Falsely High High-Density Lipoprotein Triglyceride Values by the Heparin–Manganese Precipitation Method

I. Eva Simo, Zelma Klaas, and Teik C. Ool

Recent evidence indicates that high-density lipoprotein triglyceride (HDL-Tg) may be a predictor of coronary artery disease. We examined three methods for HDL-Tg measurement, comparing results obtained by measurement of Tg in the supernate after heparin–manganese chloride (heparin–Mn) precipitation of EDTA-treated plasma (I) with results obtained after preparative ultracentrifugation (II and III). In II, we used heparin–Mn precipitation of low-density lipoprotein (LDL) from the infranate after ultracentrifugation at d 1.006 to remove very-low-density lipoprotein (VLDL). In III, we performed sequential flotation ultracentrifugation at d 1.006 and 1.063, then measured Tg in the d >1.063 fraction. Method I gave significantly higher HDL-Tg results than II and III, which gave essentially identical results. The difference in results between I and II was not caused by the presence of heparin or manganese chloride, because these were used in both methods. Prior removal of VLDL in II and III resulted in lower HDL-Tg values, and subsequent removal of LDL by precipitation or ultracentrifugation did not alter final HDL-Tg values. The higher values obtained in I were the result of the presence of VLDL-rich unsedimented precipitate in the supernate.

Numerous lipid variables have predictive value in coronary artery disease, including total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, apolipoprotein A-I (apo A-I), and apolipoprotein B (apo B) in serum. The value of HDL-triglyceride (HDL-Tg) was shown in a study in which myocardial infarction survivors had a significantly higher concentration of HDL-Tg than did healthy controls (1). Also, decreases in HDL-Tg as a result of medical treatment were shown to correlate with regression of atheroma (2).

In view of the recent interest in HDL-Tg, a convenient method is needed for isolating HDL to quantify its Tg component. The most widely performed technique for isolating HDL in the clinical laboratory is precipitation of apo B-containing lipoproteins with sulfated polyanions and divalent cation, heparin and manganese being commonly used as the precipitation agents (3). This precipitation is followed by low-speed centrifugation, which isolates HDL in the supernate. Incomplete sedimentation of the precipitate will lead to overestimation of HDL. Overestimation is expected in supernates showing obvious turbidity; however, as we previously showed, HDL-cholesterol is slightly overestimated even when the HDL supernate looks nonturbid (4). Because 80–90% of the total Tg in plasma is found in very-low-density lipoprotein (VLDL) and LDL, the presence of these lipoproteins in the supernate would result in overestimation of HDL-Tg. We investigated clear-looking HDL supernates to determine if this is the case.

Ultracentrifugation has been recommended (5) to remove floating precipitates from both turbid and nonturbid supernates. However, ultracentrifugation cannot be used when Tg is to be measured, because they contain glycerol, which interferes in the Tg assay (6). Thus we used preparative ultracentrifugation as the comparison method for determining HDL-Tg. Our results show that HDL-Tg measured in clear heparin–Mn supernates is overestimated.

Materials and Methods

Specimen Selection and Collection

Blood was collected in EDTA-containing Vacutainer Tubes (Becton Dickinson Canada Inc., Mississauga, Ont., Canada) and the plasma was separated within 15–30 min and stored at 4 °C. We selected 30 specimens for this study, all of which gave visibly clear supernates after heparin–Mn precipitation. Specimens that gave turbid supernates were not selected for the study because they had unsedimented precipitates and would be expected to have factitiously high concentrations of triglycerides and cholesterol. The Tg concentrations in 19 specimens were <2.75 mmol/L (mean 1.66, range 0.55–2.69 mmol/L) and in 11 were >2.75 mmol/L (mean 3.93, range 2.80–5.96 mmol/L). The mean concentrations of triglyceride and cholesterol for the whole group were 2.43 and 6.84 mmol/L, respectively.

