compounds (such as globulins) to produce chromogens absorbing light at 560 nm. Purification of acetic acid, however, is inconvenient and does not enhance reagent stability. Klungsöyr et al. (24) substituted ethyl acetate for the acetic acid and extracted the serum with alcohol–ethyl acetate. Their values, however, were 14% greater, on the average, than those obtained by the Schoenheimer-Sperry (25) method.

Bowman and Wolf (26) first extracted serum with ethanol, and then subjected the extract to the ferric chloride–sulfuric acid reaction, with ethanol in place of glacial acetic acid. They reported satisfactory agreement with the Schoenheimer-Sperry method (25), and claimed no interference from hemoglobin, bilirubin, or lipemia. Franey and Amador (27) also extracted serum with ethanol, but reacted the cholesterol in the extract with a mixture of ferric chloride, ethyl acetate, and concentrated sulfuric acid. Bilirubin had no influence on the results, which agreed with those obtained by the method of Abell et al. (28).

When these latter investigators substituted ethyl acetate and (or) ethanol for glacial acetic acid, the results were in good agreement with those for reference methods, but still included an extraction step and the use of an unstable combined reagent. We therefore proceeded to investigate the use of ethanol and ethyl acetate as substitutes for glacial

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\(^1\) Also available from address in footnote 2, or use a size of vial compatible with the colorimeter or spectrophotometer to be used.
acetic acid in the combined reagent used in our first experiments. The reagent containing ethyl acetate was more stable than the one with ethanol, but after six weeks of storage at room temperature the reagent containing ethyl acetate visibly darkened. The latter reagent without ferric chloride did not darken with storage, and if it was completed just before use by adding ferric chloride, the results were identical to those obtained with a freshly prepared complete reagent.

Since ferric chloride was apparently the major contributor to reagent instability, we investigated the substitution of other metal ions. Altescu (29) used cupric chloride but found that tryptophan and bilirubin interfered. Of the ferric salts we tried, ferric perchlorate gave the most stable reagent; the finally selected reagent being that described under Reagents. This reagent is stable for at least a year when stored in an amber bottle at 25°C. We obtained all of the following data with use of this combined reagent.

Conditions for Color Development

When 50 μl of serum or standard is added to 5.0 ml of the cholesterol reagent and the reaction allowed to proceed at room temperature (25°C), a good amount of color develops by 30 min but continues to increase slowly. However, serum cholesterol concentrations calculated from the 30-min readings were lower than those obtained by the Kessler semiautomated method. This, plus the impractical 30-min waiting period, prompted us to investigate other conditions.

Sufficient color develops when the reaction mixture is heated for 1.5 min in a heating block set at 100°C. This heating time is satisfactory if the temperature of 5.0 ml of cholesterol reagent reaches 70°C in 1.5 min. Heating times between 1 and 2 min do not affect the results, since sample and standard developed color proportionately. Heating for less than 1 min or more than 2 min gave low and high results, respectively. Absorbancies of standard and unknowns should be read promptly after color development, because the color intensity increases slowly with time. If many samples are being analyzed in a group, measure the absorbancies of the standard and any given unknown within 5 min of each other.

Another reason for adopting the short heating period is an increased specificity of the method. This was suggested by the absorption spectra of the chromogen(s), which showed multiple peaks if color was allowed to develop for 30 min at room temperature (Figure 1), whereas there was only one major absorption peak when the color was developed during 1.5 min at the higher temperature (Figure 2). Furthermore, different concentrations of hemoglobin and γ-globulin showed a proportionately greater interference when color was developed during 30 min at room temperature as compared with the short heating period at 100°C (Tables 2 and 3). The effect of bilirubin (Table 4) was the same for both methods of color development.

Specificity and Precision

Results by the present method were compared with those by the generally accepted reference procedure of Abell et al. (24). The correlation coefficient (Figure 3) was 0.987, and a paired t test revealed no statistical significance of difference between the two means (t = 0.928; critical t = 1.999, 95% confidence limits).
Table 2. Effect of Hemoglobin on Values Obtained for Total Cholesterol in Serum

<table>
<thead>
<tr>
<th>Hemoglobin added, g/liter</th>
<th>Conditions for color development</th>
<th>25°C, 30 min</th>
<th>%</th>
<th>100°C, 1.5 min</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol, g/liter</td>
<td>Apparent increase</td>
<td></td>
<td>Cholesterol, g/liter</td>
<td>Apparent increase</td>
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<tr>
<td>0</td>
<td>2.12</td>
<td>...</td>
<td>2.15</td>
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<tr>
<td>2</td>
<td>2.25</td>
<td>6.1</td>
<td>2.18</td>
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<tr>
<td>4</td>
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<td>14.0</td>
<td>2.18</td>
<td>1.4</td>
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</tr>
<tr>
<td>6</td>
<td>...</td>
<td>...</td>
<td>2.24</td>
<td>4.2</td>
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</tr>
<tr>
<td>8</td>
<td>...</td>
<td>...</td>
<td>2.36</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>...</td>
<td>...</td>
<td>2.39</td>
<td>11.2</td>
<td></td>
</tr>
</tbody>
</table>

a Sufficient hemoglobin solution was added to a pool of serum to give the desired hemoglobin concentrations.

