High-Density-Lipoprotein Cholesterol in Heparin–MnCl₂ Supernates Determined with the Dow Enzymic Method after Precipitation of Mn²⁺ with HCO₃⁻

Paul S. Bachorik, Robert E. Walker, and Donna G. Virgil

Manganese interferes with enzymic cholesterol methods. In this study, we enzymically measured high-density-lipoprotein (HDL) cholesterol in heparin–Mn²⁺ supernates that had been treated with NaHCO₃ (91 mmol/L) to precipitate Mn²⁺, and compared results with those by an automated Liebermann–Burchard method. For untreated supernates of 96 fresh plasma samples, the enzymic values were 10.4% higher than comparison-method values, a bias that declined to +2.3% for treated supernates. For 72 sera promptly frozen and stored after collection, the enzymic values for untreated and treated supernates were, respectively, 6.0% and 0.5% higher than comparison-method values. In all cases, the magnitude of the bias was independent of the concentrations of cholesterol, triglyceride, and HDL-cholesterol. Enzymic HDL-cholesterol measurements in NaHCO₃-treated heparin–Mn²⁺ supernates prepared from four pooled serum controls agreed within 21 mg/L with values established for these pools by the Centers for Disease Control. We conclude that the accuracy of enzymic HDL-cholesterol measurements in heparin–Mn²⁺ supernates is considerably increased by treatment with NaHCO₃.

Additional Keyphrases: plasma vs serum - variation, source of - triglycerides

A decrease in the concentration of high-density lipoproteins (HDL) in plasma is associated with increased risk for ischemic heart disease (1–3). Thus, measurement of plasma HDL has become important. The HDL concentration, generally expressed in terms of HDL-cholesterol, is usually measured in plasma after other lipoproteins have been precipitated with a polyamion–divalent-cation combination. The heparin–MnCl₂ procedure of Burstein and Samaille (4) has been the most widely used and extensively studied of the precipitation methods, and much of the data relating HDL-cholesterol concentration with cardiovascular risk have been obtained with this method (reviewed in ref. 5). The heparin–MnCl₂ method gives HDL-cholesterol values that agree quite well with those determined in HDL fractions that were prepared with the ultracentrifuge (6, 7).

Earlier, cholesterol was measured chemically in heparin–MnCl₂ supernates and the precipitants did not interfere. However, the chemical methods are now being replaced by completely enzymic methods for cholesterol, because of their accuracy, precision, and convenience. Because MnCl₂ interferes positively with the enzymic analysis of HDL-cholesterol in heparin–MnCl₂ supernates (8–10), various workers have tried using other noninterfering precipitating agents such as dextran sulfate (11), phosphotungstate (12), and polyethylene glycol (13) to remove apolipoprotein (apo) B-containing lipoproteins.

Because different precipitating agents can lead to results for HDL-cholesterol that are as much as 5–10% higher or lower than those obtained with the heparin–MnCl₂ method (14), we considered it desirable, when we converted to an enzymic cholesterol method, to retain the heparin–MnCl₂ precipitation method if possible. We examined the enzymic measurement of HDL-cholesterol directly in heparin–MnCl₂ supernates and in supernates from which Mn²⁺ was first removed by precipitation with NaHCO₃. We observed a positive bias for enzymic HDL-cholesterol values in heparin–MnCl₂ supernates compared with those determined with the chemical method used by the Lipid Research Clinics Program, but this bias was diminished considerably by NaHCO₃ treatment.

Materials and Methods

Fresh plasma samples. The subjects were 96 patients, sampled during routinely scheduled visits to the Johns Hopkins Lipid Referral Clinic. All subjects had fasted for at least 12 h. Of the 96 subjects, 54 had lipoprotein concentrations outside the age- and sex-adjusted 5 to 95 percentile limits established in the Lipid Research Clinics Program Prevalence Studies (15). Of these, 51 had types IIa (32), IIb (3), III (1), IV (11), V (1), or hyperalpha (3) lipoprotein patterns. Three subjects had low-density lipoprotein (LDL)-cholesterol concentrations below the 5th percentiles.