Lipid and Apolipoprotein Measurement

We measured the Tg content of plasma and of separated lipoprotein fractions by an enzymatic colorimetric method, "GPO-PAP" (Boehringer-Mannheim Canada, Dorval, PQ Canada). For HDL-Tg measurement we used 0.1-mL samples; we measured the absorbance against distilled water and used a reagent-blank correction in the calculations. The intra-assay CV of 10 triglyceride measurements was 2.3% at 0.23 mmol/L, the interassay CV was 4.1% at 0.20 mmol/L (n = 10). Cholesterol was measured by an enzymatic colorimetric method (CHOD-PAP "high-performance reagent"; Boehringer-Mannheim Canada). The intra-assay CV of cholesterol measurement was 4.06 mmol/L was 0.5% (n = 10), the interassay CV was 1.33% at 4.75 mmol/L (n = 10). The intra-assay CV for the heparin–Mn precipitation followed by cholesterol measurement was 0.9% at 1.24 mmol/L (n = 10), the interassay CV was 1.91% at 0.96 mmol/L (n = 10).

Apo A-I was measured with an RIA kit (Ventrex Laboratories Inc, Portland, ME), in which a monoclonal antibody is used. The intra-assay CV was 1.6% (n = 12) and the interassay CV was 3.4% at 1.07 g/L (n = 12).

Isolation of HDL

We used three methods for isolating HDL.

Method I. LDL and VLDL were precipitated from total plasma with heparin–Mn according to the method of War-

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Metabolism Laboratory, Division of Endocrinology & Metabolism, Ottawa Civic Hospital, University of Ottawa, 1053 Carling Avenue, Ottawa, Ontario, Canada K1Y 4E9.

1 Nonstandard abbreviations: HDL, LDL, VLDL, high-, low-, and very-low-density lipoproteins, respectively; apo, apolipoprotein; Tg, triglyceride(s); C, cholesterol; ANOVA, analysis of variance; d, relative (to water) density.

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nick and Albers (7) with MnCl$_2$ at a final concentration of 92 mmol/L. Cholesterol and triglycerides measured in the supernate were reported as HDL-C and HDL-Tg.

Method 2. VLDL was removed from total plasma by ultracentrifugation according to the Lipid Research Clinics manual (9). Plasma was ultracentrifuged at $d \approx 1.006$ for 18 h at 10 $^\circ$C and 105,000 x g in a Model L8 60M ultracentrifuge with a 50T rotor (Beckman Instruments, Inc., Palo Alto, CA). The separated lipoprotein fractions were recovered after tube slicing. We precipitated an aliquot of the infrate (containing LDL and HDL) with heparin–Mn, to remove LDL. After separating the precipitated LDL by low-speed centrifugation, we determined the cholesterol and triglyceride content of the HDL-containing supernate.

Method 3. HDL was isolated by sequential flotation ultracentrifugation. The density of the infrate from the first ultracentrifugation (Method 2) was adjusted to 1.063 kg/L with sodium bromide, and the specimen was recenterfuged for 20 h at 15 $^\circ$C and 105,000 x g (7). Cholesterol and triglycerides were measured in the $d > 1.063$ infrate.

All fractions from each specimen derived from all three methods were stored at 4 $^\circ$C until both centrifugations were completed in Method 3 (two days after the blood was drawn), so that all cholesterol and triglyceride assays could be performed in the same batch.

Analytical Recovery for the Ultracentrifugation Procedure

We assessed analytical recovery by measuring cholesterol in the separated lipoprotein fractions, then compared the sum with the total cholesterol content of the original plasma. Mean recovery ($n = 39$) of cholesterol for plasma specimens was 100% (SD 2.6%) after one centrifugation ($d = 1.006$) and 96.4% (SD 3.4%) after two (at $d = 1.006$ and 1.063).

Statistical Analysis

Results are expressed as mean ± SD. For all data analysis we used the Statistical Analysis System (SAS, Cary, NC). One-way analysis of variance (ANOVA) with an alpha significance level set at 0.05 and linear regression analysis were used.

