Six Methods for Isolating High-Density Lipoprotein Compared, With Use of the Reference Method for Quantifying Cholesterol in Serum

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Using 90 serum specimens, we compared six routine procedures for high-density lipoprotein (HDL) isolation to determine the biases, if any, of each. Use of the Reference Method for cholesterol (Duncan et al., Centers for Disease Control, Atlanta, GA) and automated dispensing equipment helped ensure the accuracy of the cholesterol measurements and minimized errors from sample and reagent manipulations. Regression analysis of the results showed significant differences between most HDL isolation methods, except for those involving precipitation with heparin–MnCl₂ (1.0 mol/L) or polyethylene glycol 6000, which yielded comparable results with a slope close to one and a zero intercept. The dextran sulfate (M, 500 000)–MgCl₂ method had the largest proportional and constant bias with respect to those two methods. All the methods produced comparable results in the clinically important low HDL-cholesterol range (250 to 350 mg/L), but biases were significant at high concentrations. We conclude that these increased biases in the upper ranges of HDL-cholesterol concentrations are the result of increased heterogeneity of HDL and the different mechanisms involved in forming the insoluble complexes between lipoproteins and the various precipitation reagents.

Additional Keyphrases: analytical error · heart disease

Selective precipitation of lipoproteins that contain apoprotein B is the technique most frequently used to isolate high-density lipoproteins (HDL) for subsequent analysis for cholesterol in the resulting supernates.2 The precipitation methods are fast, simple, and inexpensive, which accounts for their popularity. Several precipitation procedures are available for HDL isolation, each with variations or modifications; however, not all methods yield the same result for high-density-lipoprotein cholesterol (HDL-C).

The Centers for Disease Control (CDC) conducted a survey designed to assess the performance of HDL-C measurements, both within and among laboratories, in serum pools distributed for comparative analysis (1). Most participating laboratories (121 of 130) used a precipitation procedure to isolate HDL, and 70% of them used enzymic analyses for cholesterol to quantify it. From this survey, HDL-C quantification as performed in many laboratories was considered to be unsatisfactory, judged by the criteria for either imprecision or inaccuracy set by CDC. These criteria were based on HDL-C reference values determined by HDL isolation with heparin–MnCl₂ and the Reference Method for cholesterol (2). Therefore, all values reported in the survey were compared with those obtained with this method.

Several comparisons of HDL-C methods have been reported (3–13), many comparing two or more precipitation procedures with the ultracentrifuge method, which has been regarded as the Reference Method for HDL. A few of these studies were comprehensive evaluations, so measurements of apoprotein A-I and B were included to assess incomplete precipitation of low-density lipoprotein (LDL) or coprecipitation of HDL with the various reagents (3, 8, 9, 11, 12). Regardless of sample type (serum or EDTA-treated plasma), source (normal lipemic or hyperlipemic subjects), or method of HDL-C quantification (chemical or enzymic), in all these studies the results obtained by use of the selective precipitation procedures were highly inter-correlated. But systematic biases were found between the various methods, and the magnitude and direction of these deviations were not consistent among these reports.

Here we have compared six methods for HDL isolation and quantified the cholesterol in the respective supernates, using the Reference Method (2). This is the first report of a study in which the accurate and precise Reference Method, automated dispensing equipment, and selected methods for HDL were used to determine the potential differences or biases between methods.

1 The use of trade names is for identification only and does not constitute endorsement by the Public Health Service of the U.S. Department of Health and Human Services.

References

Materials and Methods

Samples

Serum samples. Serum specimens were obtained from the CDC Serum Bank. Specimens of whole blood were collected in glass containers from 90 healthy fasting donors, and left for no longer than 1 h at ambient temperature to clot. The clotted blood was centrifuged (1500 × g, 30 min, ambient temperature), and the serum was aseptically transferred to Wheaton bottles and stored at −20 °C, except for those samples analyzed on the same day they were collected, which were stored at 4 °C until processed. Frozen serum samples were used for HDL isolation within a week of collection. The supernates obtained were analyzed for cholesterol within two weeks. Aliquots of these serum samples were analyzed for total cholesterol and triglyceride content (Table 1).

Human serum reference pool. This pool was prepared by mixing several serum specimens we collected from normal donors. The donors were requested to be fasting, and the individual specimens were assayed for total cholesterol and triglyceride to ensure that we used only sera with values within the established normal range. We analyzed this pool for HDL-C, using the heparin–MnCl₂ technique for isolating HDL and the cholesterol Reference Method (2). The HDL-C reference value for this pool was determined to be 508.3 (SD 6.15) mg/L. Aliquots of the pool, maintained at −70 °C, were thawed immediately before use in the HDL isolation procedure.