Blood, collected with disodium EDTA (final concentration 1.5 g/L) as anticoagulant, was cooled and transported to the laboratory in an ice bath. Cells were removed within 3 h of venipuncture. The HDL-containing fractions, prepared without delay by the heparin–MnCl₂ precipitation method (see below), were stored at 4 °C until analysis. We analyzed samples in groups of 10 to 15 over a three-month period.

Fresh-frozen serum samples. These samples were from 72 volunteers who were participating in a survey, a randomly selected sample of free-living (nonhospitalized) Hispanics who reside in the United States. Thirty of the 72 samples were from subjects who had fasted for at least 12 h before venipuncture. Aliquots of serum were promptly frozen, shipped to the laboratory on solid CO₂, and stored frozen for two to five days before they were thawed and analyzed. Lipid and lipoprotein concentrations of 43 of the samples were within the age- and sex-specific 5th to 95th percentiles (15). Of the remaining 29 samples, the lipoprotein patterns...
were type IIa in two, type IV in 13, and "above-normal" HDL in five. Nine samples had values for LDL below the 5th percentile. Samples were analyzed in groups of 10 to 15 over a two-month period.

Control sera. For standardization and quality control, we used specimens of pooled sera provided by the Clinical Chemistry Standardization Section, Centers for Disease Control (CDC), Atlanta, GA 30333. We used two kinds of control pools. The first, prepared with low concentrations of total cholesterol, was used to control cholesterol measurements in the HDL-cholesterol concentration range (250 to 750 mg/L). We used the second to prepare heparin-MnCl₂ supernates, which we analyzed as described below. The cholesterol concentrations of the control sera were determined by the CDC by a reference method based on the procedure of Abell et al. (16).

Lipid and lipoprotein determinations. We measured cholesterol colorimetrically (Liebermann–Burchard reaction) and triglycerides fluorometrically according to the methods of the Lipid Research Clinics Program (17), using the AutoAnalyzer II (Technicon Instruments, Tarrytown, NY 10591) for both analyses.

We prepared HDL-containing fractions by using heparin-MnCl₂ as described previously (17), with a MnCl₂ concentration of 46 mmol/L. For fresh plasma samples we prepared the HDL fractions on the day the blood was drawn, and for frozen serum samples and control pools we prepared the fractions as soon as the samples were thawed and mixed. Cholesterol in the heparin–MnCl₂ supernate was measured with the Lipid Research Clinics method, the comparison method in this study. Aliquots of the heparin–MnCl₂ supernates were also analyzed enzymically (Cholesterol Reagent Set, cat. no. 46650; Dow Chemical Co., Indianapolis, IN 46208) in a reaction system that contained 10 μL of sample and 1000 μL of enzyme reagent. Each sample was analyzed once with each method. Both the comparison and the enzymic cholesterol methods were standardized according to criteria developed for the Lipid Research Clinics Program (18).

Treatment of heparin–MnCl₂ supernates with NaHCO₃. To clear heparin–MnCl₂ supernates, we added 1.0 mol/L NaHCO₃ at one-tenth the volume of the supernate, allowed the mixture to stand at room temperature for 30 min, and sedimented the precipitate by centrifuging for 5 min at 10,000 × g. After removing the clear supernate from the tightly packed precipitate, we measured its cholesterol content enzymically.

Results

When we added the heparin–MnCl₂ supernate to the enzyme reagent, we noticed a wisp of turbidity that disappeared upon mixing. The source of the turbidity was the enzyme reagent buffer, because we also saw it when the enzymes were omitted from the system. We performed several experiments to establish the conditions for NaHCO₃ treatment. First, we treated aliquots of the heparin–MnCl₂ supernate with NaHCO₃ (final concentration, 0 to 91 mmol/L). We also added these same concentrations of NaHCO₃ directly to plasma. A precipitate formed immediately in the heparin–MnCl₂ supernate, but not in the plasma.

After removing the precipitates from the heparin–MnCl₂ supernates, we added 100 μl of each supernate to 900 μl of the enzymic reagent buffer. In the control experiment we substituted NaHCO₃-treated plasma for NaHCO₃-treated heparin–Mn²⁺ supernate. After letting them stand for 15 min at room temperature, we mixed the samples and measured their absorbance at 830 nm as an index of turbidity. Untreated heparin–Mn²⁺ supernate produced a marked turbidity (A830nm = 0.750). Turbidity was reduced 10-fold by the addition of 35 mmol of NaHCO₃ per liter, and at 91 mmol/L the absorbance was 0.022, compared with 0.017 in the NaHCO₃-treated plasma controls. The NaHCO₃ did not interfere with the enzymic determination of cholesterol.

