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An Enzymic and Centrifugal Method for Estimating High-Density Lipoprotein Cholesterol

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Enzymic measurement of high-density lipoprotein cholesterol with a centrifugal analyzer is described. We used polyethylene glycol (M_r 6000), final concentration 100 g/L, to precipitate low-density and very-low-density lipoproteins, thereby eliminating the difficulties of the commonly used heparin/Mn^{2+} precipitation method and facilitating the use of ethylenediaminetetraacetate-stabilized plasma. As measured by rocket immunoelectrophoresis, this final concentration of polyethylene glycol completely precipitates β-lipoproteins, leaving the α-lipoproteins in solution. Between-run reproducibility (CV) was 3.6%, within-run reproducibility (CV) 0.8%. Reagent costs currently are $US 0.13 per test and large numbers of samples can be handled conveniently. Normal ranges were compiled for 539 men and 444 women. The high-density lipoprotein cholesterol for men was 1.20 ± 0.31 (SD) mmol/L and for women 1.52 ± 0.38 (SD) mmol/L.

Additional Keyphrases: *normal values* · *centrifugal analyses* · *economics of lab. operation* · *enzymatic methods*

Increasing epidemiological and clinical interest in the estimation of high-density lipoprotein (HDL) or HDL-cholesterol (HDL-C), which correlate negatively with the risk of coronary artery disease (*1–3*), has led to a need for a method capable of handling large workloads conveniently and economically. We have adapted the method of Viikari (*4*) for HDL-C for use in the centrifugal enzymic analysis of cholesterol.

Like other commonly used methods, this one is based on precipitation of lipoproteins other than HDL, followed by measurement of cholesterol in the supernatant fluid after centrifugation. This precipitation is commonly achieved by using divalent cations, such as Mn^{2+} or Mg^{2+}, together with a polyanion such as heparin or dextran sulfate (*5, 6*). However, these techniques depend on the presence of divalent metal ions, and ethylenediaminetetraacetate (EDTA), necessary as a lipoprotein stabilizing agent, must be used with care. Further, Liedtke et al. (*7*) found that the heparin/Mn^{2+} technique interferes with enzymic cholesterol estimation and so we turned to polyethylene glycol (M_r 6000; PEG-6000) as a precipitant. The final procedure is given below.

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Materials and Methods

Apparatus

A CentrifiChem 300 (Union Carbide, Tarrytown, NY) and a Mistral 4L centrifuge (MSE, Crawley, U.K.) were used. PEG-6000 reagent was dispensed with a Compu-Pet Automatic Dilutor (Alphamedics, Levittown, PA). Plasma was dispensed with air-displacement Lancer Pipettes (Sherwood Medical Industries, St. Louis, MO).

Reagents

PEG-6000: Polyethylene glycol, average relative molecular mass 6000 daltons (British Drug House), in distilled water, 200 g/L.

Cholesterol standards: Preciset (Boehringer Mannheim GmbH).

Cholesterol reagents: Cholesterol was measured enzymically with the Boehringer Mannheim "CHOD-PAP Kit" the reaction of the sample with cholesterol esterase and cholesterol oxidase produces hydrogen peroxide, which, in the presence of horseradish peroxidase (EC 1.11.1.7), reacts with 4-aminophenazone and phenol to yield 4-(*p*-benzoquinone-monoimino)phenazone (*8, 9*).

Method

Add 200 μL of EDTA-treated plasma to 200 μL of the PEG-6000 solution in a plastic centrifuge tube, vortex-mix, allow to precipitate for 5 min, then centrifuge at 2000 × *g* for 20 min. The supernate is aspirated with a Pasteur or air-displacement pipette for cholesterol analysis. All of these operations are done at room temperature. The CentrifiChem pipetter is set to take 15 μL of supernate, 45 μL of water, and 350 μL of combined "CHOD-PAP" reagent. The reaction conditions are: temperature, 30 °C; wavelength, 500 nm; initial absorbance reading at 5 s, followed by readings at 2-min intervals for 12 min. Calculate the HDL-C concentration with the use of a 1.30 mmol/L (50 mg/100 mL) and a 2.59 mmol/L (100 mg/100 mL) cholesterol standard, allowing for the two-fold dilution of the original sample.

