Cholesterol Determination in High-Density Lipoproteins Separated by Three Different Methods

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We describe a simplified method for measuring high-density lipoprotein cholesterol in serum after very-low- and low-density lipoproteins have been precipitated from the specimen with sodium phosphotungstate and Mg$^{2+}$. Values so obtained correlate well with values obtained with the heparin-Mn$^{2+}$ precipitation technique ($r = 0.95$, CV <5% in 68% of the subjects studied and between 5 and 10% in the remaining ones) or by ultracentrifugal separation ($r = 0.82$, CV <5% in 80% of the subjects studied and between 5 and 10% in the remaining ones). Our precipitation technique is more appropriate for routine clinical laboratory use.

Measurement of HDL cholesterol in patients with coronary heart disease is of increased interest.\(^1\) There is evidence of an inverse relationship between HDL cholesterol and coronary heart disease (1, 2), and HDL may be important for the transport of cholesterol from tissues to liver (3). Accordingly, a simplified and reliable method is needed for measuring HDL-cholesterol.

Several methods have been proposed for measuring lipoproteins by measuring their cholesterol content. The first widely used method was preparative ultracentrifugation (4), which is time consuming and requires both expensive equipment and highly trained personnel.

The Lipid Research Clinic Program of the NIH proposed a simplified analysis of lipoprotein classes that is currently widely used in the United States. Basically, ultracentrifugation is used to separate VLDL from LDL + HDL without special adjustments of sample density. The HDL are obtained after precipitation of LDL and VLDL from the whole serum with heparin-Mn$^{2+}$ and their cholesterol content measured (5).

There has been considerable interest in developing methods that would not require ultracentrifugation. A recently proposed method is based on a successive precipitation with sodium dodecyl sulfate and heparin-Mn$^{2+}$ (6). However, precipitation of LDL and VLDL with heparin-Mn$^{2+}$ presents special problems, because not all heparin preparations are equally efficient and thus the completeness of VLDL and LDL precipitation must be checked each time a different lot of heparin is used.

Burnstein and Samaille (7) described an apparently simpler method in which phosphotungstate and Mg$^{2+}$ were used to precipitate LDL and VLDL. This technique has been used for isolation procedures but has not been adapted for routine lipoprotein quantification.

Here we report the results of a study in which HDL cholesterol was determined by these three different methods (4, 5, 8). We find the last method (8) to be simple, reliable, precise, and inexpensive and that it gives results comparable to those obtained by older, more cumbersome methods.

Materials and Methods

Plasma and serum samples. Plasma and serum specimens were obtained from adults after an overnight fast. The subjects were from the diabetic clinic (n = 30) or normal individuals (n = 17). The diabetic group included 17 patients with increased and 13 with normal lipid concentration in their serum. Values for total cholesterol ranged from 1.07 to 4.22 g/liter and for triglycerides from 0.35 to 29.9 g/liter in the diabetic population.

Venous blood was drawn into two separate tubes, one for serum and another for plasma. The latter tube contained disodium ethylenediaminetetraacetate (1 g/liter, final concentration) as anticoagulant. Plasma and serum were separated by low-speed centrifugation at 4 °C and samples were stored at 4 °C. All analyses were done within 48 h of collection.

Separation of HDL. We used the two different precipitating methods with all 47 specimens. For 20 of them, the three methods were used simultaneously. With specimens from six individuals, the two precipitating methods were used simultaneously on plasma and serum obtained from the same blood specimen.

(a) Ultracentrifugation. The nonprotein solvent density of plasma was adjusted to a relative density of 1.063 g/ml with a saturated solution of NaCl (d = 1.18) and the ultracentrifuge tube was filled with a NaCl solution, relative density 1.063. Pycnometry was used in making the final density adjustments.

After centrifugation, the lipoproteins of solvent density 1.063 were removed after slicing the tube in the middle of the clear zone separating the top fraction from the infranatant. The infranatant was removed to a graduated flask, the centrifuge tube walls carefully washed with a sodium chloride solution (d = 1.063 g/ml), and the volume adjusted to the initial volume of plasma.

(b) Sodium phosphotungstate-Mg$^{2+}$ precipitation. We followed the method of Burnstein et al. (8). To 2 ml of serum or plasma we added 200 μl of a sodium phosphotungstate solution [40 g of phosphotungstic acid (Fisher) per liter of a

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\(^{1}\) Nonstandard abbreviations used: HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); VLDL, very-low-density lipoprotein(s).

