Comparison of Two Micromethods for Determination of Lipoprotein Cholesterol in Plasma

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We compared the results obtained by a micromethod for the determination of plasma lipoprotein cholesterol, in which electrophoresis is used to separate the lipoprotein fractions ($\beta-$, pre-$\beta-$, and $\alpha$-lipoproteins), with those determinations with ultracentrifugation (low-density, very-low-density, and high-density lipoproteins). Precision of determination (coefficient of variation, CV, %) was the same for $\beta$- and low-density lipoproteins (1.6%), and for pre-$\beta$- and very-low-density lipoproteins (3.7%); however, determination of $\alpha$-lipoprotein cholesterol was more precise (1.4%) than that of high-density lipoprotein cholesterol (3.1%). Analytical recovery of lipoprotein cholesterol was the same for both methods (98–100%) and the results were closely correlated ($r = 0.943$). The procedure has been used to determine the cholesterol content of plasma lipoprotein fractions of apparently healthy adults (both sexes). Lipoprotein cholesterol concentrations in our population sample compare well with those reported for other groups of similar age, in particular Stanford long-distance runners.

Additional Keyphrases: electrophoresis · ultracentrifugation · intermethod comparison · atherogenesis

Disorders in lipoprotein (LP)1 metabolism are closely related to the risk of atherogenesis (1–6). Accordingly, the determination of plasma cholesterol concentrations is a widely used tool in the diagnosis and therapeutic management of this disease. Because atherogenesis appears to start in childhood, early detection of LP disorders is desirable (7, 8). However, this requires precise yet simple methods suitable for large-scale studies. Currently, ultracentrifugation and precipitation techniques are used (9, 10), but these do not permit the simultaneous separation of the main LPs. Furthermore, large sample volumes are needed, the analytical steps are time consuming, and the equipment is expensive. Finally, the results, computed with nomograms, are only approximate (10).

The separation of LPs by electrophoresis followed by lipid analysis has been described elsewhere. However, neither extraction procedures nor radiochemical or gas-chromatographic detection of LP cholesterol is suitable for routine analysis on a large scale (these methods were reviewed in refs. 10 and 11).

In our laboratory, the LPs in 15 $\mu$L of plasma or serum are separated by electrophoresis on agarose gel, and the cholesterol is released by dissolving the agarose in HCl. After neutralization with tris(hydroxymethyl)methylamine (Tris) buffer, cholesterol is determined enzymatically. The precision of determination (coefficient of variation, CV, 1–3%) and the analytical recovery of cholesterol (nearly 100%) are very good (10–12). This procedure was also found to be suitable for analysis of capillary blood plasma (13).

In this study we compare the results with our procedure with those with ultracentrifugation. Moreover, we compare the results obtained with this procedure on a German population sample with the corresponding data reported by authors who used ultracentrifugation.

Materials and Methods

Plasma Samples

Blood was obtained by venipuncture from fasting subjects (12–16 h) and was anticoagulated with dipotassium ethylenediaminetetraacetate (EDTA). Plasma samples were cooled at 4°C and LPs separated on the same day. Plasma samples from hyperlipidemic patients were classified into LP phenotypes according to WHO recommendations (14).

Separation and Determination of LPs

Electrophoresis of plasma LPs was carried out as follows. A sheet of polyester film (22 x 15 cm) was placed on a glass plate in such a way that the upper surface of the plastic was the inner surface on the plastic roll (Cronar Type P-7 R) supplied by duPont de Nemours & Co. Inc., Wilmington, DE 19898. This was heated at 100°C for 15 min, which counter the tendency of the plastic sheet to curl during use. A few drops of water were put in the middle of the glass plate to keep the plastic sheet in position.

We heated 0.6 g of agarose (Serva, Heidelberg, F.R.G.) to boiling in 75 mL of barbital buffer (sodium barbital, 0.05 mol/L, pH 8.6, in EDTA, 0.94 mmol/L). The solution was then cooled to 50°C in a water bath. We mixed 70 mL of this agarose solution with 1 mL of a bovine albumin solution (100 g/L of distilled water) at 50°C and poured it onto the polyester film, which was kept at 50°C on a level surface. Using a glass rod or a plastic tip, we spread the agarose solution over the entire surface of the film. After 10 min, a well-former was positioned on the coated film. After a further 5 min, the solution had set into a gel, and was then transferred to the refrigerator (4°C) for another 15 min. The well-former was taken out carefully, and excess fluid was removed from the slots with a pipette connected to a suction pump.

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1 Nonstandard abbreviations used: LP, lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; Tris, tris(hydroxymethyl)methylamine; EDTA, ethylenediaminetetraacetate.

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A 50-µL Hamilton syringe and the plasma samples were warmed in a water bath at 50°C. The plasma samples were mixed with equal volumes of the agarose solution and 30-µL aliquots of these mixtures were applied to the agarose slots; the slots were then covered with agarose. We transferred the polyester sheet with the agarose layer to the Boscamp microphor system (Boscamp/IL, Hersel, F.R.G.), dipping the edges into buffer on the electrophoresis cell was kept in the refrigerator at 4°C. The LPs were separated at 150 V for 70 min.

