Separation of Serum High-Density Lipoprotein for Cholesterol Determination: Ultracentrifugation vs Precipitation with Sodium Phosphotungstate and Magnesium Chloride

Larry Seigler and Wei T. Wu

We compared two methods for separation of serum high-density lipoprotein: selective precipitation with sodium phosphotungstate and magnesium chloride, and ultracentrifugation in sodium chloride solution (relative density 1.063). When the cholesterol content (determined enzymically with a centrifugal analyzer) of fractions obtained by each method was compared (ultracentrifugation = x), the correlation coefficient was 0.97; y = 1.01 x - 11.2 mg/L; p < 0.05; n = 54. The within-day and between-day coefficients of variation for this method were 1.1 and 4.0%, respectively. Reference intervals for high-density lipoprotein cholesterol in subpopulations categorized by age and sex were based on data obtained from volunteer blood donors.

Additional Keyphrases: reference interval · coronary heart disease

The concentration of high-density lipoprotein cholesterol (HDLC)2 in serum is reported to be inversely related to the incidence of coronary heart disease (1-8). Consequently, the measurement of HDLC has become popular as an adjunct in assessing individual risk to this disease. In the modified procedure recommended by the Lipid Research Clinic Program (9-12) heparin and manganese are used to precipitate very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) selectively, the extent of precipitation being in some dispute (10, 13). The manganese ion interferes with enzymic determination of cholesterol (14, 15), so ethylenediaminetetraacetate (EDTA), a chelator, must be added (15, 16). Moreover, because the precipitating ability of heparin varies with the supplier and the biological source, each new lot of heparin must be standardized to assure maximum precipitation of the apolipoprotein B-containing lipoproteins (14, 17).

Sodium phosphotungstate (NaPT) and magnesium ion have been used as an alternative to heparin-manganese in the selective precipitation of VLDL and LDL (12, 13, 17, 18). The pH of the NaPT solution, however, must be lower than 7.6 for the precipitation of VLDL and LDL to be complete (18).

We have compared the NaPT-Mg2+ procedure with ultracentrifugation in the isolation of HDL, and have found the former to be suitable for routine use by the clinical laboratory in the determination of serum HDLC concentration.

Materials and Methods

Specimens

Blood specimens were collected at Charity Hospital of Louisiana at New Orleans from laboratory personnel and students who had fasted overnight. For serum specimens, blood was collected from an arm vein into 7-mL Vacutainer Tubes (Becton-Dickinson, East Rutherford, NJ 07073) containing no anticoagulant, and was allowed to clot at room temperature. For plasma specimens, blood was collected into 7-mL Vacutainer Tubes containing 10.5 mg of K3EDTA or 143 USP units of sodium heparinate and mixed thoroughly. Cells were separated by centrifugation (1500 x g, 15 min) and the plasma or serum was stored at 4 °C. The assay was done within 48 h.

Specimens for the reference interval study were obtained from the Blood Bank of Charity Hospital of Louisiana at New Orleans.

Reagents

Sodium phosphotungstate, 40 g/L, pH 7.4: Eight grams of phosphotungstate acid (reagent grade; Sigma Chemical Co., St. Louis, MO 63178) was added to 160 mL of water. After addition of 32 mL of 1 mol/L NaOH, the pH of the solution was adjusted to 7.4 with 1 mol/L HCl and it was diluted to 200 mL with water.

Magnesium chloride, 0.5 mol/L: Freshly opened reagent-grade sodium chloride hexahydrate, 10.17 g (Sigma Chemical Co.), was dissolved in deionized water in a volumetric flask and diluted to 100 mL.

Sodium chloride, relative density 1.063 or 1.177: These solutions were prepared by the method of Havel et al. (19) from reagent-grade sodium chloride (Mallinckrodt, Inc., St. Louis, MO 63147).

Cholesterol reagent: Worthington Cholesterol Reagent (cat. no. 27059; Worthington Diagnostics, Freehold, NJ 07728) was reconstituted in 14.0 mL of the Tris buffer supplied with the reagent.

