Enzymic Serum Cholesterol Measurement with a Basic AutoAnalyzer and the Du Pont aca Method

Benjamin Fingerhut

I compared enzymic methods for total serum cholesterol as used with discrete (Du Pont aca and reagent packs) and continuous-flow (Boehringer Mannheim reagents and the Technicon AutoAnalyzer I) analysis of normal, icteric, and lipemic sera. The regression equation for 24 clear, non-icteric sera was: \( y = 0.944x \) (aca) + 10.69; \( r = 0.971 \). \( S_{xy} \) ± 53.7 mg of cholesterol per liter. The continuous-flow system indicated no significant interference when as much as 350 mg of bilirubin was added per liter. Results with the aca method indicated a decrease in apparent cholesterol of about 5 mg/liter per milligram of added bilirubin. Serial diluting of lipemic sera resulted in falsely higher values with the aca method but with no clinically significant effect on results with the AutoAnalyzer procedure. Apparent cholesterol as measured with the aca became proportionately greater than AutoAnalyzer values with increasing serum triglyceride concentration.

The recent development of enzymic methods for determining total cholesterol in serum has led to their use with various automated analytical systems such as the aca (Du Pont Instruments, Wilmington, Del. 1989),\(^1\) the ABA (Abbott Laboratories, North Chicago, Ill. 60064) (I), centrifugal analyzers (such as the CentrifiiChem from Union Carbide Corp., Rye, N.Y. 10580) (2), and AutoAnalyzers (Technicon Instruments Corp., Tarrytown, N.Y. 10591).\(^2\) Reports of results with these systems are conflicting with respect to the reliability of values obtained in the case of icteric and lipemic sera (2-5).

Here I compare results obtained on using the Du Pont aca system and its cholesterol reagents and the Technicon AutoAnalyzer I system with Boehringer Mannheim Corp. (Bio-Dynamics/bmc, Indianapolis, Ind. 46250) reagents and flow diagram.\(^3\) I also determined the effect of bilirubin and triglycerides on the results for cholesterol given by these methods.

Material and Methods

The Du Pont aca analyzer and reagent packs were used as recommended by the manufacturer. Each reagent pack contained, per liter, 48.0 U of cholesterol oxidase (EC 1.1.3.6), 2290 U of horseradish peroxidase (EC 1.11.1.7), 286 U of cholesterol esterase (EC 3.1.1.13), 0.78 mmol of 4-aminoantipyrine, 34.0 mmol of morpholineethanesulfonic acid buffer (pH 6), 1.43 mmol of sodium cholate (emulsifier), 0.51 g of Triton X-100 surfactant, and 1.56 mmol of \( N,N \)-diethylylammoniumbromide.

In the continuous-flow method, I used basic modules consisting of a Sampler (Model III), Proportioning Pump (Model

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Results and Discussion

Figure 2 indicates the relationship between results given by the methods compared for clear, non-icteric sera. From the regression equation the calculated AutoAnalyzer I values are about 50 mg/liter greater than the aca results for a cholesterol concentration of 1.0 g/liter, and the same for 2.0 g/liter. These differences are not clinically significant. Precision for within-run determinations yielded a CV of 3.0% and 2.9% by the aca and AutoAnalyzer I methods, respectively. For day-to-day precision the CV with the aca procedure was 4.8%, and 5.0% with the AutoAnalyzer I method.

When icteric sera were analyzed by both methods, significant differences were noted (Table 1). There was a consistent trend towards higher results with increasing serum bilirubin concentration by the AutoAnalyzer I method. This difference does not appear to be strictly proportional to the bilirubin concentration. Investigation of the effect of bilirubin on the results of cholesterol analysis by continuous flow indicated a great dependence on the colorimeter filters used. Results are highest when measured with 460-nm filters and decrease with increasing filter wavelength up to 520 nm (Table 2). There is no difference in results with use of 520- and 550-nm filters (Figure 3). These findings would be expected from the absorbance spectrum of bilirubin, which has a maximum absorbance in the 460-nm range.