After electrophoresis, we transferred the agarose sheets to a dextran sulfate–CaCl₂ solution (dextran sulfate 500, 6 g/L; CaCl₂, 0.33 mol/L) for 15 min. When the LP fractions became visible, they were cut out with a scalpel. The agarose samples containing the LP fractions were transferred into plastic tubes (82 × 16 cm) and dried overnight under reduced pressure in a desiccator containing blue gel (11).

These LP fractions were dissolved in 300 µL of HCl (6 mol/L) by shaking the samples vigorously for 5 min. Addition of 600 µL of saturated Tris solution at room temperature produced a Tris-buffered set of samples with pH 7. We added 1 mL of a reaction mixture (see below) to the cholesterol samples in the Tris-buffered solution. The samples were mixed, incubated at 37°C for 15 min, and centrifuged at 3000 rpm for 10 min. The supernates were reacted with oxidase and measured against the corresponding sample blanks.

For cholesterol determination we prepared a reaction mixture from a modified “solution 5” of the Biochemica Test Combination (No. 124086; Boehringer, Mannheim, F.R.G.) as follows: 10 parts of solution 1 (ammonium phosphate buffer, 0.6 mol/L, pH 7; methanol, 1.7 mol/L; catalase, >700 kU/L); 1 part of solution 2 (acetylacetone, 0.42 mol/L; methanol, 2.5 mol/L; hydroxypropyl-β-cyclodextran, 21 g/L); 0.08 part of solution 3 (cholesterol ester, >7 kU/L); 0.52 part of methanol; 0.0052 part of catalase (1 g/50 mL; No. 15674, Boehringer) (12).

The plasma LPs were also ultracentrifuged with an Airfuge (Beckman Instruments, Palo Alto, CA 94304). To separate the LPs into plasma density classes, we used a modified procedure after Bronzert and Brewer (15). Very-low-density LPs (VLDL) were separated at density 1.006 kg/L; 100 µL of plasma was mixed with 75 µL of KBr solution (9 g/L). High-density LPs (HDL) were separated at density 1.060 kg/L, and 100 µL of plasma and 75 µL of KBr solution (184 g/L) were mixed. The samples were centrifuged at 160 000 × g for 2.5 h. We examined each step of centrifugation by electrophoresis with a microelectrophoresis system (Corning/IMA, Giessen, F.R.G.).

The cholesterol concentrations of plasma, HDL, and VLDL were analyzed according to Roeschlauf et al. (16) with the Biochemica Test Combination Cholesterol (Boehringer). Low-density LP (LDL) cholesterol was computed as follows: plasma cholesterol (HDL + VLDL) cholesterol = LDL cholesterol.

Student’s t-test and linear regression analysis were used for statistical examination of the results.

### Results and Discussion

#### Analytical Procedure

Table 1 presents data on the precision of determination (CV, %) and the analytical recovery of LP cholesterol by both methods. The CV’s for the β-LPs (1.5%) and pre-β-LPs (3.7%) were essentially the same as in the corresponding LP density classes (LDL = 1.7%; VLDL = 3.6%). The determination of the α-LPs (CV = 1.4%), however, was more precise than that of the HDL cholesterol (CV = 3.1%). The analytical recovery of cholesterol was nearly 100% in both methods.

Furthermore, we used both methods to analyze the LPs of plasma samples of 42 hyperlipidemic and apparently healthy subjects (adults and children). The average values for total cholesterol and LP cholesterol were nearly identical, as shown in Table 1, and the correlation of the individual measurements was quite good (r = 0.990; r² = 0.943), as shown in Figure 1.

We conclude that results with our microprecedure, in which electrophoresis for the separation of the LPs in 15 µL of serum or plasma is followed by the enzymatic determination of cholesterol (12), are comparable with the results obtained by ultracentrifugation. Indeed, the determination of α-LP cholesterol with our micromethod is more precise than with HDL analysis. Furthermore, the very small sample volumes needed for our procedure and the simple equipment, available in most laboratories, make our method suitable for large-scale studies.

#### Application

We analyzed plasma cholesterol and the corresponding LP cholesterol of apparently healthy adults. This sample group consisted of 39 voluntary subjects (students and laboratory personnel) aged 20 to 40 years, with no restrictions as to diet or athletic activity. The subjects fasted for 12–16 h before venipuncture.

