Heparin–Magnesium Chloride–Albumin Method for Enzymic Measurement of Cholesterol in High-Density Lipoprotein

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We describe a new method for measuring high-density-lipoprotein cholesterol in sera, with use of heparin, MgCl₂, and albumin to precipitate very-low- and low-density lipoproteins. Lipoprotein electrophoresis and immunochromatographic methods show that high-density lipoprotein is completely and specifically isolated in the supernate, the cholesterol concentration of which is estimated by the enzymic method of Allain et al. (Clin. Chem. 20, 470, 1974). The precision of the method is excellent (CV = 1%) and the response is linear. Values so obtained correlate well with those obtained with the heparin–Mn²⁺ (r = 0.98) or the phosphotungstate–Mg²⁺ (r = 0.98) procedures. The technique is appropriate for routine clinical laboratory use.

An inverse relationship has been demonstrated between increased HDL cholesterol concentrations in plasma and risk of cardiovascular disease (1). Generally HDL is separated either by physical methods—ultracentrifugation or electrophoresis—or by selective precipitation methods, which are more suitable as a routine procedure. Multi-laboratory comparisons have established a good correlation between HDL as measured after selective precipitation with phosphotungstate–Mg²⁺ or heparin–Mn²⁺ and results by ultracentrifugation (2). However, in some samples with very high triglyceride concentrations neither of these precipitation techniques results in total precipitation of LDL, VLDL, and chylomicrons (2). In a recent study, electrophoresis indicates that some VLDL contaminates the supernates of hypertriglyceridemic (3.5 g/L) samples after precipitation with heparin–Mn²⁺ or phosphotungstate–Mg²⁺ reagents (3). Moreover, these procedures require relatively large volumes of plasma (1 mL). Thus they are less than ideal for use in estimating HDL cholesterol, especially in lipemic plasma. Here we report a heparin–Mg²⁺–albumin procedure that requires only 0.1 mL of serum, from which LDL and VLDL are completely precipitated. We compared this new method to both the phosphotungstate–Mg²⁺ and the heparin–Mn²⁺ methods, and tested the efficiency of HDL separation at various lipoprotein concentrations by electrophoresis of the supernate on polyacrylamide gel.

Materials and Methods

Samples

Venous blood was sampled from 300 ostensibly normal and hyperlipoproteinemic adults. All subjects had been fasting for 12 to 15 h. The serum was promptly separated and stored (both at 4 °C) until analysis.

Reagents

Reagent I: Dissolve 30 mL of heparin (5000 kilo-int. units/mL; Riker Labs.), 20 mmol of MgCl₂, and 10 g of bovine serum albumin in a final volume of 1 L of distilled water. Stored at 4 °C, this reagent can be used for at least one month.

Reagent II: Dissolve 4.0 g of phosphotungstic acid in 100 mL of water and adjust the pH to 6.15.

Procedures

Lipoprotein precipitation by heparin Mg²⁺–albumin: Add 0.1 mL of serum to 2 mL of Reagent I and immediately mix. Allow the mixture to stand for exactly 15 min at 20 °C, then separate the precipitate by centrifugation (3500 × g, 20 min, 4 °C).

Heparin–Mn²⁺ procedure: Precipitate 1 mL of serum by adding 40 μL of heparin and 50 μL of 1 mol/L MnCl₂ (final Mn²⁺ concentration, 46 mmol/L) (4).

Phosphotungstate–Mg²⁺ procedure: Add 100 μL of Reagent II and 25 μL of 2.0 mol/L MgCl₂ solution to 1.0 mL of serum (5). Incubate the samples obtained by the heparin–Mn²⁺ and phosphotungstate–Mg²⁺ procedures for 30 min at 25 °C, then centrifuge (1500 × g, 10 min, 4 °C).

Cholesterol determination: Measure cholesterol in the supernate according to the enzymic procedure of Allain et al. (6) by continuous-flow analysis. We used an AutoAnalyzer II with scale expansion modified to increase sensitivity; the response was linear from 0 to 2.5 mmol/L. A standard reference solution (Technicon) was diluted 40-fold and used for calibration.