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mixture of NaOH (1 mol/liter) and distilled water (16/84 by volume) and 50 μl of MgCl₂ (2 mol/liter). After mixing in a vortex-type mixer, the sample was centrifuged (1500 × g, 30 min, 4 °C) and cholesterol determined in the supernate.

(c) Heparin–Mn²⁺ precipitation. We followed the protocol used by the Lipid Research Clinic Program (5). To 2 ml of plasma or serum we added 80 μl of a heparin solution (5000 kilounits/liter) and immediately mixed. We then added 100 μl of MnCl₂ solution (1 mol/liter), also with immediate vortex mixing. After the sample had stood for exactly 30 min in an ice bath, the precipitate was separated by centrifugation (1500 × g, 30 min, 4 °C). Cholesterol was determined in the supernate.

Occasionally, with either precipitation method, we encountered lipemic plasmas with which the supernates were turbid after the initial precipitation. These we diluted before adding the precipitating agents, to more completely precipitate the VLDL and LDL. This turbidity was seen more frequently with the heparin–Mn²⁺ method and rarely with the phosphotungstate–Mg²⁺ precipitation.

In all such cases where a twofold dilution of the plasma was made for one of the methods, the same dilution was also used for the other two methods.

Lipid analyses. Samples were extracted with isopropanol and the extracts assayed for cholesterol and triglyceride in a continuous-flow analyzer (9, 10). HDL cholesterol was measured in a serum/isopropanol (1/10 by volume) extract instead of the usual 1/20 extract.

We used a correction factor of 1.09 for the heparin–Mn²⁺ method and 1.125 for the phosphotungstate–Mg²⁺ method, to correct for the dilution introduced by the precipitating agents (5).

Results

Figure 1 shows our results for HDL-cholesterol on the HDL obtained by both precipitating methods, in 47 subjects. The correlation coefficient (r) was 0.95 (slope, 1.036; y-intercept, -0.00497). The CV for both methods was less than 5% for 31 subjects. For the other 16 the CV was between 5 and 10%.

only one case, for whom the plasma triglycerides concentration was 29.90 g/liter, was the CV significantly greater (22.2%).

The CV was similar in 17 of 47 subjects who showed above-normal lipid concentrations (r = 0.96). In Figure 2 we compare the values of cholesterol content in HDL obtained by ultracentrifugal separation and sodium phosphotungstate–Mg²⁺ precipitation (r = 0.82). Only in four of 20 subjects the CV exceeded 6% but was less than 10%.

Figure 3 shows our results for the values of cholesterol
content in HDL obtained by ultracentrifugal separation and heparin-Mn\(^{2+}\) precipitation \(r = 0.88\). The CV for 11 of 20 subjects was less than 8% and for the remaining ones between 6 and 10%. Table 1 presents the mean values of cholesterol content in HDL separated by the three methods stated. We determined concurrently the cholesterol content of HDL separated by phosphotungstate and heparin methods in the serum and plasma of six subjects. The correlation coefficient was 0.99 and the CV was less than 3%.

### Discussion

Our main purpose was to compare the cholesterol content for HDL separated by different methods.

Our results show similar results for the cholesterol content of HDL separated by phosphotungstate–Mn\(^{2+}\), by heparin–Mn\(^{2+}\), and by ultracentrifugation methods. The CV in individual samples for the cholesterol content of HDL separated by ultracentrifugation and by phosphotungstate–Mg\(^{2+}\) are within the limits of variation expected in duplicate analysis.

There are considerable practical advantages in the phosphotungstate–Mg\(^{2+}\) method. Solutions of sodium phosphotungstate are very stable, and reproducible results are obtained on using the same solution for as long as six months. LDL and VLDL are totally precipitated with undiluted hyperlipemic plasma, while a dilution of twofold is almost invariably needed to obtain adequate precipitation with heparin.

In some cases with very high triglyceride concentrations, heparin precipitation of LDL and VLDL is impossible without a preliminary ultracentrifugation at \(d = 1.006\), to eliminate chylomicrons and VLDL. In contrast, a single twofold dilution will allow total precipitation of LDL, VLDL, and chylomicrons by the phosphotungstate–Mg\(^{2+}\) method.

It seems reasonable to conclude that although the three methods used in this study give comparable results, precipitation with phosphotungstate–Mg\(^{2+}\) is technically simpler and does not require the use of expensive equipment or the commitment of very skillful personnel and as such is a more suitable method for routine procedures.

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### References


