Development and Evaluation of a Method for Quantitation of Plasma High-Density-Lipoprotein Cholesterol

Evan A. Stein,1,2 Paula M. Steiner,2 Peter S. Gartside,2 and Charles J. Glueck2

An improved method for separating high-density lipoprotein with use of "Alphachol" tubes containing pre-dispensed and lyophilized heparin and MnCl2 is described. Results for high-density lipoprotein cholesterol (C-HDL) isolated by ultracentrifugation from a 360 mg/liter and a 510 mg/liter pool were compared with those for the same pools by the Alphachol (358 and 506 mg/liter) and standard Lipid Research Clinic (LRC) (354 and 504 mg/liter) procedures. The within-day coefficient of variation (CV) for the Alphachol method was 1%, as compared to 1.3% for the standard LRC method. The CV for between-day precision studies conducted over 20 days was 5.3% for the Alphachol, 4.2% for the standard LRC procedures. Analysis of C-HDL in 100 patients by both the Alphachol and the standard LRC method showed mean C-HDL values to be slightly (23 mg/liter) higher by the standard LRC method (P < 0.001). A similar mean difference (24 mg/liter, P < 0.001) was observed by comparing 75 patients sera analyzed by the Alphachol and modified LRC (final MnCl2 concentration, 92 mmol/liter) methods. It is unlikely that the 20 mg/liter difference between methods would have clinical significance. 

High-density lipoprotein cholesterol (C-HDL) concentration is inversely associated with the presence of atherosclerotic coronary heart disease (1-4). Currently it is difficult to quantitate high-density lipoprotein (HDL) directly (5-7), therefore most methods depend on the measurement of C-HDL. HDL may be separated from other lipoproteins by ultracentrifugation (8) in the density range 1.063-1.21, and its cholesterol content then determined separately. Ultracentrifugation requires expensive instrumentation and considerable technician time and so is impractical for routine clinical laboratory application.

Most methods for measuring C-HDL are based on polyanion precipitation of the low- and very-low-density lipoproteins (LDL, VLDL) (9). The Lipid Research Clinics' (LRC) modification of the Burstein method (9) requires ultracentrifugation to remove VLDL, precipitation of LDL with heparin-MnCl2, and measurement of C-HDL in the remaining supernate (10). Alternatively, ultracentrifugation can be omitted (10, 11), and VLDL and LDL precipitated from whole plasma by heparin-MnCl2, with measurement of C-HDL in the supernate. These methods must be performed with considerable care, because the final MnCl2 concentration (46 mmol/liter) is barely adequate to completely precipitate LDL and VLDL (11-13).

Indeed, one problem with the use of heparin-Mn2+ is incomplete precipitation of VLDL and LDL in hypertriglyceridemic samples (12). Also, half of nonturbid supernates obtained by precipitation with heparin-MnCl2 (46 mmol/liter), contain measurable apoB-associated cholesterol (12). A higher (92 mmol/liter) concentration of MnCl2 has been reported to more completely precipitate LDL and VLDL, with improved accuracy (11, 12). The use of alternative polyanions such as phosphotungstate and MgCl2 has been proposed (14, 15).

All of these precipitation methods (11-15) are limited in their use in routine laboratories by the following factors:

1. Consistent, accurate constitution and delivery of small volumes of separate heparin and MnCl2, or other polyanion solutions of specific concentrations.
2. A dilution correction factor must be applied to the final HDL cholesterol value obtained (10), to account for the volume of liquid reagents added.

Modifications to the heparin-MnCl2 method were developed to alleviate these problems. Here, we describe, evaluate, and validate these modifications and compare the results to those by more established methods.

Materials and Methods

Method Modification: Lyophilized Heparin-MnCl2 in Disposable Tubes

Disposable 10 × 75 mm glass tubes containing 226 USP units of sodium heparin and 100 μmol of MnCl2 (lyophilized and sealed; Alphachol) were developed with Standard Scientific (385 Conners Lane, Hebron, Ky. 41048). Quality control was carried out on every tenth tube; the coefficient of variation for the amount of heparin-MnCl2 dispensed per tube (by weight) was 0.51%.