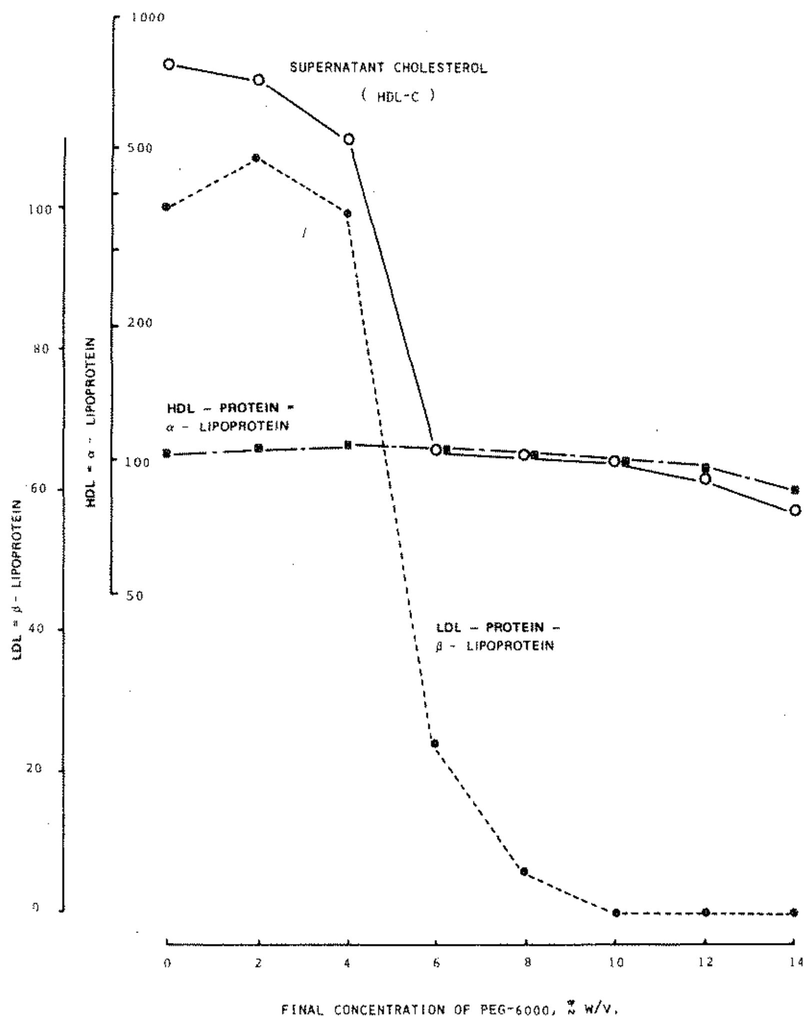

Fig. 1. Lipoprotein present, as a function of polyethylene glycol concentration

Five specimens of EDTA-treated plasma were precipitated with final concentrations of PEG-6000 ranging from 0 to 140 g/L. These specimens had HDL-C varying from 0.33 to 2.18 mmol/L and total cholesterol varying from 5.3 to 7.6 mmol/L. To compare results, the values of HDL-C and α-lipoprotein were expressed as a percentage of the value at 100 g/L PEG-6000. β-lipoprotein is expressed as a percentage of the value at 0 g/L PEG-6000. The means are shown for HDL-C (—); α-lipoprotein (- - -), and β-lipoprotein (- - -)

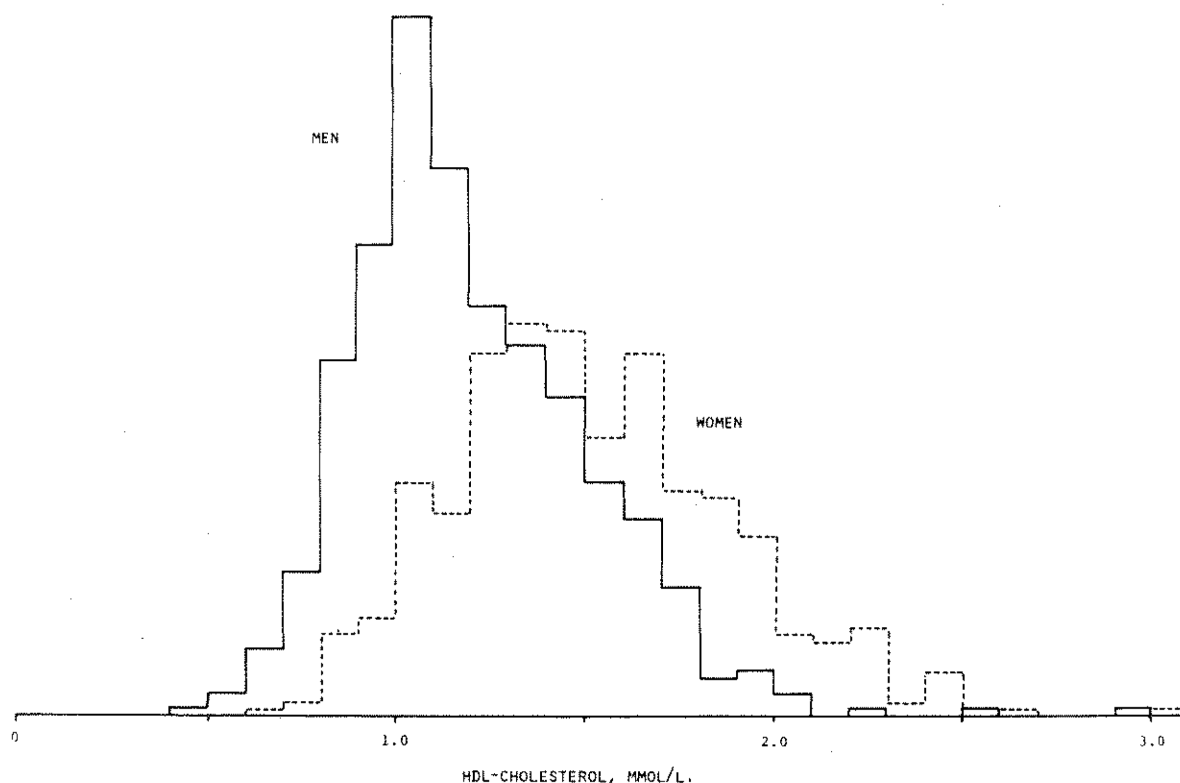

Fig. 2. Distributions of HDL-C concentrations in plasma of 539 men and 444 women attending a multiphasic health-screening center