As shown in Table 3, the amount of total cholesterol per subject in our population sample was largely consistent with that reported for male elite runners at Stanford (17); it was higher than in young U.S. Air Force recruits (18) but less than in Swedish and English population samples (17, 18). In all

### Table 2. Comparison of Cholesterol Determination by Both Methods

<table>
<thead>
<tr>
<th>Total cholesterol</th>
<th>Ultracentrifugation</th>
<th>Micromethod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.74 ± 0.93</td>
<td>4.72 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>LDL (β-LP)</td>
<td>2.58 ± 0.70</td>
<td>2.56 ± 0.66</td>
</tr>
<tr>
<td>VLDL (pre-β-LP)</td>
<td>0.71 ± 0.38</td>
<td>0.65 ± 0.31</td>
</tr>
<tr>
<td>HDL (α-LP)</td>
<td>1.44 ± 0.31</td>
<td>1.51 ± 0.35</td>
</tr>
<tr>
<td>Recovery %</td>
<td>99.8</td>
<td>100</td>
</tr>
</tbody>
</table>

Samples from plasma of 42 hyperlipidemic and apparently healthy adults and children.
<table>
<thead>
<tr>
<th>Country, Year</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Total Cholesterol, mmol/L (mean ± SD)</th>
<th>LDL (β-LP) Cholesterol, mmol/L</th>
<th>VLDL (pre-β-LP) Cholesterol, mmol/L</th>
<th>HDL (α-LP) Cholesterol, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uppsala, 1975 (19)</td>
<td>30-39 M</td>
<td>6.32 ± 0.28</td>
<td>4.19 ± 0.23</td>
<td>0.54 ± 0.08</td>
<td>1.37 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>London, 1974 (20)</td>
<td>20-39 M</td>
<td>5.54 ± 0.49</td>
<td>3.58 ± 0.60</td>
<td>0.54 ± 0.21</td>
<td>1.49a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.47 ± 0.65</td>
<td>3.29 ± 0.52</td>
<td>0.31 ± 0.16</td>
<td>1.57a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.73 ± 1.04</td>
<td>2.05 ± 1.24</td>
<td>0.36 ± 0.26</td>
<td>1.50 ± 0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.50 ± 0.93</td>
<td>1.89 ± 0.94</td>
<td>0.31 ± 0.23</td>
<td>1.53 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>US Air Force recruits, 1973 (18)</td>
<td>18-22 M</td>
<td>4.53 ± 0.68</td>
<td>2.80 ± 0.63</td>
<td>0.28 ± 0.13</td>
<td>1.45 ± 0.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.47 ± 0.79</td>
<td>2.67 ± 0.55b</td>
<td>0.61 ± 0.15</td>
<td>1.47 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Stanford, 1977 (elite runners) (17)</td>
<td>21-34 M</td>
<td>4.74 ± 0.79</td>
<td>2.67 ± 0.55b</td>
<td>0.61 ± 0.15</td>
<td>1.47 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Duesseldorf, 1979</td>
<td>20-40 F</td>
<td>4.49 ± 0.85</td>
<td>2.34 ± 0.58b</td>
<td>0.59 ± 0.23</td>
<td>1.57 ± 0.33</td>
<td></td>
</tr>
</tbody>
</table>

All results based on ultracentrifugation except Duesseldort, which is by the micromethod.

* SD not reported.
* Difference between men and women is significant by Student’s t-test.

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**Figure 1.** Comparison of total and lipoprotein cholesterol in plasma, as determined by the reference method (x, 16) and by the micromethod (y).

A, total cholesterol by both methods; B, LDL cholesterol by ultracentrifugation vs. β-LP cholesterol by the micromethod; C, VLDL cholesterol by ultracentrifugation vs. pre-β-LP cholesterol by the micromethod; D, HDL cholesterol by ultracentrifugation vs. α-LP cholesterol by the micromethod. $y = bx + a$, regression line; $r$ = correlation coefficient.
groups investigated, the cholesterol concentration was higher in men than in women.

The differences in total cholesterol amounts are primarily in the \( \beta \)-LP fractions. The \( \beta \)-LP cholesterol concentration in our men was nearly identical with that of the Stanford elite runners. The sex difference observed for the \( \beta \)-LP cholesterol of our German group was significant. The same tendency may be seen in the data from other authors.

Although the total cholesterol content in our subjects was lower, the pre-\( \beta \)-LP cholesterol determined in our group was within the same ranges reported for the Swedish and English groups.

Interestingly, the concentrations of total cholesterol, as well as \( \beta \)-LP (LDL) and pre-\( \beta \)-LP (VLDL) cholesterol, vary markedly among the various population samples. The \( \alpha \)-LP (HDL) cholesterol concentrations, however, vary only within narrow limits. As in the other female groups, the women in our study also carry a slightly higher amount of cholesterol in the \( \alpha \)-LP fraction than men do.

The microprocedure for the determination of plasma LP cholesterol without ultracentrifugation (12) was reliable for routine analysis and gave nearly identical results to those with ultracentrifugation. The application of the method to the investigation of apparently healthy adults led to data that are in good agreement with those reported by other authors who used ultracentrifugation.

The reliability of our method is further supported by results obtained from a large group of kindergarten children (8). Normal values obtained for serum lipids and LPS were largely consistent with data reported by Starr (21) and by Lauer et al. (22). Among these children (\( n = 335 \)), who were defined as "normal" according to anthropometric criteria, we found 13 children with increased values for cholesterol. Another group of overweight children (\( n = 22 \)) had lower amounts of \( \beta \)-LP cholesterol than are reported for adiposity in adults. Among 117 socially underprivileged children, 93 were "normal" according to anthropometric criteria, but had reduced concentrations of pre-\( \beta \)- and \( \alpha \)-LP cholesterol. The latter result was reported for protein malnutrition.

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References