Standards: Monitrol II (Dade, Miami, FL 33152) and Cholesterol Calibrated Reference Sera (Data Medical Associates, Arlington, TX 76011) were used as reference cholesterol standards. After reconstitution according to the supplier's directions, these solutions were diluted fourfold and sixfold, respectively, with isotonic (8.5 g/L) saline. The cholesterol concentrations in these diluted solutions, as calculated from information supplied by the manufacturers, were 612.5 and 617 mg/L, respectively. Preciset® Cholesterol Standard (Boehringer-Mannheim Biochemicals, Inc., Indianapolis, IN 46250), 500, 1000, 1500, 2000, 3000, and 4000 mg/L solutions of cholesterol in methanol/water (10/90 by vol), were used as supplied.

Procedures

Precipitation of VLDL and LDL: The LDL and VLDL
Table 1. Operational Parameters for Quantification of High-Density Lipoprotein Cholesterol on the GEMSAEC Analyzer

<table>
<thead>
<tr>
<th>Vol, μL</th>
<th>Pump, μL</th>
<th>%</th>
<th>Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>24</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>Flush</td>
<td>42</td>
<td>200</td>
<td>21</td>
</tr>
<tr>
<td>Reagent</td>
<td>420</td>
<td>1000</td>
<td>42</td>
</tr>
<tr>
<td>Sample tip: Polypropylene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank switch: Reagent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cuvet no. and contents: 1, water; 2, cholesterol standard; 3, control; 4-16, unknown samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reaction temperature: 30 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength: 500 nm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter position: 430-560 nm</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Reaction mode: Auto/Rate</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IR (initial reading)</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RI (read interval)</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR (number of readings)</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC (standard concn)</td>
<td>* (enter concn of standard)</td>
<td></td>
<td></td>
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</tbody>
</table>

were precipitated with NaPT–Mg²⁺ by a modification of the method of Burstein (20, 21), as follows:

Add 50 μL of 40 g/L NaPT and 50 μL of 0.5 mol/L MgCl₂ sequentially, with thorough vortex-mixing after addition of each reagent, to 500 μL of serum or plasma. Then centrifuge the mixture (1500 × g, 15 min) and promptly remove the supernatant fraction with a Pasteur pipette for cholesterol analysis.

Lipoprotein fractionation by ultracentrifugation: Adjust to relative density 1.063 by adding 2.0 mL of NaCl solution (relative density 1.177) to a cellulose nitrate tube containing 4.0 mL of serum; then centrifuge the tubes (105 000 × g, 5 °C, 72 h). Quantitatively recover the infranat fractions containing the HDL by a tube-slicing technique, adjust the volume of each fraction to 5.0 mL with isotonic saline, and store at 5 °C until analysis for cholesterol. Observed concentrations of cholesterol should be multiplied by 1.25 to compensate for dilution.

Quantitation of HDLC: We set up the centrifugal analyzer (GEMSAEC, Electro-Nucleonics, Fairfield, NJ 07006) as shown in Table 1. A multiple-data printout obtained after each run recorded the final absorbance (at 12 min) of each cuvet.

A blank absorbance value for each sample was obtained by using a separate distribution disc. The procedure for loading this disc was identical to that for loading the assay disc except that the reagent wells were filled with water instead of cholesterol reagent. The control and analyzer module settings were the same as those for the assay except: RI = 5 and NR = 1.

The blank absorbance for each cuvet was obtained from a multiple-data printout after each blank run.

(Note: We used the Auto/Rate mode to obtain absorbance information at 60-s intervals, which can be useful in evaluating the overall performance of the reactions. If this information is not desired, the Auto/End Point reaction mode may be used with the additional alteration: IR = 720 and NR = 1.)

Calculation: The concentration of HDLC in the original serum or plasma specimen was calculated as follows:

\[
\text{HDLC (mg/L)} = \frac{\Delta A_{\text{unknown}}}{\Delta A_{\text{standard}}} \times C_{\text{standard}} \times 1.2
\]

C_{standard} is the concentration of standard used, in mg/L, and 1.2 is the dilution factor.