Table 3. Effect of γ-Globulin on Values Obtained for Total Cholesterol in Serum

<table>
<thead>
<tr>
<th>Globulin added, g/liter</th>
<th>Conditions for color development</th>
<th>25°C, 30 min</th>
<th>%</th>
<th>100°C, 1.5 min</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol, g/liter</td>
<td>Apparent increase</td>
<td></td>
<td>Cholesterol, g/liter</td>
<td>Apparent increase</td>
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<tr>
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<td>2.12</td>
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<tr>
<td>15</td>
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<td>6.4</td>
<td>2.19</td>
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<td>2.65</td>
<td>31.0</td>
<td>2.24</td>
<td>5.7</td>
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<tr>
<td>60</td>
<td>3.26</td>
<td>61.0</td>
<td>2.26</td>
<td>6.6</td>
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</tr>
<tr>
<td>90</td>
<td>...</td>
<td>...</td>
<td>2.27</td>
<td>7.1</td>
<td></td>
</tr>
</tbody>
</table>

a Sufficient powdered γ-globulin was added to a pool of serum to give the desired globulin concentrations.
b The original serum contained 30 g of globulins per liter.

Table 4. Effect of Bilirubin on Values Obtained for Cholesterol in Serum

<table>
<thead>
<tr>
<th>Bilirubin added, mg/liter</th>
<th>Conditions for color development</th>
<th>25°C, 30 min</th>
<th>%</th>
<th>100°C, 1.5 min</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol, g/liter</td>
<td>Apparent increase</td>
<td></td>
<td>Cholesterol, g/liter</td>
<td>Apparent increase</td>
</tr>
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<td>2.09</td>
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<td></td>
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<tr>
<td>25</td>
<td>1.94</td>
<td>1.0</td>
<td>2.10</td>
<td>0.5</td>
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<td>50</td>
<td>1.96</td>
<td>2.0</td>
<td>2.11</td>
<td>0.7</td>
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<tr>
<td>100</td>
<td>2.04</td>
<td>6.0</td>
<td>2.27</td>
<td>8.0</td>
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</tr>
<tr>
<td>200</td>
<td>2.12</td>
<td>10.0</td>
<td>2.34</td>
<td>11.5</td>
<td></td>
</tr>
</tbody>
</table>

a Sufficient bilirubin solution was added to a pool of serum to give the desired bilirubin concentrations.

Table 5. Absence of a Bilirubin Effect on Total Cholesterol in Six Different Sera

<table>
<thead>
<tr>
<th>Bilirubin concn, mg/liter</th>
<th>Cholesterol concn, g/liter</th>
<th>Proposed method</th>
<th>Semi-automated method (5)</th>
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</thead>
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<tr>
<td>116</td>
<td>2.82</td>
<td>2.73</td>
<td></td>
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<tr>
<td>60</td>
<td>2.62</td>
<td>2.71</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>3.53</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>3.80</td>
<td>3.85</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>1.88</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>1.94</td>
<td>2.05</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Proposed method compared with the Abell method for determination of total serum cholesterol

Cholesterol measurements were made on serum samples selected without conscious bias. Correlation coefficient, r; number of assays, n; standard error of the estimating equation, \( S_y \).

Effects of hemoglobin, bilirubin, and γ-globulin, each of which show a marked positive interference with other direct methods, were studied. The following experiment was performed: Three pools of serum were collected, and the baseline cholesterol concentration was determined for each pool. To portions of the first serum pool was added sufficient hemoglobin solution to give hemoglobin concentrations as shown in Table 2. Likewise, γ-globulin and bilirubin were added to the second and third serum pools, respectively, as shown in Tables 3 and 4. The data from analysis of these samples show that hemoglobin and bilirubin at concentrations as large as 600 mg/100 ml and 5 mg/100 ml, respectively, have a negligible effect on the results for cholesterol when the color was developed at 100°C for 1.5 min. Even at a concentration of 10 mg of bilirubin per 100 ml, the error was only +8%. γ-Globulin, present in a twice-normal concentration, resulted in a positive error of only 5.7%.

The bilirubin effect was studied further by a different approach. Patient sera containing abnormally high amounts of bilirubin were analyzed by the proposed method and by the semiautomated method of Kessler. The results (Table 5) show no apparent difference.

Inter-run precision of the method is about ±3.0% (95% confidence limits) for serum cholesterol concentrations between 1 and 4 g/liter. This coefficient of variation was calculated from data obtained by analyzing 20 patient sera, selected without conscious bias, on two consecutive days.

References


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