HDL isolation Procedures

Small sets of serum samples (six samples plus one reference pool sample) were concurrently treated by the six HDL isolation procedures, to minimize potential bias originating from extended storage, the sample and reagent dispensing process, and cholesterol analysis. Serum aliquots of 1.0 or 2.0 mL, depending on the specific HDL method requirements, were delivered into disposable 15-mL conical centrifuge tubes with an automated dispenser. Appropriate volumes of the HDL isolation reagents were added to the serum aliquots with the automated dispenser and thoroughly mixed. After being incubated at either 4 °C or ambient temperature—again, depending on the specific method requirements—for at least 30 min, the mixtures were centrifuged (1500 × g, 30 min, 4 °C). The HDL-containing supernates were carefully transferred to labeled glass vials without disturbing the pelleted material, then stored at 4 °C until analysis for cholesterol.

Heparin–MnCl₂ (46 mmol/L) (HM1M). The stock solution was prepared by diluting a sodium heparin solution (Li-quaemin® Sodium, 4 × 10⁹ USP units/L; Organon Inc., West Orange, NJ 07082) eightfold with isotonic saline (NaCl, 0.15 mol/L). The HDL isolation reagent was prepared by mixing four volumes of the heparin stock solution with five volumes of a 1.0 mol/L MnCl₂ solution. An 180-μL aliquot of this mixture was dispersed into 2.0 mL of serum sample. The resulting mixtures were processed as previously described (14). The final concentrations of heparin and MnCl₂ used to precipitate the VLDL and LDL were 183 units and 46 mmol/L, respectively.

Heparin–MnCl₂ (92 mmol/L) (HM2M). This HDL isolation reagent was prepared by the same scheme as the HM1M reagent except that the concentration of the stock MnCl₂ solution was increased to 2.0 mol/L. Thus, the final concentration of MnCl₂ was 92 mmol/L.

Dextran sulfate (Mₙ 500 000–Mₘ 380 (D500). A dextran sulfate stock solution was prepared by dissolving 2.0 g (Mₙ 500 000; Pharmacia, Piscataway, NJ 08854) in 100 mL of de-ionized water. The D500-HDL isolation reagent was prepared immediately before being used by mixing equal volumes of the stock dextran sulfate solution and a 2.0 mol/L MnCl₂ solution. A 200-μL aliquot of the working reagent was dispensed into 2.0 mL of serum sample. The resulting mixtures were mixed, incubated at 4 °C, and processed as previously described (16). The final concentrations of dextran sulfate and MnCl₂ were 1.8 μmol/L and 91 mmol/L, respectively.

Dextran sulfate (Mₙ 50 000–Mₘ 580 (D50). We used a lower-molecular-mass dextran sulfate (Mₙ 50 000; Sochibo SA, Boulonge, France 92100) to prepare the isolation reagent (17) in this procedure. In addition, the concentration of the MnCl₂ solution was 1.0 mol/L, a lower concentration than used to prepare the D500 reagent. Immediately before use, the isolation reagent was prepared by mixing equal volumes of the D50 stock solution (2.0 g/100 mL of de-ionized water) and the MnCl₂ solution. The isolation reagent used was in the same manner as the D500 reagent. Therefore, the final concentrations of dextran sulfate and MnCl₂ in the HDL supernates were 1.8 μmol/L and 45 mmol/L, respectively.

Sodium phosphotungstate–MgCl₂ (NAP). For HDL isolation with this reagent (12, 18) we prepared a stock solution of sodium phosphotungstate, dissolving 4.0 g of phosphotungstic acid (Fisher Scientific Co., Fair Lawn, NJ 07410) in 16.0 mL of 1.0 mol/L NaOH solution, then adding de-ionized water to a final volume of 100 mL. Again, we prepared the isolation reagent immediately before use, mixing four volumes of the stock solution with one volume of 2.0 mol/L MgCl₂ solution. A 250-μL aliquot of the reagent was dispensed into 2.0-mL aliquots of the serum samples, and the resulting mixtures were incubated at ambient temperature.

Polyethylene glycol 6000 (PEG). The PEG-HDL isolation reagent (7) was prepared by dissolving 20 g of polyethylene glycol 6000 (approximate Mₙ 6000; Fisher Scientific Co.) in glycerin buffer (0.2 mol/L, pH 10.0) to give a final volume of 100 mL. VLDL and LDL were precipitated by mixing 1.0 mL of the PEG reagent with 1.0 mL of serum and incubating the resulting mixture at ambient temperature before centrifugation. The isolation reagent was prepared freshly once a month.

Other Procedures

Dispensing serum samples and HDL isolation reagents. To minimize imprecision originating from sample processing, we used a Model 23024 automated reagent dispenser (Micromedic Systems, Inc., Horsham, PA 19044), equipped with a 20-mL reagent cartridge and a 0.50-mm (i.d.) Teflon deliv-
ery tip, to dispense all serum samples and HDL isolation reagents. This dispenser is a microprocessor-controlled instrument that accurately and precisely dispenses selected volumes of reagents from 1 μL to 9.999 mL, in 1-μL increments.