Results and Discussion

Modified enzymic cholesterol assay: The ratio of specimen volume to total reaction mixture volume for the cholesterol assay was increased from 1:100, as recommended by the reagent manufacturer, to 1:20, (a) to keep the absorbance change of most specimens (those containing 300 to 700 mg of HDLC per liter) within an absorbance range where the most nearly accurate photometric measurements could be made and (b) to reduce the effect of absolute volume errors in pipetting.

Precipitation: Unlike precipitation procedures in which heparin–Mn²⁺ is used (14–16), this NaPT–Mg²⁺ procedure does not produce "pseudocholesterol" turbidity in the presence of enzymic cholesterol reagents. Addition of as much as fourfold excess NaPT and MgCl₂ (compared with the concentrations normally used in this precipitation procedure) to the Preciset 1500 mg/L cholesterol standard caused no more than 0.6% higher cholesterol values than was expected. This implies that NaPT and MgCl₂ neither inhibit nor enhance these enzyme reactions.

Sample blanks: Absorbance values of sample blanks observed at 500 nm in 66 serum supernates ranged from 0.0002 to 0.0430, with a mean of 0.0122. Because this absorbance corresponds to an HDLC concentration of 18 mg/L, we included specimen blanks in the procedure. The absorbance data obtained 26 s after reaction initiation (the earliest possible reading on our GEMSAEC) could not be used for the sample blank absorbances because a significant portion of the reaction had occurred by that time. The separate run we found necessary for obtaining appropriate blank absorbances would not be needed if the GEMSAEC were equipped with early reading capability.

Linearity: To assess the linearity of the modified cholesterol assay procedure, we used volumetric dilutions of a 1150 mg/L cholesterol standard. The results of the assay are linear with concentration up to 1150 mg/L (y = 1.02x - 13.4 mg/L; r = 0.9999; p < 0.01).

Precision: The coefficients of variation obtained on using pooled sera as the reference with this HDLC procedure were: within-run, 0.9% (x = 392 mg/L; SD = 3 mg/L; n = 14); between-run, 1.1% (x = 359 mg/L; SD = 4 mg/L; n = 12); and between-day, 4.0% (x = 364 mg/L; SD = 14 mg/L; n = 31). The pooled sera used for determination of the within-run coefficient of variation differed from those used for the other determinations. Preconditioning of the polypropylene loader sample tip was found to be unnecessary, in contrast to results obtained with a stainless steel tip (22). HDLC concentrations observed for 14 replicate specimens in each of two successive runs were randomly distributed about the mean concentration. No consistent trend was noted from the first to the 28th specimen.

Correlation with ultracentrifugation: The HDLC concentration data obtained after lipoprotein fractionation by ultracentrifugation and by NaPT–Mg²⁺ precipitation are compared in Figure 1. The slope y-intercept, and coefficient of correlation obtained in a study with 54 split serum samples were 1.01, −11.2 mg/L, and 0.97, respectively.

Premixing the NaPT and MgCl₂: Incomplete precipitation of the VLDL and LDL fractions resulted when NaPT and MgCl₂ were mixed before addition to serum or plasma. The order in which the NaPT and MgCl₂ were added, however, had no effect on the precipitation of VLDL and LDL, an observation in agreement with that of Grove (18). We believe that the divalent cation, Mg²⁺, forms a bridge between LDL-phospholipids and the polyanion, phosphotungstate. Premixing the polyanion and the divalent cation leads to the
formation of a stable matrix that will not react with the LDL-phospholipids. Formation of such matrices significantly reduces the concentration of polyanions and divalent cations available for interaction with the lipoproteins. Support for such a mechanism is provided by Bernfield and Kelly (23), who observed that LDL treated with proteolytic enzymes retained its ability to interact with polyanions in the presence of divalent cations. They concluded that the protein moiety of LDL is not essential for the formation of insoluble complexes. Srinivasan et al. (24) observed that LDL precipitation with heparin and Ca\(^{2+}\) is inversely proportional to the degree of LDL-phospholipid hydrolysis by phospholipase C. They concluded that insoluble LDL–divalent cation–polyanion complexes form through the phospholipid moiety of LDL. Presumably, polyanions and divalent cations interact with VLDL and HDL through a similar mechanism.