We also examined the time course of formation of the Mn(HCO₃)₂ precipitate after addition of 91 mmol/L NaHCO₃ reagent. After 2 to 30 min, the precipitates were collected, washed once with distilled H₂O, lyophilized, and weighed. Precipitation was complete within 20 to 30 min.

Analysis of HDL-cholesterol in plasma. Heparin–MnCl₂ supernates were prepared from 96 fresh plasma samples. We measured HDL-cholesterol in untreated supernates with the enzymic and comparison methods and in NaHCO₃-treated supernates with the enzymic method. The enzymic values in untreated supernates were 10.4% higher than values obtained with the comparison method (Table 1). The linear regression equation relating the two methods was: HDLcomp. = 0.966 (HDLcomparison) + 69.2 (r = 0.902). This equation predicts an almost constant bias (60.7 to 43.7 mg/L) over the concentration range 250–750 mg/L, and there was no significant relation between the magnitude of bias and the concentration of plasma cholesterol or triglyceride.

In the NaHCO₃-treated supernates, the enzymically determined values averaged 2.3% higher than the comparison-method values, the regression equation being: HDLcomp. = 0.970 (HDLcomparison) + 27.0 (r = 0.944). That is, there was a predicted positive bias of 19.5 to 4.5 mg/L over the HDL cholesterol concentration range 250–750 mg/L. Again, there was no relation between bias and cholesterol or triglyceride concentration.

Analyses of HDL-cholesterol in fresh-frozen serum. We carried out a similar comparison in 72 fresh-frozen serum samples. The enzymic values in untreated supernates averaged 6.0% higher than the comparison method values (Table 1). NaHCO₃ treatment reduced the positive bias to 0.5%. The regression equation for untreated samples was HDLcomp. = 1.031 (HDLcomparison) + 16.7 (r = 0.960), and that for NaHCO₃-treated samples was HDLcomp. = 0.956 (HDLcomparison) + 4.7 (r = 0.964). These equations predicted positive biases of 24.3 to 39.6 mg/L over the concentration range 250–750 mg/L in untreated supernates, as compared with 4.0 to 2.0 mg/L in NaHCO₃-treated supernates. There was no relation between bias and cholesterol or triglyceride concentration in either case.

<table>
<thead>
<tr>
<th>n</th>
<th>Comparison method</th>
<th>Enzymic method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh plasma</td>
<td>Untreated</td>
</tr>
<tr>
<td>96</td>
<td>509.2 (138.4)</td>
<td>562.4* (149.4)</td>
</tr>
<tr>
<td>72</td>
<td>563.3 (137.0)</td>
<td>597.2* (147.1)</td>
</tr>
</tbody>
</table>

* Lipid content, mean (and SD), mg/L: plasma, total cholesterol 2521 (655), triglycerides, 1426 (776); serum, total cholesterol, 1975 (414), triglycerides, 1361 (562).

* Significantly different from results by the comparison method at p < 0.001 or * p < 0.05 (by Student’s paired t-test).

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Table 2. Enzymic Analysis of HDL-Cholesterol in Untreated and NaHCO₃-Treated Heparin–Mn²⁺ Supernates of Three Control Pools

<table>
<thead>
<tr>
<th>Reference value, mg/L</th>
<th>No. of analyses</th>
<th>Untreated, mean (SD)</th>
<th>Treated, mean (SD)</th>
<th>Δenz-ref (%)</th>
<th>Δenz-ref (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>265</td>
<td>4</td>
<td>273 (13)</td>
<td>255 (13)</td>
<td>+ 8 (+3.0)</td>
<td>-10 (-3.8)</td>
</tr>
<tr>
<td>351</td>
<td>3</td>
<td>383 (42)</td>
<td>347 (21)</td>
<td>+32 (+9.1)</td>
<td>- 4 (-1.1)</td>
</tr>
<tr>
<td>630</td>
<td>4</td>
<td>676 (13)</td>
<td>638 (17)</td>
<td>+45 (+7.1)</td>
<td>+ 8 (+1.3)</td>
</tr>
</tbody>
</table>

*Enzymic measurements were made directly in heparin–Mn²⁺ supernates.  
*bThe heparin–Mn²⁺ supernates were treated with NaHCO₃ before analysis.