Results

Figure 1 depicts the Tg content of the HDL fraction isolated by Methods 1, 2, and 3 on 30 plasma specimens. The mean Tg concentrations of the HDL fraction by Methods 1, 2, and 3 were 0.20 (SD 0.06), 0.16 (SD 0.06), and 0.16 (SD 0.06) mmol/L, respectively. Univariate analysis indicated that the Tg data were normally distributed. ANOVA revealed a significant overall F ratio ($F = 5.46, P < 0.006$). Thus, we found significant differences in HDL-Tg values among the three methods. We carried out a Newman–Keuls post hoc analysis, to determine which methods significantly differed from the others. Method 1 gave a significantly higher Tg than either Method 2 or Method 3, but there was no significant difference between results by Methods 2 and 3. Linear regression analysis revealed significant correlation among the three methods (Figure 2).

HDL-C values derived from the three methods were compared (Figure 3). The mean cholesterol concentrations of the HDL fraction separated by methods 1, 2, and 3 were 1.14 (SD 0.29), 1.14 (SD 0.28), and 1.16 (SD 0.32) mmol/L, respectively. Univariate analysis indicated that the HDL-C response was normally distributed. ANOVA revealed no significant difference among the three methods for HDL-C.

We compared the mean ($n = 10$) apo A-I content of total plasma (1.09, SD 0.31 g/L), the mean apo A-I concentration of the infrate after one ultracentrifugation at $d = 1.006$ (1.07, SD 0.30 g/L), and that after two ultracentrifugations at $d = 1.006$ and 1.063 (1.06, SD 0.26 g/L). Univariate analysis indicated that the data were normally distributed. ANOVA revealed no significant difference among any of the methods for apo A-I.

Discussion

Recent interest in HDL-Tg as a predictor of coronary artery disease prompted this study to assess the validity of the heparin–Mn method for HDL-Tg quantitation. This method is used for HDL-C measurement but is largely untested for HDL-Tg.

In our study we found that HDL-Tg concentrations obtained by this method are higher than those obtained by sequential ultracentrifugation or when VLDL is removed by ultracentrifugation before precipitation. A possible explanation is loss of HDL during ultracentrifugation, but this is unlikely, because we obtained good analytical recovery of cholesterol and apo A-I after ultracentrifugation. Because apo A-I is associated only with HDL in the absence of chylomicrons, we conclude that there was no significant loss of HDL throughout the ultracentrifugal procedures.

Another possible explanation for the higher HDL-Tg value after heparin–Mn precipitation of the total plasma is the presence of an interfering substance in the supernate after low-speed centrifugation. Heparin–Mn itself or lipoproteins containing Tg could be responsible. Heparin–Mn is unlikely to have caused interference in the Tg assay because Method 2, with use of heparin–Mn on $d > 1.006$ plasma fraction, yielded the same results for HDL-Tg as the sequential ultracentrifugation method.

The most likely cause for overestimation of HDL-Tg in Method 1 is the presence of lipoproteins in the heparin–Mn supernate. These were not LDL because there was no
overestimation of Tg in the d > 1.006 fraction (which has LDL) that underwent heparin–Mn precipitation. Only when total plasma underwent heparin–Mn precipitation was HDL-Tg overestimated. We conclude that the substance present in the HDL supernate is VLDL. VLDL is composed of 56% triglycerides and 18% cholesterol by weight. Therefore its presence in nonturbid HDL supernates greatly overestimates Tg without affecting cholesterol to the same extent. This explains the greater degree of HDL-Tg overestimation (up to 25%) than that seen for HDL-C (up to 4.5%) in our previous work (4).

The three methods used to isolate HDL-Tg have advantages and disadvantages. Method 1 has good precision and also is the most convenient for the clinical laboratory. However, researchers should be aware that it overestimates HDL-Tg. The major disadvantage of Methods 2 and 3 is that they require ultracentrifugation. Method 2 is less cumbersome because it requires only one ultracentrifugation. Method 3 can produce poor recovery of HDL in inexperienced hands. We recommend Method 2—precipitation after ultracentrifugation to remove VLDL—as the best method for isolating HDL-Tg.

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References