We determined performance characteristics of the dispenser by using the manufacturer's recommended procedures. We evaluated the accuracy for dispensing certain volumes—0.1, 1.0, and 2.0 mL—and calculated the relative error to be 0.213, 0.040, and 0.002%, respectively. On the basis of 10 consecutive weight measurements with a five-place analytical scale, the CV for the dispenser was estimated to be 0.241% at 0.1 mL.

Lipid analysis. We used an AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, NY 10591) and colorimetric and fluorometric methods (14) to determine the cholesterol and triglyceride concentrations, respectively, in the serum samples. The Reference Method (2) was used to analyze duplicate aliquots of the HDL supernates for cholesterol.

Statistical methods. We used analysis of variance (ANOVA) to allocate measurement variability in methods, samples, and replicates. Estimates of these individual variance components were expressed as a percentage of the total variance, to show the relative error originating from each source. Differences among the six methods in the overall mean concentration found were compared by an ANOVA. Differences in means for the methods considered on a pairwise basis were tested by the Bonferroni technique (19).

Results

Figure 1 illustrates the range of HDL-C values obtained for the 90 serum samples with each of the HDL methods. For all methods 230 mg/L was the lowest HDL-C value, but the high values varied considerably—from 850 mg/L for HM1M and PEG to 680 mg/L for D500. (Histograms of the HDL-C values obtained with each method illustrated the same results.)

Precision and accuracy of HDL-C quantification. A sample from the human serum reference pool was included in every analytical run. Its assigned HDL-C value was 508.3 mg/L, as determined by the CDC HDL-C Reference Procedure (HM1M). Repetitive analysis of the serum pool allowed us to assess the precision of each of the HDL-C methods and the accuracy of the HM1M procedure. Results presented in Table 2 illustrate the precision of each of the HDL-C methods as judged from the standard deviations, which ranged from a low of 6.61 mg/L for the PEG method to 12.80 mg/L for the D500 method and the D50 method. For all HDL-C methods the precision is well within acceptable limits. The accuracy of the HM1M method was within acceptable limits as established by CDC.

Analysis of variance for individual HDL-C methods. A one-way ANOVA (with samples as the factor) was performed for each HDL-C method, and the results are summarized in Table 3. The percent of total variance among samples is greater than 99% in each case. The within-sample CV ranges from 1.05% (HM2M) to 2.15% (D50). The HM2M method has the smallest variance of the six methods. The D50 method has a variance about fourfold greater than that of the HM2M method.

Overall analysis of variance. An overall analysis of variance was done to further compare the six methods. Of the total variance in 1080 values, 2.8% was among-methods, 96.8% was among replicates (within methods and samples). The overall among-replicate CV was 1.6%, with an overall mean HDL-C concentration of 467.8 mg/L. This analysis of variance indicated a significant difference among means for the six methods (p<0.01)—which is not surprising, given nearly 1000 error degrees of freedom. Individual means for all six methods were compared (15 comparisons) by using the Bonferroni i-test and an experiment-wide error rate of 5%. All comparisons except PEG vs HM1M were significant.

Discussion

Several of the previous comparisons of methods for HDL-C have established the relative accuracy of the various methods by comparison with the classic ultracentrifugation procedure for isolating HDL. However, HDL isolated between the hydrated densities of 1.063–1.210 kg/L by ultracentrifugation presents several problems: (a) the HDL is contaminated with non-HDL lipoproteins in this density range; (b) the recovery of lipoproteins is often less than 90%; and (c) the use of high salt concentrations alters lipoprotein characteristics. Therefore, ultracentrifugation—or, in fact, any other method—is not appropriate as a reference procedure. Our goal was to determine the relative relationships between these specific methods for isolating HDL.

![Fig. 1. The mean and range of HDL-C values for 90 serum samples tested by the six HDL isolation procedures.](image-url)

| Table 2. Analytical Variation for the HDL-C Methods Based on Results with Pooled Human Serum* |
|----------------------------------------|----------------------------------------|----------------|----------------|
| HDL isolation method | Cholesterol concn, mg/L | CV, %<br>(total) |                     |
| HM1M | 507.8 | 7.39 | 1.46 |
| HM2M | 475.9 | 10.88 | 2.28 |
| D500 | 456.6 | 12.90 | 2.82 |
| D50 | 473.4 | 12.90 | 2.72 |
| NAP | 486.3 | 8.57 | 1.78 |
| PEG | 520.0 | 6.61 | 1.27 |

