Effect of Reagent pH on Determination of High-density Lipoprotein Cholesterol by Precipitation with Sodium Phosphotungstate–Magnesium

Thomas H. Grove

When determining high-density lipoprotein cholesterol by use of sodium phosphotungstate–magnesium precipitation method, I found that the pH of the sodium phosphotungstate reagent was a critical factor in the method. Unless the pH of the reagent was less than 7.6, the precipitation of low-density lipoprotein and very-low-density lipoprotein was incomplete. When the specimen pH was between 7.35 and 8.65, the pH of the serum or plasma did not influence the completeness of precipitation. Optimum concentrations of precipitation reagents, determined after the pH of the sodium phosphotungstate reagent was standardized to pH 6.15, were 40 g/L for sodium phosphotungstate and 2 mol/L for MgCl₂. The distribution of high-density lipoprotein cholesterol in a healthy adult population was skewed to the left for women (n = 34; mean = 660 mg/L) and bi-modal for men (n = 44; mean = 460 mg/mL). The central 95% reference interval was 280 to 880 mg/L for women and 250 to 750 mg/L for men.

Partitioning total cholesterol into specific lipoprotein fractions is becoming increasingly important. In particular, the concentration of high-density lipoprotein (HDL) cholesterol has been shown to strongly reflect the risk of developing coronary heart disease (7–4). Decreased concentrations of HDL cholesterol are associated with increased risk of developing coronary heart disease. Several methods are available to measure HDL cholesterol, including ultracentrifugation analysis (5), radioimmunoassay of apolipoprotein A-I in HDL (6, 7), electrophoresis in various media (8–10), and selective precipitation of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) with polyaniols and divalent cations (11–15). In the precipitation method, HDL cholesterol remains in solution and can be quantitated by measuring the residual cholesterol concentration.

I report the results of a study in which HDL cholesterol was determined by a sodium phosphotungstate–magnesium (NaPT–Mg²⁺) precipitation method (12, 14, 15). In previous publications, the control of pH in the NaPT–Mg²⁺ precipitation method was not addressed. I have found that the pH of the NaPT reagent is a critical factor in the method and that it must be less than 7.6 for the precipitation of LDL and VLDL to be complete. (All pH measurements were made at room temperature.) The resulting pH of specimen plus NaPT reagent is less critical. Failure to precipitate LDL and VLDL completely will lead to an overestimation of HDL cholesterol and the misidentification of clinically relevant low values.

Materials and Methods

Reagents

Analytical grade sodium hydroxide, magnesium chloride, and phosphotungstic acid (n-hydrate), were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Sodium heparin (10 000 USP units/mL in NaCl, 154 mmol per liter) was obtained from Organon Inc., West Orange, NJ 07052. Manganese chloride was obtained from Fisher Scientific Co., Medford, MA 02155. Cholesterol concentrations were determined by a cholesterol esterase/cholesterol oxidase/peroxidase method with Autoflo reagents from Bio-Dynamics/bmc, Indianapolis, IN 46250. The single-vial reagent was reconstituted and used as prescribed by the manufacturer. Lipoprotein electrophoresis with the Redi-Disc polyacrylamide gel system from Ames Co., Elkhart, IN 46514 was performed according to the manufacturer’s instructions. Lipoproteins were prestained before electrophoresis, by incorporating the Sudan Black B dye in the stacking gel.

Procedure

Sodium phosphotungstate–magnesium precipitation of LDL and VLDL was performed as described by Lopes-Virella et al. (15).

To 1 mL of serum or plasma add 25 μL of 2 mol/L MgCl₂ and 100 μL of a solution of phosphotungstic acid (40 g/L) that has been pre-adjusted to the indicated pH with 1 mol/L NaOH. Determine the amount of NaOH to add to the phosphotungstic acid solution by measuring the sodium concentration with an Model 143 or 343 flame photometer (Instrumentation Laboratory, Lexington, MA 02173).

Mix the specimen by vortexing, then centrifuge for 30 min at 1500 × g and 4 °C. Separate the supernatant fluid from the pellet by decanting; use the enzymic cholesterol procedure to determine the residual cholesterol concentration in the clear supernate. Analyses can be done with either an Abbott ABA-100 Bichromatic Analyzer (sample dilution 1:101) or a Gilford System 3500 Analyzer (sample dilution 1:76). Multiply the residual cholesterol concentration by a dilution factor of 1.125 to calculate the HDL cholesterol concentration. Heparin–manganese precipitation was done according to the protocol used by the Lipid Research Clinic Program (16).

