Lipoprotein Cholesterol in the Serum of Children, as Determined Independently by Two Different Methods

Sathanur R. Srinivasan, Ralph D. Ellefson, Carl F. Whitaker, and Gerald S. Berenson

We separately evaluated split specimens of serum for lipoprotein cholesterol in 38 five- to 14-year-old children, by the heparin/Ca\(^{2+}\) precipitation method and the ultracentrifugation/dextran sulfate precipitation method. Statistical analysis of the results indicated an excellent agreement (especially in \(\beta\) - and \(\alpha\)-lipoprotein cholesterol values) between results by the two methods. The percentage of \(\alpha\)-lipoprotein cholesterol (mean ± SD: 38.6 ± 5.0) in children further confirmed the earlier observations that cholesterol derived from \(\alpha\)-lipoprotein in children constitutes a relatively greater part of the serum total cholesterol than is true of adults.

Additional Keyphrases: lipoproteins • polyanionic precipitation method • ultracentrifuge method • pediatric chemistry • screening

Because increased concentrations of certain serum lipoproteins relate to susceptibility to coronary artery disease, practical methods are greatly needed for quantitating serum lipoproteins in the general population, especially in children. Several such methods that have been proposed are based on complexing with polyanionic macromolecules (1-5) and are currently being used in clinical as well as population studies. Sulfated polysaccharides—i.e., heparin or dextran sulfate—are used because these materials react to form insoluble complexes with pre-\(\beta\) and \(\beta\)-lipoproteins in the presence of certain divalent cations.

Recent studies indicate that the characteristics and concentrations of glycosaminoglycans, the lipoproteins in serum, and the divalent metal ions (e.g., Ca\(^{2+}\) or Mn\(^{2+}\)) are all critical for quantitative and selective precipitation of the lipoproteins (6, 7). Based on these observations, a simple and inexpensive procedure for quantitating the serum lipoproteins was developed (8, 9), the results of which correlated reasonably well with studies by analytical ultracentrifugation (10, 11). Although it gave similar values for adults (mostly men) when compared to results obtained with a heparin/Mn\(^{2+}\) precipitation method (12-14), the heparin/Ca\(^{2+}\) precipitation method consistently gave higher values for \(\alpha\)-lipoproteins in children (12, 15, 16). Therefore, we have attempted to compare values for serum lipoprotein cholesterol concentration obtained in children by our method (LSU method) with another method developed independently at the Mayo Clinic (Mayo method). The latter procedure, developed from the observations reported by Burstein et al. (17), is a combination of preparative ultracentrifugation and polyanionic (dextran sulfate) precipitation that permits quantitation of cholesterol content of pre-\(\beta\), \(\beta\), and \(\alpha\)-lipoproteins by direct measurements.

Materials and Methods

Serum

Sera were collected in five different batches (March–July 1974) from 38 school children (18 boys and 20 girls), ages 5 to 14 years, who were among the 3524 children screened during the 1973–1974 school year as part of the Bogalusa Heart Study (Bogalusa, La.). Each child was asked to fast for 12–14 h and fasting compliance was determined by interview on the morning of the examination. Venous blood was collected and was allowed to clot. After centrifugation, sera were collected in tubes containing thimerosal, an antibacterial agent (Aldrich Chemical Co., Inc., Milwaukee, Wis. 53233), placed in a shipping box containing frozen packs, and sent by bus to New Orleans, where they were refrigerated at 4 °C overnight. The next day the samples were divided into two parts for analyses at LSU and at the Mayo Clinic. The samples were shipped airmail to the Mayo Clinic in containers packed with ice. Twelve of the 38 samples were randomly selected and the split sample included as blind duplicates.

LSU Method

Serum cholesterol. Serum cholesterol was determined with an AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, N. Y. 10591) according to the protocol developed by the Lipid Research Clinics in collaboration with the Center for Disease Control in Atlanta, Georgia. An isopropanol extract of the sample (0.2 ml of whole serum or \(\beta\) + pre-\(\beta\)-lipoprotein fraction) was used for the determination. We used a serum
calibrator provided by the Center to convert the cholesterol value obtained by the AutoAnalyzer II to values consistent with the method of Abell-Kendall (18). This laboratory participates in the Cooperative Lipid Standardization Program of the CDC.