Plasma Samples

Plasma samples were obtained from fasting subjects attending the outpatient lipid clinic of the General Clinical Research Center. Blood was collected into evacuated collection tubes containing powdered EDTA, and plasma was subsequently separated according to the Lipid Research Clinics Manual (10). Plasma samples that were visibly lipemic were not included, to minimize cases of incomplete precipi-
tation of VLDL and LDL (12). These samples were analyzed within 24 h, by both the Alphachol and LRC methods.

Two pools (C-HDL 360 and 520 mg/liter) were prepared from plasma of normotriglyceremic subjects. Portions were removed for ultracentrifugation (8), and the remainder divided into portions, frozen in sealed bottles, and stored at -20 °C. These pools were used to compare the C-HDL obtained by Alphachol precipitation to that by the LRC method (10). We analyzed the previously frozen aliquots over a period of five days, after which time increasing variability in the pool C-HDL was observed.

For more stable pools for use in longer-term studies of precision, serum from normotriglyceremic subjects was pooled, divided, sealed, and stored at -20 °C until used.

Separation of HDL, Quantitation of C-HDL

HDL was separated by ultracentrifugation at density 1.063 (8) by use of the Alphachol tubes (final MnCl₂ concentration, 0.1 mol/liter) and by standard LRC methods (10), (46 mmol of MnCl₂ per liter, final concentration) and a modified LRC method (92 mmol/liter).

Ultracentrifugation was used as the method for HDL isolation against which the results of polyanion precipitation methods were compared. A 5-ml plasma aliquot was adjusted to background density 1.063 g/ml with solid KBr (16). In a cellulose nitrate tube, the density-adjusted sample was overlaid with KBr solution, d = 1.063, to fill the tube and then centrifuged at 100 000 × g for 18 h at 10 °C. The d > 1.063 lipoprotein fraction was recovered with a tube slicer and reconstituted to 5 ml.

Standard LRC method (10). Specimens were precipitated with heparin and MnCl₂ as described in the Manual of Laboratory Operations of the Lipid Research Clinics Program (10) with a final Mn²⁺ concentration of 46 mmol/liter.

Modified LRC method. Plasma was handled as in the standard LRC method, but the final concentration of MnCl₂ was doubled (to 92 mmol/liter).

Alphachol method. A 1-ml aliquot of plasma was added to the prepared Alphachol tube, which was stoppered with the provided plastic closure and vortex mixed for 5 s (final MnCl₂ concentration, 0.1 mol/liter). After standing for 10 min at room temperature, the sample was centrifuged in a cold room (4 °C) at 1500 × g for 30 min. The HDL-containing supernate was then recovered for cholesterol quantitation.

Lipid Measurements

All cholesterol and triglyceride estimations were done with the Technicon AutoAnalyzer II by the LRC procedure (10), with use of the serum calibrator supplied by the Center for Disease Control, Atlanta, Ga. A correction factor of 1.09 was applied to the cholesterol values quantitated in HDL supernates obtained by the standard or modified LRC methods. No correction for dilution by liquid reagents was required for the Alphachol method.

Lipoprotein electrophoresis was done with use of a commercially available ACI-Corning kit. The agarose films were stained with Fat Red 7B.

Recovery of ¹²⁵I-labeled HDL

HDL was isolated by ultracentrifugation, and then labeled (by the Chloramine T technique) with ¹²⁵I.

Statistical Methods

We compared C-HDL in plasma samples by using two-way analysis of variance, and paired t-tests (17). Standard re-

<table>
<thead>
<tr>
<th>Table 1. Plasma C-HDL as Quantitated by Ultracentrifugation, the Standard Lipid Research Clinics Precipitation (Final Mn²⁺ Concentration, 46 mmol/liter) Method, and by the Alphachol Method</th>
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<tbody>
<tr>
<td>C-HDL 360 mg/liter</td>
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<tr>
<td><strong>High-density lipoprotein cholesterol, mg/liter</strong></td>
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<td>LRC</td>
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<td>Mean ± SD</td>
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<td>CV, %</td>
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* Quantitation by ultracentrifugation.

gression analysis was also used to assess relationships between various analytical methods (17).

Results

C-HDL by Alphachol Compared with Standard LRC and Ultracentrifugal Methods

HDL was separated ultracentrifugally from two plasma pools, with C-HDL values being 360 and 510 mg/liter. These values were used for the comparison study. Agarose lipoprotein electrophoresis of the fraction d > 1.063 showed that sinking prebeta-lipoprotein was absent from both pools.