To 1 mL of serum add 40 μL of a sodium heparin solution, 5000 USP units/mL, in 154 mmol/L NaCl. Mix the solution
immediately by vortexing, and add 50 μL of 1 mol/L MnCl₂. Vortex-mix and store for 30 min at 4°C. Clarify the suspension by centrifuging for 30 min at 1500 × g, at 4°C. To prevent the manganese from interfering with the enzymic determination of cholesterol (17), add 50 μL of ethylenediaminetetraacetic acid (EDTA, 0.06 mol/L) per 100 μL of supernate. Measure the cholesterol concentration in the EDTA-supplemented supernate with an Abbott ABA-100 Analyzer (sample dilution of 1:101). Multiply the residual cholesterol concentration in the EDTA-supplemented supernate by a dilution factor of 1.635 to calculate the HDL cholesterol.

Results

Effect of Reagent and Specimen pH

Table 1 shows the effect of pH on the determination of HDL cholesterol. Single-donor sera were precipitated with a series of NaPT reagents (40 g/L) that varied in pH. The concentration of the MgCl₂ stock reagent was 2 mol/L in each case. Results were identical when single-donor citrate plasma or fresh serum pooled from 45 individuals was used as the source of lipoproteins. LDL and VLDL were precipitated only when the pH of the NaPT reagent was less than 7.6. The conditions in experiment F are similar to previously published NaPT–Mg²⁺ methods (12, 14, 15). The corresponding lipoprotein electropherograms of the HDL-containing supernates obtained in experiments A–K are shown in Figure 1. The hazy band in the VLDL region in gels B–F was not lipoprotein but an artifact band from dye migration; it was also present in blank gels. The lipoprotein electrophores show that LDL and VLDL are precipitated only when the pH of the NaPT reagent is less than 7.6.

To ascertain whether it was the pH of the NaPT reagent or the resultant pH of the specimen plus NaPT reagent that determined the completeness of LDL and VLDL precipitation, I repeated the protocol followed in Table 1 on pooled specimens with different pH’s. The pH of the specimens was varied by storing freshly pooled plasma or serum for 16–18 hours at either room temperature or at 2–8°C. The results (Table 2) demonstrate that the completeness of precipitation of LDL and VLDL was determined primarily by the pH of the NaPT reagent when the specimen had a pH between 7.35 and 8.65. In all cases, NaPT reagent with a pH of 7.50 or lower precipitated LDL and VLDL, but NaPT reagent with a pH of 7.81 or higher precipitated little or no LDL and VLDL.

Because NaOH was used to adjust the pH of phosphotungstic acid, the sodium concentration of the various NaPT reagents differed. To determine whether the sodium content of the NaPT reagent influenced LDL and VLDL precipitation, I fortified serum with NaCl. The pH 6.15 NaPT reagent completely precipitated LDL and VLDL, even when the final sodium concentration was 230 mmol/L. This sodium concentration was greater than when the pH 12.35 NaPT reagent was used on unfortified serum (sodium = 224 mmol/L).

Selection of NaPT Reagent

The mean concentration of HDL cholesterol and the within-run precision of the method were determined by using a series of NaPT reagents with pH values ranging from 1.57 to 7.53. The lipoprotein source was a human serum pool composed of samples from several thousand blood donors (Pool A) (18). The serum pool was stored before use at −20°C for four to five months (19, 20). The mean concentrations of HDL cholesterol determined by this method were very similar (Table 3), with values ranging from 470 to 490 mg/L. The coefficients of variation were also similar and ranged from 1.8 to 2.4%.

I selected the pH 6.15 NaPT reagent for routine use in determining HDL cholesterol because the reagent (a) is close to neutrality, (b) does not precipitate other serum proteins, and (c) allows a convenient range of pH for the successful precipitation of LDL and VLDL. However, any other NaPT reagents can be used in this method if pH 6.15 is not suitable.

Figure 1. Representative polyacrylamide gels of lipoproteins in the supernates of single-donor sera treated with sodium phosphotungstate reagents with various pH values

Gels labeled A–K correspond to experiments A–K described in Table 1. High-density lipoprotein and albumin migrate to the same vicinity.
agent within the pH range of 1.5 to 7.6 could also be used if it was first properly evaluated.

I determined the optimum concentrations of NaPT (pH 6.15) and MgCl₂ stock reagents by first varying the concentration of MgCl₂ at a fixed concentration (40 g/L) of NaPT, pH 6.15, and second varying the concentration of NaPT, pH 6.15, at a fixed concentration (2 mol/L) of MgCl₂ (Figure 2). The lipoprotein source was human serum Pool A. The reagent concentrations selected from Figure 2 for routine use were 40 g/L NaPT (pH 6.15) and 2 mol/L MgCl₂, concentrations in agreement with those selected by Burstein et al. (12), who used a pH 7.6 NaPT reagent.