Serum lipoprotein cholesterol. Beta + pre-\(\beta\)-lipoprotein cholesterol values were determined after selective precipitation of serum \(\beta\)- and pre-\(\beta\)-lipoproteins with heparin and \(\mathrm{Ca}^{2+}\) (6, 8). Briefly, this method consists of mixing serum (0.2 ml), distilled water (3.2 ml), beef lung heparin (0.1 ml of 2.5 g/liter solution, \(\approx 140\) USP units/mg; Upjohn Co., Kalamazoo, Mich.), and \(\mathrm{CaCl}_2\) (0.5 mol/liter, 0.5 ml), in the order given. After the mixtures were allowed to stand for 15 min, the precipitate was obtained after centrifugation (1500 \(\times\) g, 30 min) and analyzed for the corresponding \(\beta\)- and pre-\(\beta\)-lipoprotein cholesterol content by dissolving it in 0.2 ml of 0.15 mol/liter NaCl.

The value for \(\alpha\)-lipoprotein cholesterol was obtained by subtracting \(\beta\) + pre-\(\beta\)-lipoprotein cholesterol from total cholesterol (15, 16).

Electrophoretic ratio of \(\beta\)- and pre-\(\beta\)-lipoprotein. Serum (10–20 \(\mu\)l) was electrophoresed on agar-agarose gel plates (8.3 \(\times\) 10 cm), with use of a barbital buffer (pH 8.6, 0.05 mol/liter) and 22 mA per plate (8, 19). Staining was done with Oil Red 0 for 6 h or overnight, followed by washing successively with alcohol/water (5/3 by vol) for 5 min and then distilled water. The lipoprotein bands were scanned in a densitometer to assess the relative proportion of \(\beta\) - and pre-\(\beta\)-lipoprotein. The dye uptake (per unit weight of lipoprotein) is known to differ among the lipoprotein classes (20) and their protein and lipid content differ, and so we corrected the densitometric ratios of \(\beta\)- and pre-\(\beta\)-lipoprotein as described previously (15), assuming that 1 mg of \(\beta\)-lipoprotein takes up the same amount of dye as does 0.86 mg of pre-\(\beta\)-lipoprotein (15).

Indirect estimation of \(\beta\)- and pre-\(\beta\)-lipoprotein cholesterol. The estimation of serum \(\beta\)- and pre-\(\beta\)-lipoprotein concentrations were based on the densitometric ratio of \(\beta\)- to pre-\(\beta\)-lipoprotein, \(\beta\) + pre-\(\beta\)-lipoprotein cholesterol concentration, and the reported mean values of cholesterol contained in \(\beta\)-lipoprotein (46.9%) and pre-\(\beta\)-lipoprotein (22.2%) molecules (8, 9). Any changes in lipoprotein estimations due to variations in cholesterol content of these molecules in normal individuals including children were considered to be small (10). The \(\beta\)- and pre-\(\beta\)-lipoprotein cholesterol concentrations were estimated indirectly as follows: \(\beta\)-lipoprotein cholesterol = mg \(\beta\)-lipoprotein \(\times\) 0.469; pre-\(\beta\)-lipoprotein cholesterol = mg pre-\(\beta\)-lipoprotein \(\times\) 0.222.

Mayo Clinic Method

Serum cholesterol. Cholesterol in serum was measured with a Technicon AutoAnalyzer, redesigned to produce equivalent color yields from cholesterol and cholesteryl esters (21). An optimized acetic acid/sulfuric acid/ferric chloride reagent was used, and the instrument was calibrated with pure cholesterol obtained from the National Bureau of Standards. As a preliminary to quantitation, cholesterol was extracted from 0.5-ml aliquots of serum or lipoprotein fractions with isopropanol and interfering substances were removed from the extracts by absorption to a zeolite preparation (22). This method produced values that agreed closely with values obtainable with the manual Abell–Kendall method (18). Accordingly, the Mayo Laboratory, like the LSU Laboratory, became "standardized" in the Cooperative Lipid Standardization Program of the Center for Disease Control.

Serum lipoprotein cholesterol. Briefly, the method included isolation of very-low-density lipoproteins (VLDL, pre-\(\beta\)-lipoprotein) by ultracentrifugation, precipitation of low-density lipoproteins (LDL, \(\beta\)-lipoprotein) with dextran sulfate (SOCHIBO, Boulogne, France; mol wt >2 000 000) and \(\mathrm{Ca}^{2+}\), and precipitation of high-density lipoproteins (HDL, \(\alpha\)-lipoprotein) with additional dextran sulfate and \(\mathrm{Mn}^{2+}\). The conditions used for the selective quantitative precipitations of the LDL and HDL have been described by Burstein et al. (17). The selectivities of the conditions for precipitations of LDL and HDL were ascertained by radial immunodiffusion against anti-sera raised in rabbits, and by electrophoresis. VLDL fractions and each of the precipitates of LDL and HDL, dissolved in sodium chloride solution (50 g/liter), were analyzed for cholesterol by the method described above.