On each of five days, a frozen aliquot of each plasma pool was thawed and C-HDL determined by the standard LRC precipitation and by the Alphachol method (Table 1). For the 360 and 510 mg/liter pools (by ultracentrifugation) the mean C-HDL values by both methods—354 and 504 (LRC), 358 and 506 (Alphachol)—were highly similar (Table 1). C-HDL in either pool measured by Alphachol did not differ from that measured by the standard LRC method, P > 0.1.

Precision Studies

The within-day coefficient of variation (CV) for the Alphachol method was 1% for 20 precipitations of a serum pool, with a mean C-HDL of 505 mg/liter (SD = 5.1 mg/liter). For 20 precipitations of a separate serum pool with mean C-HDL of 464 mg/liter, the CV for the standard LRC method was 1.3% (SD, 6.0 mg/liter).

The between-day CV for the Alphachol method for analysis of a frozen serum pool on 20 separate days was 5.3% (mean C-HDL, 393 ± 21), while the CV for the standard LRC method for the same 20 days for the same pool was 4.2% (mean C-HDL, 438 ± 18 mg/liter).

Comparison of C-HDL by Precipitation Methods in a Patient Population

As summarized by Table 2, C-HDL values in 100 subjects were quantitated by the standard LRC and Alphachol methods. The mean total plasma cholesterol and triglyceride in these 100 subjects were 2380 ± 590 and 1280 ± 540 mg/liter, with a cholesterol range of 1200-4940 and triglyceride range of 420-2740 mg/liter. C-HDL values by the standard LRC method (Table 2) were slightly but consistently higher (mean difference ± SD, 23 ± 2 mg/liter). The difference between the two methods was significant by paired t-test (t = 10.3, P <
Table 2. C-HDL Values by the Standard and Modified LRC Methods and the Alphachol Method

<table>
<thead>
<tr>
<th>No. plasma samples</th>
<th>C-HDL by LRC mg/liter</th>
<th>C-HDL by Alphachol</th>
<th>C-HDL by LRC minus C-HDL by Alphachol</th>
<th>Mean diff.</th>
<th>SD of diff.</th>
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<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>SD</td>
<td>( \bar{x} )</td>
<td>SD</td>
<td>( \bar{x} )</td>
</tr>
<tr>
<td>100 (standard LRC)</td>
<td>503</td>
<td>107</td>
<td>480</td>
<td>99</td>
<td><em>22.6</em></td>
</tr>
<tr>
<td>75 (modified LRC)</td>
<td>495</td>
<td>131</td>
<td>471</td>
<td>119</td>
<td><em>24.1</em></td>
</tr>
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</table>

* \( P < 0.001 \) (two-way analysis of variance, C-HDL by LRC compared to C-HDL by Alphachol).

C-HDL values for 75 subjects were quantitated by the modified LRC and Alphachol methods (Table 2). These 75 patients had mean total plasma cholesterol and triglyceride values of 2080 ± 630 and 1210 ± 80 mg/liter, with a cholesterol range of 1210–3800 and triglyceride range of 440–3700 mg/liter. C-HDL measured by the modified LRC method was consistently higher, with a mean (± SD) difference between the two methods of 24 ± 4.5 mg/liter (Table 2). The difference between the two methods was significant by paired \( t \)-test (\( t = 5.42, P < 0.001 \)), and by two-way analysis of variance (\( F = 29.3, df = 1,72, P < 0.01 \)).

There was a close correlation between C-HDL as measured by the Alphachol method, and by either the standard LRC method (\( r = 0.98, P < 0.001 \)) or the modified LRC method (\( r = 0.96, P < 0.001 \)). The spread of points about the line of equality for the two methods was narrow, as reflected in the high correlation coefficients. The respective slopes were .901 and .866, the respective y-intercepts were 28 and 42 mg/liter.

C-HDL Quantitation in Plasma with Increased Triglyceride Values

In the group of 100 subjects (Table 2), 15 had plasma triglyceride concentrations of >1700 mg/liter. In these 15 subjects, the mean ± SD C-HDL by the standard LRC method (434 ± 89 mg/liter) was higher than by the Alphachol method (421 ± 97 mg/liter), paired \( t = 2.8, P < 0.02 \).