Electrophoretic Evaluation of Method

Eight serum samples with normal lipoprotein phenotypes (20) were selected without conscious bias. The present pH 6.15 NaPT–Mg²⁺ method was used to precipitate lipoproteins, and the resulting suspensions were clarified by centrifugation. The supernates containing HDL were separated from the pellets by decanting. Lipoprotein electrophoresis of the supernates showed no LDL or VLDL present. Thus the precipitation of LDL and VLDL was judged to be complete. The pellets were rinsed several times with a NaCl solution (154 m mol/L) and each pellet was suspended in 0.2 mL of the NaCl solution. Twenty-five microliters of a pellet suspension were dissolved in polyacrylamide stacking gel before photopolymerization was started. The lipoprotein electrophoretic pattern of the pellets had dense LDL and VLDL bands and no HDL band.

Reproducibility with the pH 6.15 NaPT Reagent

The within-run precision of the method was estimated from 20 replicate determinations of HDL cholesterol in human serum Pool A. The mean concentration of HDL cholesterol was 490 mg/L. The coefficient of variation was 2.1% and the standard deviation was ±10 mg/L.

The between-run precision of the method over a period of one month was evaluated by determining the HDL cholesterol concentration in human serum Pool A, apportioned into 10-mL tubes and stored at −20 °C. A separate tube of serum was thawed each day for 20 consecutive working days and analyzed for HDL cholesterol. The mean concentration of HDL cholesterol was 530 mg/L. The coefficient of variation was 3.6% and the standard deviation was ±20 mg/L.

Comparison with Heparin–Mn²⁺ Method

The concentration of HDL cholesterol was determined in 32 sera by using the present pH 6.15 NaPT–Mg²⁺ method and the heparin–Mn²⁺ method of the Lipid Research Clinic (16). None of the samples was lipemic. The results are shown in Figure 3. HDL cholesterol determined by the pH 6.15 NaPT–Mg²⁺ method averaged 8.6% lower than that deter-

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**Table 3. Within-run Precision for HDL Cholesterol Determined by Using Sodium Phosphotungstate Reagent of Various pH's**

<table>
<thead>
<tr>
<th>NaPT reagent pH</th>
<th>n</th>
<th>HDL cholesterol (mg/L) Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.57 a</td>
<td>10</td>
<td>450</td>
<td>12</td>
<td>2.4</td>
</tr>
<tr>
<td>2.70</td>
<td>10</td>
<td>470</td>
<td>10</td>
<td>2.0</td>
</tr>
<tr>
<td>4.53</td>
<td>10</td>
<td>470</td>
<td>9</td>
<td>1.8</td>
</tr>
<tr>
<td>6.15</td>
<td>10</td>
<td>490</td>
<td>11</td>
<td>2.3</td>
</tr>
<tr>
<td>7.53</td>
<td>10</td>
<td>470</td>
<td>9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*a* Phosphotungstic acid pH; no NaOH added to the reagent.

*b* No. specimens.
mained by the heparin–Mn²⁺ method. The mean concentration of HDL cholesterol in the sera was 530 mg/L by the pH 6.15 NaPT–Mg²⁺ method and 580 mg/L by the heparin–Mn²⁺ method. The correlation coefficient was 0.974. The equation of the regression line was $y = 1.012x + 5.3$ where $y$ is the results obtained by the pH 6.15 NaPT–Mg²⁺ method and $x$ is the results obtained by the heparin–Mn²⁺ method.

Six pairs of samples were selected without conscious bias from the 32 pairs used in the comparison study. The results of lipoprotein electrophoresis on these pairs are shown in Figure 4. For each pair of samples, the pH 6.15 NaPT–Mg²⁺ procedure completely precipitated LDL and VLDL, whereas the heparin–Mn²⁺ procedure failed to completely precipitate VLDL. The higher mean concentration of HDL cholesterol determined by the heparin–Mn²⁺ method was probably the result of the incomplete precipitation of VLDL.

Reference Interval

Serum samples were obtained from 78 healthy non-fasting, white adult residents from central Connecticut (34 women, 44 men), who had a variety of employment backgrounds. The mean age of the men was 35 years (range, 20 to 58), that of the women 32 years (range, 18 to 58). Individuals were not included in the study if they were on any medication (including oral contraceptives) or if their serum was lipemic. The HDL cholesterol concentration, as determined by the pH 6.15 NaPT–Mg²⁺ method, gave a mean concentration for men of 460 mg/L (SD ±130; range = 240 to 740) and for women, 660 mg/L (SD ±150; range = 240 to 890). The reference interval for HDL cholesterol, as determined by the pH 6.15 NaPT–Mg²⁺ method, compared very well with the reference intervals determined by other precipitation methods (Table 4).