Isolation of VLDL. 2.0-ml aliquots of serum were transferred in polycarbonate centrifuge tubes (11 \(\times\) 77 mm) and diluted with 1.0 ml of saline, relative density 1.006. The contents were centrifuged in a type SW 28 rotor fitted with adaptors (Model L2-65B preparative ultracentrifuge; Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif. 94304) for 15 h at 100 000 to 120 000 \(\times\) g. About 0.5 ml of the top layer containing the VLDL was collected with a transfer pipette with a capillary tip. The pipettes were rinsed with isotonic saline and the VLDL fraction was diluted with saline to 1.0 ml. When much VLDL is present in a specimen, the VLDL may stick to the tip of the centrifuge tube in the form of a waxy layer. A quantitative transfer of VLDL requires a transfer of the floating VLDL as described above, followed by quantitative removal of the LDL and HDL from the tube, then quantitative rinsing of the remaining VLDL from the tube. In the experience of one of us (R.D.E.), the procedure described above has been as quantitative as the procedure that includes the use of a thin-wall centrifuge tube and tube slicer.

Isolation of LDL. The remaining infranatant solutions were quantitatively transferred to graduated tubes and diluted to 3.0 ml with distilled water. To each tube, 330 \(\mu\)l of 1.0 mol/liter \(\mathrm{CaCl}_2\) solution and 33 \(\mu\)l of 100 g/liter dextran sulfate solution were added, the contents mixed thoroughly, and allowed to stand for 1 h. The precipitates of LDL were centrifuged, followed by a rinse with 2.0 ml of distilled water, and the supernatant fluids were collected in another set of graduated centrifuge tubes. The precipitate of LDL was dissolved in 1.0 ml of 50 g/liter NaCl solution prior to cholesterol analysis.

Isolation of HDL. The above supernatant fluids containing HDL were diluted to 6.0 ml with distilled water, and 1600 \(\mu\)l of 1 mol/liter \(\mathrm{MnCl}_2\) solution and 560 \(\mu\)l of 100 g/liter dextran sulfate solution were added. The contents were mixed thoroughly and allowed to stand for 1 h, or preferably overnight. The HDL precipitates were centrifuged, rinsed once with 2.0 ml of distilled water, and dissolved in 1.0 ml of 50 g/liter NaCl solution prior to cholesterol analysis.

Results

We assessed the reproducibility of the two laboratories by analyzing blind duplicate samples, presented in no particular order. Although the LDL and VLDL cholesterol were determined separately by the Mayo method, we combined the cholesterol values of the two classes of lipoproteins, to enable a comparison with the direct determination of \(\beta\) + pre-\(\beta\)-lipoprotein cholesterol by the LSU method. Table 1 shows the results of blind duplicate analyses for total cholesterol, \(\beta\) + pre-\(\beta\)-lipoprotein cholesterol, and \(\alpha\)-lipoprotein cholesterol. Duplicate analyses by LSU gave a \(\pm 0.9\)% mean difference for total cholesterol, \(\pm 0.8\)% for \(\beta\) + pre-\(\beta\)-lipoprotein cholesterol, and \(\pm 2.0\)% for \(\alpha\)-lipoprotein cholesterol, as compared to Mayo's \(\pm 0.4\)%, \(\pm 1.4\)% and \(\pm 1.9\)% respectively. For all specimens compared between the LSU and Mayo methods, the sums of the cholesterol values determined for the lipoprotein fractions agreed within 60 mg/liter with values determined directly for whole serum.
Table 1. Mean Percent Difference (±) between Blind Duplicate Analyses of Serum Total Cholesterol and Lipoprotein (LP) Cholesterol (mg/liter)

<table>
<thead>
<tr>
<th>No. of pairs</th>
<th>Total cholesterol</th>
<th>β + pre-β-LP cholesterol</th>
<th>α-LP cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSU</td>
<td>Mayo</td>
<td>LSU</td>
</tr>
<tr>
<td>12</td>
<td>0.9 (0.4)</td>
<td>0.8 (1.4)</td>
<td>2.0 (1.9)</td>
</tr>
<tr>
<td></td>
<td>(0.2.5)²</td>
<td>(0.2.3)</td>
<td>(0.5.5)</td>
</tr>
</tbody>
</table>

* Range.