Solutions obtained from precipitated lipoproteins: The precipitates obtained by the various procedures named above were washed twice with 0.15 mol/L NaCl solution, then dissolved in 5 mL of a 100 g/L sodium citrate solution according to Burstein and Scholnick (7). This solution was then dialyzed for 24 h against 0.02 mol/L tri(hydroxymethyl)aminomethane/0.17 mol/L NaCl buffer, pH 7.7, to remove citrate and metal ions.

Lipoprotein separation by ultracentrifugation: Ultracentrifuge 10 mL of serum at 100 000 × g for 24 h at 4 °C (Model L5-65 centrifuge, rotor Ti 65; Beckman, 93220 France). Dialyze the supernate against running water and concentrate it 20-fold (we used a "Rotavapor" apparatus) to obtain VLDL. Subsequently, adjust the relative density of the subnatant fluid to d = 1.21 with dried KBr (8) and centrifuge at 100 000 × g for 24 h. Dialyze the supernate and concentrate it 20-fold to obtain the HDL–LDL fraction.

Lipoprotein electrophoresis: Concentrate by 20-fold the supernates and the dissolved precipitates on a Minicon (Aminco, 75020 France). Prestain the sample (50 μL) with Sudan Black B and electrophorese on polyacrylamide gel for 30 min with a continuous current of 4 mA per column (Redi-
disc Reagent; Ames, 75755 France). Electrophoresis of serial dilutions of lipoproteins showed that the lower limit of sensitivity of this method was 0.05 g/L for HDL, 0.06 g/L for LDL, and 0.2 g/L for VLDL.

Results

Precision: The precision was examined for both normal and hyperlipoproteinemic sera. The respective variances (± SD) were as follows (mmol/L): 1.06 ± 0.01 (CV = 1.2%) and 1.18 ± 0.03 (CV = 2.5%) for intra-assay precision, and 1.04 ± 0.03 (CV = 2.9%) and 1.20 ± 0.06 (CV = 5%) for inter-assay precision. The linear response of the heparin–Mg²⁺–albumin method was studied by varying various dilutions of a pool of sera. Samples, undiluted and diluted with 0.155 mol/L NaCl, were subjected to precipitation and cholesterol in the supernatant fluid was measured in quintuplicate. The response was linear from 0 to 2.5 mmol/L with the AutoAnalyzer apparatus.

Comparison study: The three methods we used to estimate HDL cholesterol were standardized with the same reference solution. For both the heparin–Mn²⁺ and the heparin–Mg²⁺–albumin methods, the coefficient of correlation was \( r = 0.98 \) (n = 100). The slope obtained by linear regression analysis and the y-intercept values were 0.94 and 0.03, respectively. On the other hand, there was a significant difference when a statistical analysis of results was performed by Student's t-test for paired data (t = 4.5). The results obtained by comparison between our method and the phosphotungstate–Mg²⁺ method were as follows: \( r = 0.98 \) (n = 82); slope 1.01; y-intercept = 0.07; and t = 7.8.

The average differences in results, expressed in percent, were about 5% (0.06 mmol/L) between our method (average, 1.30 mmol/L) and the heparin–Mg²⁺ technique, and 9% (0.1 mmol/L) between our method and the phosphotungstate–Mg²⁺ procedure.

To define the limits of our method, we concentrated by 20-fold the supernates and the dissolved precipitates obtained from normal sera. Electrophoresis revealed a single band corresponding to \( \alpha \)-lipoprotein in the supernates and two bands corresponding to pre-\( \beta \) and \( \beta \)-lipoproteins in the precipitates. VLDL (8 g/L) or LDL (10 g/L) were also added to normal sera, further to test the validity of the method. Electrophoresis showed that both these fractions were entirely and specifically precipitated by addition of heparin–Mg²⁺–albumin.