The data presented in Table 3 indicate the lack of change with 520-nm filters, but large changes with 480-nm filters, in results of continuous-flow analysis on addition of up to 350 mg of National Bureau of Standards bilirubin per liter to clear, non-icteric serum. aca measurement of cholesterol in serum to which bilirubin was added indicated that results are decreased by approximately 5 mg/liter for each milligram of added bilirubin (Table 4).
The Du Pont aca cholesterol methodology information sheet\(^1\) lists no measurable effect of as much as 120 mg of bilirubin per liter on cholesterol results, but no information is given concerning the extent or direction of interference from greater bilirubin concentrations. It has been suggested (4) that the negative interference from bilirubin in some enzymic cholesterol methods may be related to the concentration of peroxidase used. Thus the negative interference from bilirubin I observed in the aca method may be due to the greater concentration (2 kU/liter) of peroxidase in this system compared to that used in the continuous-flow procedure (1.2 kU/liter). To test this hypothesis, I added 7.5 kU of additional peroxidase per liter to the BMC reagent used with the AutoAnalyzer I system. The results (Table 5) indicate no significant effect on either icteric and non-icteric serum cholesterol values with the additional peroxidase. It is therefore unlikely that the greater peroxidase concentration used in the aca method accounts for the negative interference form bilirubin. It has been reported that bilirubin depresses cholesterol results determined enzymatically with manual techniques (3) with serum and reagent blanks or with the Union Carbide Corp. Centrifichem (2), but not with the Technicon AutoAnalyzer II or SMA 12/60 systems.\(^2\) One factor present in the aca system is the use of a filter to measure the absorbance of the reaction product and an additional filter absorbing light at a different wavelength to correct for sample pigmentation or turbidity. The Centrifichem has a built-in sample-blank correction. All the Technicon AutoAnalyzer methods (AAI, AAI, and SMA 12/60) do not correct for serum color or turbidity interference. Generally those methods using sample blank corrections in their absorbance measurements yield results affected by above normal serum bilirubin concentrations. The reason for this is not clear at this time.

Bilirubin interference in the manual technique (3) is possibly explained by the choice of 500-nm filters used in measuring the absorbance of the reaction product. Bilirubin absorbance is greater at 500 nm than at 520 nm. Curves C and D of Figure 2 in reference 3, which are absorption spectra for the cholesterol reaction product with and without added bilirubin, indicate appreciable absorbance differences at 500 nm, which become almost negligible at 520 nm. Since the bilirubin effect in the manual procedure was reported to be a small, negative one at 500 nm, it would be much less at 520 nm, where curves C and D are almost superimposable. Therefore the difference in effect of bilirubin in the continuous-flow procedure from that in the manual method used in reference 3 can be attributed to measurement of the reaction product absorbance at 500 nm in the latter procedure instead of 520 nm as in the former method.

The effect of serum triglyceride concentration (turbidity) on cholesterol results obtained by both methods is summarized in Tables 6 and 7. There is an increased difference in

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**Table 3. Effect of Added Bilirubin on Cholesterol Results with the AutoAnalyzer I**

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Added bilirubin Conc., mg/liter</th>
<th>Before added bilirubin</th>
<th>After added bilirubin</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>520</td>
<td>125.0</td>
<td>2040</td>
<td>2060</td>
<td>+10</td>
</tr>
<tr>
<td>520</td>
<td>218.0</td>
<td>1620</td>
<td>1610</td>
<td>+10</td>
</tr>
<tr>
<td>520</td>
<td>350.0</td>
<td>1330</td>
<td>1310</td>
<td>-40</td>
</tr>
<tr>
<td>480</td>
<td>158.0</td>
<td>1920</td>
<td>2330</td>
<td>+410</td>
</tr>
<tr>
<td>480</td>
<td>199.0</td>
<td>1870</td>
<td>2380</td>
<td>+510</td>
</tr>
</tbody>
</table>