**Effects of anticoagulants:** In an experiment designed to determine the effects of anticoagulants on HDLC determinations, specimens were collected from 38 volunteers. During a single venipuncture, separate blood samples were drawn from each volunteer into Vacutainer Tubes containing either heparin, EDTA, or no anticoagulant. The mean HDLC concentrations observed in the respective specimens were 465, 457, and 466 mg/L. The differences between the mean observed in serum and those observed in heparinized plasma and EDTA-treated plasma (8 and 9 mg/L, respectively) were within the limits of analytical error associated with the assay.

Linear regression analysis of HDLC values obtained from serum (x) and heparinized plasma (y) yielded a slope, y-intercept, and coefficient of correlation of 1.01, -11.8 mg/L, and 0.99, respectively. The corresponding values for serum and EDTA-treated plasma were similar, being 0.96, -2.3 mg/L, and 0.98, respectively.

**Specimen stability:** Aliquots of pooled serum stored at -20 °C were thawed at intervals and analyzed for HDLC. The mean concentration obtained on eight aliquots stored two months was 0.9% lower than the initial mean, a difference within the limits of analytical error of the assay. Aliquots assayed after storage for four, six, and eight months, however, yielded mean values that were less than the initial mean HDLC value by 8.3% (n = 10), 15.3% (n = 11), and 27.1% (n = 8), respectively.

**Reference intervals:** Serum HDLC concentrations with respect to age and sex were studied on a reference population comprising 210 men and 81 women blood donors at the Blood Bank at Charity Hospital of Louisiana at New Orleans. About two-thirds of the donor population was black. Although the medical histories of the donors were not available, each donor met the health criteria established by the Blood Bank for donation. The distribution of HDLC values was approximately log normal among all subpopulations except among men and women aged 18–25 years, which had an approximately gaussian distribution. Reference ranges for HDLC based on the subpopulations are shown in Table 2. The 95% range is bounded by the HDLC concentrations corresponding to the 2.5th and 97.5th percentiles of the HDLC concentration distribution for that subpopulation. The HDLC concentrations corresponding to the 5th and the 95th percentiles bound each 90% range. The lower limit of each reference range is of greater clinical interest than the upper limit because the incidence of coronary heart disease is inversely proportional to the serum HDLC concentration. Acceptance of the reference range that includes 90% rather than 95% of the reference population reduces the risk of accepting as "normal" a patient whose serum HDLC concentration value is actually "abnormal" (Type II error). The mean serum HDLC concentration observed among men of this population (451 mg/L) was comparable with values reported in studies of other reference populations (18, 22, 25–28). Nevertheless, the mean serum HDLC concentration observed among New Orleans women (468 mg/L) was considerably less than those reported in similar studies, including one in which a NaPT-Mg\(^{2+}\) precipitation procedure was used (18). This observation may reflect the higher incidence of mortality from coronary heart disease among New Orleans residents compared with the national average (29).

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**Table 2. Reference Ranges for Serum High-Density Lipoprotein Cholesterol**

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>n</th>
<th>HDL cholesterol, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>90% range</td>
</tr>
<tr>
<td>Men</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–25</td>
<td>85</td>
<td>300–600</td>
</tr>
<tr>
<td>26–35</td>
<td>49</td>
<td>280–690</td>
</tr>
<tr>
<td>36–45</td>
<td>47</td>
<td>260–660</td>
</tr>
<tr>
<td>46–55</td>
<td>29</td>
<td>240–650</td>
</tr>
<tr>
<td>Women</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–25</td>
<td>28</td>
<td>290–590</td>
</tr>
<tr>
<td>26–35</td>
<td>33</td>
<td>350–690</td>
</tr>
<tr>
<td>36–45</td>
<td>20</td>
<td>280–740</td>
</tr>
</tbody>
</table>

The 90% range includes 90% of the reference population; the 95% range includes 95% of the reference population.

**References**