Table 2. Serum Total Cholesterol and Lipoprotein Cholesterol (mg/liter) in Children as Determined by Two Different Methods in Two Laboratories

<table>
<thead>
<tr>
<th>n = 38</th>
<th>Total cholesterol</th>
<th>β + pre-β-LP cholesterol</th>
<th>α-LP cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSU</td>
<td>Mayo</td>
<td>LSU</td>
</tr>
<tr>
<td>Mean</td>
<td>1620</td>
<td>1610 ᵒ</td>
<td>990</td>
</tr>
<tr>
<td>SD</td>
<td>191</td>
<td>210</td>
<td>148</td>
</tr>
<tr>
<td>SE</td>
<td>31</td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>Range</td>
<td>(1050–1990)</td>
<td>(1050–2080)</td>
<td>(610–1280)</td>
</tr>
</tbody>
</table>

* Differences between the method were statistically not significant (paired t-test, P > .05).

Table 2 gives the values (mean ± SD, SE, and range) for total cholesterol, β + pre-β-lipoprotein cholesterol, and α-lipoprotein cholesterol in 38 children as determined by the LSU and Mayo methods. The means for the two different methods were very similar; by the paired t-test they were not significantly different.

Each child’s total cholesterol as determined by the LSU method vs. the Mayo method is plotted in Figure 1. The correlation coefficient for the two methods was 0.96. Figure 2 compares the values of β + pre-β-lipoprotein cholesterol obtained by both methods (r = 0.91), and Figure 3 compares the α-lipoprotein cholesterol concentration (r = 0.91). These observations indicate a good agreement between two independent methods.

Although β- and pre-β-lipoprotein cholesterol concentrations were determined indirectly by the LSU method, the results were compared with the direct determinations of LDL and VLDL cholesterol by the Mayo method. The mean β-lipoprotein cholesterol (LSU) and LDL cholesterol (Mayo) were very similar (mean ± SD and SE: 89 ± 14, 2.5 and 87 ± 17, 2.9). The correlation coefficient for the two methods was 0.93. While the mean values for the pre-β-lipoprotein cholesterol (LSU) and VLDL cholesterol (Mayo) were very close (mean ± SD and SE: 10 ± 5, 0.9 and 12 ± 7, 1.2), the correlation coefficient was 0.7. Since these values are low and technically more difficult to assay, when the seven outlying values in this group are excluded the correlation coefficient becomes 0.92.

Discussion

Inexpensive and simple methods for quantitating serum lipoproteins for routine clinical use would be extremely useful. Both methods applied here are being used for clinical studies on many individuals, and each has certain advantages and limitations. The LSU method is rapid, requires no expensive equipment, and can be applied to mass (population) studies. Although primarily designed for measuring β + pre-β-lipo-

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![Fig. 1](image1.png)

Fig. 1. Comparison of serum cholesterol concentrations in children, as determined by two methods
Correlation coefficient, r = 0.94

![Fig. 2](image2.png)

Fig. 2. Comparison of β + pre-β-lipoprotein cholesterol concentrations in children, as determined by two methods
Correlation coefficient, r = 0.91
protein cholesterol, it also indirectly measures the individual lipoprotein fractions. The Mayo method was designed to: (a) use small volumes of serum (1–2 ml), (b) quantify lipids in the major lipoprotein fractions by direct measurements, (c) avoid the problems of measuring HDL lipids in diluted serum preparations, and (d) so minimize procedural difficulties that 42 specimens can be processed per day. The conditions specified in this paper provide complete or very nearly complete isolations of the major lipoprotein fractions. This method also allows further electrophoretic characterization of isolated lipoprotein fractions if this is desired.

In measuring serum lipoprotein cholesterol concentrations in children, there was excellent agreement between two methods applied independently by the two different laboratories. Of particular interest is the close agreement on assays for a-lipoprotein cholesterol (a mean value of 620 mg/liter constituting 38.6 of the total cholesterol value). Previous studies indicated that cholesterol derived from a-lipoprotein in children as compared to adults constitutes a relatively greater part of the total cholesterol (12, 15, 16), which was in variance with earlier studies in which a heparin/Mn2+ precipitation method was used to quantify a-lipoprotein cholesterol (13, 14). More recent studies on serum lipoprotein-heparin interactions indicated that Ca2+ or Mg2+ is more specific in precipitation of β- and pre-β-lipoproteins than is Mn2+, which precipitates 40% of the HDL2 and 10% of the HDL3 (7). Recently it has been speculated that perhaps the other serum proteins exert a protective effect, either directly or by utilizing part of the heparin/Mn2+ (23). Addition of serum albumin (80 g/liter) to the isolated HDL fractions produced similar results. Earlier studies on abetalipoproteinemia have indicated that serum treated with heparin/Mn2+ also yields a precipitate containing albumin, γ-globulin, and a-lipoprotein as well as trace amounts of other plasma proteins (24), suggesting a nonspecificity of Mn2+ in protein precipitation. In women taking oral contraceptives the HDL cholesterol as measured by the heparin/Mn2+ method was not found (25) to differ significantly from controls, whereas the values for total HDL and HDL2 measured by analytical ultracentrifuge were higher in treated women. A degree of difference between methods using Ca2+ and Mn2+ is likely to be determined by the nature and quantity of HDL present in sera. Because a precipitation method is often used to differentiate a-lipoprotein cholesterol from β + pre-β-lipoprotein cholesterol, it is important to recognize the cation selectivity and the specificities of the current methods for quantitating lipoproteins.