The histograms of HDL cholesterol concentrations demonstrated a distribution skewed to the left for women and a bi-modal distribution for men (Figure 5). This sex-related

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**Table 4. Reference Intervals for HDL Cholesterol Determined by Precipitation Methods**

<table>
<thead>
<tr>
<th>Location</th>
<th>No. subjects</th>
<th>Ages</th>
<th>HDL cholesterol, mg/L (mean ± SD)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Connecticut</td>
<td>44</td>
<td>20-58</td>
<td>460 ± 130</td>
<td>NaPT–Mg²⁺</td>
<td>This study</td>
</tr>
<tr>
<td>Males</td>
<td>57</td>
<td>20-69</td>
<td>560 ± 120</td>
<td>Dextran S–Ca²⁺</td>
<td>25</td>
</tr>
<tr>
<td>Females</td>
<td>42</td>
<td>20-69</td>
<td>700 ± 220</td>
<td>Dextran S–Ca²⁺</td>
<td>25</td>
</tr>
<tr>
<td>London, England</td>
<td>67</td>
<td>40-49</td>
<td>490 ± 100</td>
<td>Heparin–Mn²⁺</td>
<td>26</td>
</tr>
<tr>
<td>Males</td>
<td>44</td>
<td>40-49</td>
<td>620 ± 140</td>
<td>Heparin–Mn²⁺</td>
<td>26</td>
</tr>
<tr>
<td>Females</td>
<td>506</td>
<td>50-59</td>
<td>450</td>
<td>Heparin–Mn²⁺</td>
<td>27</td>
</tr>
<tr>
<td>Bethesda, MD</td>
<td>614</td>
<td>50-59</td>
<td>590</td>
<td>Heparin–Mn²⁺</td>
<td>27</td>
</tr>
<tr>
<td>Framingham, MA</td>
<td>25</td>
<td>50-59</td>
<td>480</td>
<td>Heparin–Mn²⁺</td>
<td>27</td>
</tr>
<tr>
<td>Evans County, GA</td>
<td>27</td>
<td>50-59</td>
<td>640</td>
<td>Heparin–Mn²⁺</td>
<td>27</td>
</tr>
</tbody>
</table>

* NaPT–Mg²⁺ = sodium phosphotungstate, pH 6.15, magnesium precipitation; dextran S–Ca²⁺ = dextran sulfate–calcium precipitation; heparin–Mn²⁺ = heparin–manganese precipitation.
difference in the distribution of HDL cholesterol was expected, for the risk of developing atherosclerotic disease is sixfold greater in men than in women. The central 95% reference interval was determined by the nonparametric method of Herrera (22). Outliers were rejected if the difference between an outlier and the next value were greater than one-third of the range (23). By this criterion, no outliers were found. The central 95% reference interval for men was 250 to 700 mg/L and for women was 220 to 880 mg/L.

**Discussion**

I undertook the characterization of LDL and VLDL precipitation by NaPT–Mg2+ to optimize the conditions necessary for the accurate assessment of HDL cholesterol. HDL cholesterol concentrations are usually quite low and are masked by LDL and VLDL cholesterol concentrations. The correct concentration of HDL cholesterol can be determined only by using a specific and sensitive method. Although ultracentrifugal analysis of serum or plasma is the most accurate method for determination of HDL cholesterol, it has several drawbacks; for instance, it requires a high-priced instrument, is difficult to perform, and has a low through-put. Estimation of HDL cholesterol by precipitation methods is more suitable for routine use by a clinical laboratory. These methods require no elaborate equipment or special technique, the cost per test is low, and many samples can be quickly processed.

Precipitation of LDL and VLDL with heparin–manganese, heparin–magnesium or –calcium, dextran sulfate–magnesium or –calcium, or sodium phosphotungstate–magnesium each gives HDL cholesterol concentrations comparable to those obtained by ultracentrifugation analysis (13, 15, 24). Although the precipitation of LDL and VLDL with heparin–manganese is the most widely used procedure, precipitation with sodium phosphotungstate–magnesium has several advantages. First, the NaPT–Mg2+ reagents are stable and commonly found in the clinical chemistry laboratory. Second, because not all heparin preparations are equally effective in precipitating LDL and VLDL, the completeness of precipitation must be checked before each new lot of reagent is used (13). Third, manganese reacts with enzymatic cholesterol reagents and leads to an overestimation of the cholesterol concentration (17).

Just why the pH of the NaPT reagent is important, as I have reported here, is not known. Perhaps phosphotungstic acid must be in a certain state of ionization before it binds to LDL and VLDL. The titration curve of phosphotungstic acid had three sets of ionizing groups with pKa values of 2.3, 7.7, and 12.1 (data not shown).

Precipitation of LDL and VLDL by the pH 6.15 NaPT and MgCl2 reagents was immediate and complete. HDL cholesterol concentrations determined in samples that were precipitated and centrifuged immediately were identical to those in samples precipitated and then stored 30 min at room temperature before centrifugation (n = 10). The order of addition of pH 6.15 NaPT and MgCl2 reagents also did not influence the result.

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**References**