Discussion

Among precipitation methods, the heparin–Mn²⁺ procedure is recommended by the Lipid Research Clinic Program (9), but the use of Mn²⁺ produces HDL–heparin complexes of varying insolubility (10). The phosphotungstate–Mg²⁺ method offers considerable practical advantages (2), but slight changes in both phosphotungstate and Mg²⁺ concentrations affect the lipoprotein precipitation (4, 5, 7). We therefore suggest here a new method involving heparin–Mg²⁺–albumin for complete and selective precipitation of VLDL and LDL in normal or extremely lipemic sera. This rapid, simple, and reproducible method requires little serum. Moreover, the absence of Mn²⁺ from the reagent obviates disturbance by Mn²⁺–phosphate buffer interaction in the cholesterol determination. Indeed, cholesterol concentrations in the supernates obtained by heparin–Mg²⁺–albumin precipitation are comparable to those obtained with the same supernate supplemented with ethylenediaminetetraacetic acid according to Grove (5). Thus, MgCl₂ at 2 mmol/L did not react with the enzymic cholesterol reagent. The decrease of ionic strength by dialysis or aqueous dilution was not efficient for a selective LDL–VLDL precipitation with heparin–Mg²⁺ reagent. Under these conditions, electrophoresis of 20-fold concentrated supernates on polyacrylamide gel revealed a band with mobility corresponding to VLDL. In contrast, addition of serum albumin to heparin–Mg²⁺ reagent resulted in better sedimentation of both LDL and VLDL by a mechanism presently unknown (11). The degree of HDL precipitation depends on the albumin concentration and the ionic strength. VLDL contaminated the supernate when albumin concentration was <5 g/L, and HDL was present in the precipitate when the albumin concentration was >20 g/L. In the same way optimum ionic strength was estimated by an increase in Mg²⁺ concentration from 0 to 0.1 mol/L. To confirm that the heparin concentration was adequate, heparin concentrations from 0 to 400 kilo-int. units/L were tested at optimal MgCl₂ (0.02 mol/L) and albumin (10 g/L) concentrations. Under these conditions, checks by electrophoresis revealed only HDL in the supernate from 30 to 300 kilo-int. units of heparin per liter. These results were confirmed by Ouchterlony's method: no precipitation line was revealed when the supernate was tested against \( \beta \)-lipoprotein antiserum (Behring, 75008 France). On the other hand, less than 2% of the HDL present in the starting sera was detected in the precipitates by electrophoresis.

With hypertriglyceridemic samples, the classical precipitation methods failed to produce a clear supernate. Therefore the turbid supernates had to be clarified by filtration (12). With our reagent the floating precipitates so obtained aggregated as a pellicle at the top of a test tube. The subnatant fluid was clear and could be thus removed by gentle aspiration with a Pasteur pipette. Furthermore, addition of VLDL or LDL–HDL to tested sera showed that VLDL–triglyceride and total cholesterol concentrations had no effect on the present method. HDL separation was thus independent of both triglyceride and cholesterol concentrations.

On the other hand, there are significant systematic differences in HDL cholesterol quantitation according to the precipitation method used (4). This observation was confirmed in this study by the t-test results. Therefore we now are engaged in establishing normal values for our method. Preliminary results give an HDL cholesterol value as follows (mmol/L): \( X \pm SD = 1.21 \pm 0.30 \) (CV = 25%) for 100 army volunteers.

In conclusion, the heparin–Mg²⁺–albumin method has two limitations: it requires a sensitive cholesterol method (enzymic procedure) and it gives values 5 to 9% lower than those obtained by the heparin–Mn²⁺ or phosphotungstate–Mg²⁺ techniques. However, our method has significant advantages. First, it allows complete HDL separation from hyperlipemic sera undiluted before addition of the reagent. Second, VLDL and LDL are completely precipitated up to 7 and 10 g/L, respectively. This simple technique seems thus to be suitable for routine HDL cholesterol determination.

References