Despite the close agreement between the two methods under study in measurement of β + pre-β-lipoprotein cholesterol and β-lipoprotein cholesterol, there were differences in the values for pre-β-lipoprotein cholesterol. Because the pre-β-lipoprotein cholesterol concentration, especially in children, is very low, even small absolute errors (or differences) in measurement by either method may yield large proportionate errors (26, 27). Fortunately, this difference is of little practical significance for most clinical studies. Both methods described have considerable clinical usefulness and complement cholesterol and triglyceride analyses and electrophoretic scanning of lipoproteins when these are not quantitated.

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References

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Use of the Du Pont aca to Measure Cholesterol in High-Density Lipoprotein Fractions Prepared by the Heparin/Mn$^{2+}$ Precipitation Method

Raymond J. Liedtke, Barbara Busby, and John D. Batjer

The enzymatic cholesterol method used with the Du Pont aca has been modified to provide a reliable measurement of high-density lipoprotein cholesterol in serum after heparin/Mn$^{2+}$ precipitation of the low- and very-low-density lipoproteins. Interference by Mn$^{2+}$, equivalent to about 90 mg of cholesterol per liter, is decreased to less than 40 mg of cholesterol per liter by the presence of ethylenediaminetetraacetate (8 mmol/liter) in the diluent; the residual effect of Mn$^{2+}$ is compensated by calibrating the aca with standards containing Mn$^{2+}$ and heparin. With an 80-μl sample, the sensitivity is 236 μA/mg per liter and linearity ranges from 50 to 1500 mg/liter. Average analytical recovery of cholesterol added to the high-density lipoprotein fraction was 103%. Diluted fractions give the expected results. Between-run reproducibility (CV) is 1.3 and 1.6% at 463 and 554 mg/liter. Correlation with the Lipid Research Clinics’ procedure (25 samples) gave a regression line of $y(aca) = 1.039x - 15$, and a correlation coefficient of 0.997.

Renewed interest in the quantitation of high-density lipoproteins in serum as measured by HDL cholesterol has resulted from the demonstrated inverse correlation between HDL cholesterol concentrations in serum and cardiovascular disease (1-4). Data from the Framingham study indicate that HDL cholesterol is a major lipid risk factor (3). The Tromsø Heart Study has yielded data suggesting that a low serum HDL cholesterol concentration is a common antecedent of coronary heart disease (4).

Heparin/Mn$^{2+}$ precipitation of LDL and VLDL is the technique commonly used to isolate the HDL fraction (5, 6). Of the direct HDL quantitation methods involving a precipitation step, only this procedure has been subjected to rigorous study, and HDL cholesterol values obtained with this protocol agree well with those obtained by ultracentrifugation (7-9). Other LDL- and VLDL-precipitating agents suggested for the analysis of clinical specimens are phosphotungstic acid/Mg$^{2+}$ (10) and dextran sulfate 500/Mg$^{2+}$, which have been used with enzymatic cholesterol quantitation (11). Heparin/Ca$^{2+}$ has been used in a method that indirectly quantitates HDL cholesterol as the difference between total cholesterol and precipitated LDL plus VLDL cholesterol (12).

Cholesterol in the heparin/Mn$^{2+}$-prepared HDL fraction is usually measured by continuous-flow analysis based on the Liebermann-Burchard reaction, after extracting the serum with isopropanol and treating it with zeolite (6, 9). Steele et

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1. Nonstandard abbreviations used: HDL, high-density lipoprotein; d 1.063 to 1.21 g/ml; LDL, low-density lipoprotein; d 1.006 to 1.063 g/ml; VLDL, very-low-density lipoprotein, d < 1.006 g/ml; EDTA, ethylenediaminetetraacetate; aca, Automatic Clinical Analyzer.