For the 75 subjects for whom C-HDL was determined by the modified LRC method and the Alphachol method (Table 2), 16 had plasma triglyceride values exceeding 1700 mg/liter. In these 16, the mean ± SD C-HDL by the modified LRC method was slightly but not significantly greater than that by the Alphachol method (412 ± 11 and 390 ± 100 mg/liter), paired \( t = 1.69, P > 0.05<0.1 \).

In four subjects with triglyceride values of 3700, 4380, 4020, and 4540 mg/liter, there was incomplete sedimentation of precipitate by the Alphachol method and in two of the four by the modified LRC method.

Recovery of \(^{125}\text{I}-\)labeled HDL

Approximately 10,000 cpm/ml of \(^{125}\text{I}-\)labeled HDL were added to three plasma pools. Standard LRC and Alphachol precipitations were performed and an aliquot of the HDL-containing supernate was removed for counting. A mean of 100.35% of the added counts were accounted for after precipitation by the standard LRC method and multiplication by 1.09 to account for the volume of heparin–MnCl\(_2\) added. A mean of 98.4% of counts were recovered from the supernate after Alphachol precipitation without the application of a correction factor.

Discussion

Results of the standard and modified LRC methods were highly correlated with those of the Alphachol method over a wide range of plasma cholesterol and triglyceride concentrations. Plasma C-HDL values by the standard or modified LRC methods were 4 to 5% higher than those by the Alphachol method; the mean differences, about 20 mg/liter, are unlikely to have clinical significance. The slightly lower C-HDL values (by Alphachol) might result from precipitation of minimal amounts of HDL by the final higher Mn\(^{2+}\) concentration of 0.1 mol/liter as opposed to the more optimal 92 mmol/liter final concentration used in the modified LRC method (12). Subsequent to this report, the Mn\(^{2+}\) content of each tube has been reduced to provide a final Mn\(^{2+}\) concentration of 92 mmol/liter after addition of 1 ml of plasma. In chylomicrosome plasma samples, initial ultracentrifugation to remove lipoprotein of \( d < 1.006 \) may be necessary to allow accurate measurement of HDL cholesterol (12).

In hypertriglyceridemic samples, nonprecipitating apo B-associated materials may be decreased by increasing the final Mn\(^{2+}\) concentration to 92 mmol/liter (12). In addition, Warnick and Albers (12) produced clear supernates, essentially free of turbidity in samples with triglycerides ranging from 5.32 to 54.30 g/liter, and Mn\(^{2+}\) at 92 mmol/liter final concentration by ultracentrifugation (1500 \( \times g \), 30 min). Those samples with still-persistent turbidity, could be cleared by using increasing centrifugation force (12).

Srinivasan et al. (18) reported that up to 25% of HDL forms an insoluble complex with free heparin in the presence of Mn\(^{2+}\), which would lead to a systematic underestimation of HDL by any precipitation system in which heparin–Mn\(^{2+}\) is used. In this study, essentially all of the added radioactive labeled HDL was recovered from the supernate after standard LRC and Alphachol heparin–Mn\(^{2+}\) precipitations.

As currently utilized, heparin–Mn\(^{2+}\) precipitations involve accurate constitution of reagents, addition of heparin to plasma, thorough mixing, separate addition of MnCl\(_2\), repeat mixing, return to the ice bath for 30 min, recovery of supernate, and determination of its cholesterol content (10, 11). A final correction factor of 1.09 then must be applied to account for the dilution caused by the heparin and MnCl\(_2\) solutions. Using a 46 mmol/liter final concentration of MnCl\(_2\) may result in incomplete precipitation of LDL and VLDL (11–13). The Alphachol system will facilitate the determination of C-HDL. The lyophilized heparin and Mn\(^{2+}\)-containing tubes obviate the need for solution preparation and delivery, and provide (in their more recent version) the optimal (12) final Mn\(^{2+}\) concentration, 92 mmol/liter. No correction factor is required to account for dilution of plasma by liquid reagents. Plasma (1 ml) is added to the prepared tube, followed by a 10-min incubation at room temperature, centrifugation (in a cold room or refrigerated centrifuge), and analysis for cholesterol.

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References


6. Hulley, S. B., Cook, S. G., Wilson, W. S., et al., Quantitation of serum lipoproteins by electrophoresis on agarose gel; standardization in lipoprotein concentration units (mg/100 mg) by comparison with analytic ultracentrifugation. J. Lipid Res. 12, 420 (1971).