Table 3. Standardization of Enzymic HDL-Cholesterol Analyses

<table>
<thead>
<tr>
<th>Reference</th>
<th>Acceptable accuracy</th>
<th>Acceptable SD</th>
<th>Enzymic value, mean (SD)</th>
<th>Δenz-ref, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>265 ± 25</td>
<td>25</td>
<td>263 (13.1)</td>
<td>-0.8%</td>
</tr>
<tr>
<td>2</td>
<td>351 ± 25</td>
<td>25</td>
<td>339 (12.6)</td>
<td>-3.4%</td>
</tr>
<tr>
<td>3</td>
<td>507 ± 30</td>
<td>30</td>
<td>486 (17.2)</td>
<td>-4.1%</td>
</tr>
</tbody>
</table>

*Poools 1–3 were the same in both phases. Reference values were known to the laboratory at the time of analysis for Phase 1 but not for Phase 2.

Laboratory-determined mean had to approximate reference values within these limits.

Laboratory-determined SD had to be within these limits.

Analyses of NaHCO₃-treated heparin–MnCl₂ supernates: 18 aliquots of each pool analyzed in six runs (Phase 1) or eight runs (Phase 2). Each phase was completed during a four-week period.

Analysis of HDL-cholesterol in frozen control-serum pools. We analyzed three serum pools with the enzymic method on two occasions. The HDL-cholesterol values in untreated supernates were 3 to 9% higher than the CDC values, but the values in NaHCO₃-treated supernates were within 10 mg/L of the CDC values (Table 2).

Table 3 shows the results of analyses we performed in the CDC standardization program for HDL-cholesterol. The enzymic values were within 2 to 21 mg/L of CDC values, and the bias exceeded 12 mg/L in only one case. Accuracy and precision were well within standardization limits (Table 3). The estimates of bias were fairly reliable because each pool was analyzed 18 times over a four-week period.

Discussion

The higher enzymic HDL-cholesterol values we found for the heparin–Mn²⁺ supernates agreed with the observations of others (8, 10). Mn²⁺ was precipitated by a component of the enzyme reagent buffers, perhaps phosphate (8). The resulting turbidity did not entirely account for the interference, however, because the effect was not eliminated by subtracting appropriate blanks (8, 10); moreover, Steele et al. (8) have suggested that Mn²⁺ may interfere with color development. Our data are consistent with this possibility because the bias was virtually constant over the HDL-cholesterol concentration range, and was independent of triglyceride concentration. Steele et al. (8) eliminated the interference by including EDTA (4 mM n/dL) in the enzymic reaction system to chelate Mn²⁺. Demacker et al. (10) were unsuccessful with this procedure and found that EDTA itself interfered with the analysis. The conflicting results might have resulted from the use of different enzymic methods in the two studies or from the 2.5-fold higher EDTA concentrations Demacker et al. used (10). We found a slightly greater positive bias with respect to the comparison method in plasma than in serum; the bias in plasma was not entirely eliminated by NaHCO₃ as it was in serum. This may perhaps be due to the EDTA in the plasma, but the EDTA concentration in these assays would have been about 0.4% of that which interfered with the cholesterol analysis (10). In any event, the values in the serum pools agreed closely with CDC values, another indication that the measurements in serum were more nearly accurate than those in plasma.

HDL-cholesterol analysis consists of two steps, each with inherent sources of error: first, the HDL-containing fraction must be prepared; second, its cholesterol concentration must be measured. Of the various precipitants used to prepare HDL fractions, some do not interfere with enzymic cholesterol analysis, but all differ somewhat in their precipitating properties and do not give the same results (7, 10). The use of the two-stage precipitation procedure allowed us to retain the heparin–MnCl₂ method and confined the possible sources of bias to the cholesterol method.

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References

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