*Duplicate cholesterol analysis of the respective HDL supernates in 16 assays each.
We found fair to excellent correlation between all the HDL methods evaluated; however, regression analysis illustrated that significant biases exist between most of them, probably related to the differences in the various reagents' capacity to precipitate all lipoproteins that contain apolipoprotein B and to interact with HDL. In interpreting these results, one must realize that HDL is not a homogeneous lipoprotein family. Rather, HDL is the most heterogeneous of all lipoprotein classes, generally accepted as having at least two major subclasses, HDL2 and HDL3. In fact, HDL fractionation by density-gradient ultracentrifugation reportedly resolves the lipoprotein family into 12 subfractions (21). HDL has been similarly separated into several subfractions by isoelectric focusing and by column chromatography on diethylaminoethyl cellulose or hydroxylapatite. This heterogeneity may account for the biases between the various HDL-C methods, given that HM1M/HM2M, D500/D50, NAP, and PEG-HDL isolation procedures all complex lipoproteins by different mechanisms. In addition, changes in HDL methods, such as with HM1M/HM2M or D500/D50, can result in significant biases: it may be possible to adjust the reagent concentrations, ionic strength, buffer pH, or other characteristics of the HDL isolation reagents so that the HDL-C values will mimic those by another method, but the question will remain, which method gives the "true" HDL-C value? All the HDL-C methods gave excellent agreement in the low concentration range (200–350 mg/L), the clinical range of major importance for assessing cardiovascular risk. Most of the bias among the various HDL-C methods occurs at concentrations exceeding 450 mg/L. These findings are consistent with the heterogeneity of HDL, the differences or alterations in subclasses of which would be maximized at higher concentrations. The significant biases for the HM1M and PEG procedures relative to the other reagents could be attributed to incomplete precipitation of the lipoproteins containing apolipoprotein B. However, a recent study of the heparin–Mn⁺⁺ and dextran sulfate–Mg⁺⁺ procedures, in which the contents of the supernates were evaluated by column chromatography and measurements of apolipoprotein E, concluded that apolipoprotein E-containing HDL is precipitated by dextran sulfate–Mg⁺⁺ but not by the heparin–Mn⁺⁺ reagent (22). This may explain differences we found, especially at the greater concentrations of HDL.

The most unexpected and serendipitous result of this study was the close agreement between the HM1M and PEG methods for isolating HDL. The PEG reagent is a viscous solution that requires automated pipets for accurate and precise dispensing of aliquots. In addition, the cholesterol value for the resulting PEG supernate is calculated by including a correction factor of 2 vs a correction factor of 1.09 for the HM1M method. Despite the possibility that any analytical error in the cholesterol measurement would thus be more greatly magnified in the PEG system than in the HM1M procedure, the two methods gave excellent agreement.

In conclusion, using the Reference Method for cholesterol with six HDL isolation procedures, we showed that most methods for isolating HDL have significant biases, especially at the upper range of HDL-C values. The heterogeneity of HDL, especially the HDL containing apolipoprotein E, substantially influences the lack of agreement among these methods. We hope this report will provide the information needed to select an HDL-C procedure by showing how well the method performs in comparison with several other frequently used methods; then, after the method for isolating the HDL fraction has been selected, a compatible cholesterol method must be chosen. This report should be valuable for laboratories that participate in the CDC-HDL Standardization Program but are not using the HM1M reagent system used by the CDC to assign HDL values to the test material.

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Enzyme Immunoassay of Free Thyroxin in Dried Blood Samples on Filter Paper

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We describe a double-antibody enzyme immunoassay for determination of free thyroxin (FT₄) in dried blood samples on filter paper, with use of a T₄-alpha-D-galactosidase complex. The measurable range of FT₄ concentration in two 3-mm blood discs, each of which contained about 2.7 μL of blood, was 1.9 to 93 ng/L, as determined by comparison with concentrations of FT₄ in known serum standards. FT₄ in blood samples dried on filter paper was stable for at least four weeks when kept dry at -20°C, room temperature, or 37°C. The mean coefficients of variation were 7.6% (within assay) and 6.4% (between assays). Results for FT₄ by this method correlated well with those for serum determined by radioimmunoassay (r = 0.98). The proposed method can be used to differentiate persons with hyper- and hypothyroidism from normal subjects and those with abnormal concentrations of thyroxin-binding globulin. The procedure seems suited for screening studies.

Additional Keyphrases: thyroid status - newborns - screening

Mass screening for hypothyroidism in newborns is done throughout the world, because the irreversible mental retardation caused by this disorder can be prevented by early treatment (1). Moreover, the hypothalamus–pituitary–thyroid axis in premature babies has been extensively investigated. Among the methods developed for these purposes are measurements of total thyroxin (T₄) (2) and thyrotropin concentrations in dried blood samples on filter paper (2–7).