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**Table 4. Effect of Added Bilirubin on aca Cholesterol Results**

<table>
<thead>
<tr>
<th>Added bilirubin Conc., mg/liter</th>
<th>Apparent cholesterol Before added bilirubin</th>
<th>After added bilirubin</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>125.0</td>
<td>2040</td>
<td>1940</td>
<td>-100</td>
</tr>
<tr>
<td>158.0</td>
<td>1950</td>
<td>1840</td>
<td>-110</td>
</tr>
<tr>
<td>199.0</td>
<td>1800</td>
<td>1710</td>
<td>-90</td>
</tr>
<tr>
<td>218.0</td>
<td>1680</td>
<td>1560</td>
<td>-100</td>
</tr>
</tbody>
</table>

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**Table 5. Effect of Added Peroxidase on AAI Cholesterol Results by Continuous-Flow**

<table>
<thead>
<tr>
<th>Bilirubin Apparent cholesterol with indicated peroxidase Conc. mg/liter</th>
<th>8700 U/liter</th>
<th>1200 U/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2120</td>
<td>2090</td>
</tr>
<tr>
<td>2.0</td>
<td>1770</td>
<td>1760</td>
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<tr>
<td>4.0</td>
<td>1500</td>
<td>1510</td>
</tr>
<tr>
<td>4.0</td>
<td>860</td>
<td>830</td>
</tr>
<tr>
<td>5.0</td>
<td>1250</td>
<td>1250</td>
</tr>
<tr>
<td>11.0</td>
<td>1390</td>
<td>1390</td>
</tr>
<tr>
<td>25.0</td>
<td>1810</td>
<td>1830</td>
</tr>
<tr>
<td>46.0</td>
<td>1680</td>
<td>1670</td>
</tr>
<tr>
<td>55.0</td>
<td>920</td>
<td>940</td>
</tr>
<tr>
<td>62.0</td>
<td>2300</td>
<td>2360</td>
</tr>
<tr>
<td>70.0</td>
<td>1170</td>
<td>1160</td>
</tr>
<tr>
<td>155.0</td>
<td>1380</td>
<td>1390</td>
</tr>
<tr>
<td>160.0</td>
<td>2920</td>
<td>2900</td>
</tr>
<tr>
<td>310.0</td>
<td>1040</td>
<td>1030</td>
</tr>
</tbody>
</table>

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**Fig. 3. Comparison of AAI cholesterol results with 520-nm and 550-nm filters**
values between the AutoAnalyzer I and aca methods with increasing serum triglyceride concentrations (Table 6). However, the differences are generally not clinically significant with triglyceride concentrations up to about 5 g/liter. From serial dilution experiments with a very lipemic serum (18.20 g of triglycerides per liter) it appears that lipemia does not interfere with the AutoAnalyzer I method. The data in Table 7 indicate a 98% analytical recovery of cholesterol when lipemic serum was diluted with an equal volume of isotonic saline or with two parts of saline. However, lipemic sera appear to interfere with the aca method, because measurement of the same lipemic serum and its serial dilutions gave only 86% and 83% recovery with serum dilutions of 50 and 33%, respectively (Table 7).

Both the aca and BMC-AutoAnalyzer I cholesterol methods are claimed to show no interference from hemolysis. Data in the present study tend to confirm this. A comparison of cholesterol results with these methods indicated no clinically significant difference in results for several hemolyzed samples with hemoglobin concentrations ranging from 1.75 to 4.5 g of hemoglobin per liter. In addition the continuous-flow determination of cholesterol in several hemolyzed samples with hemoglobin concentrations ranging from 2.5 to 3.5 g/liter was in close agreement on use of 480-nm and 550-nm interference filters. This indicates lack of spectral interference from hemoglobin in this range; its absorbance spectrum has a maximum at 555 nm and a minimum at 480 nm.

Addendum

After this report was submitted for publication, results confirming the negative interference effect of bilirubin on the aca cholesterol procedure were reported [Clin. Chem. 24, 108 (1978)].

References