Table 1. HDL-Cholesterol Concentrations Reported for Several Populations

Population	No.	Mean	SD	Reference range	Skewness	Kurtosis
		mmol/L				
Medicheck, men, ages 19–79	539	1.20	0.31	0.7–1.9	0.86	1.96
Medicheck, women, ages 18–78	444	1.52	0.38	0.9–2.4	0.72	1.37
Framingham Study, men (*3*)	1025	1.19				
Framingham Study, women (*3*)	1445	1.48				
Norwegian men (*11*), ages 40–59	247	1.46	0.31			
Norwegian women (*11*), ages 19–59	23	1.54	0.29			
Trømso, Norway, women (*2*)	11	1.33	0.42			

Results

Choice of PEG-6000 Concentration

Viikari (*4*) used a final concentration of 120 g/L of PEG-6000, but when increasing concentrations of PEG-6000 were used, from 0 to 140 g/L, a plateau in supernate cholesterol concentration was found between 60 and 120 g/L. These supernates were examined by rocket immunoelectrophoresis (*10*) with use of agarose gels dually impregnated with antibodies against human α- and β-lipoproteins (Behringwerke AG). β-Lipoprotein was still present at 60 g/L PEG-6000, with traces remaining at 80 g/L and none at higher concentrations of PEG-6000. α-Lipoprotein was present in all supernates at the concentration found in the original plasma (Figure 1). A final concentration of 100 g/L PEG-6000 was chosen. Because equal volumes of sample and reagent were used, the reagent itself contained 200 g of PEG-6000 per liter.

Precision

Between-run precision was assessed by repeated analysis of the specimens collected for the establishment of reference ranges. These were kept at 4 °C between analyses, which were done on 20 successive working days. Within-run precision was assessed by using a freeze-dried preparation of human origin (Precilip; Boehringer Mannheim). The results were: between-run 1.28 ± 0.046 (SD) mmol/L, CV 3.6%, n = 20; and within-run 0.78 ± 0.006 (SD) mmol/L, CV 0.8%, n = 20.

Cost of Analysis

The direct reagent costs per patient specimen (allowing for a proportion of standards and controls) currently amount to $A 0.12 ($US 0.13). One person can assay 80 specimens in half a working day.

Reference Ranges

These were compiled separately for men and women, from 983 people attending a multiphasic health-screening center (Medicheck Referral Centre, Sydney) who had blood collected for biochemical analyses. All specimens were from people who had been fasting at least 12 h. There were 539 men ranging in age from 19 to 79 years (average age, 43.5 years) and 444 women ranging in age from 17–78 years (average age, 42.0 years). Plasma from blood collected into ethylenediaminetetraacetate was kept at 4 °C until analyzed within three days of collection. The reference range was taken as that range that excluded 2.5% at the upper and 2.5% at the lower boundary. Estimates of mean, standard deviation, skewness, and kurtosis are also given in Table 1. The frequency distributions for men and women are shown in Figure 2. It is interesting to compare these ranges with those reported by others using heparin/Mn^{2+} precipitation, as this gives an assessment of any bias that might be present. Our values are very similar to those found in Framingham, Massachusetts (*3*), as shown in Table 1.

We conclude that this method is both convenient and economical. Results are comparable to those of other methods and the precision more than fulfills the criterion of Tonks (*12*) that the analytical standard deviation should be less than a quarter of the population standard deviation. The use of enzyme reagents is more convenient than the strong acids otherwise necessary and also has the potential advantage that free and esterified cholesterol in HDL could be measured by omitting cholesterol esterase from the reagent.

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References

1. Miller, G. J., and Miller, N. E., Plasma high-density lipoproteins and development of ischaemic heart disease. *Lancet* **i**, 16–19 (1975).

2. Miller, N. E., Forde, O. H., Thelle, D. S., and Nijos, O. D., The Trømso heart-study. High-density lipoprotein and coronary heart disease; a prospective case-control study. *Lancet* **i**, 965–968 (1977).

3. Gordon, T., Castelli, W. P., Hjortland, M. C., et al., High-density lipoprotein as a protective factor against coronary heart disease. *Am. J. Med.* **62**, 707–717 (1977).

4. Viikari, J., Precipitation of plasma lipoproteins by PEG-6000 and its evaluation with electrophoresis and ultra-centrifugation. *Scand. J. Clin. Lab. Invest.* **36**, 265–268 (1976).

5. Albers, J. J., Warnick, G. R., Wiebe, D., et al., Multi-laboratory comparison of three heparin–Mn^{2+} precipitation procedures for estimating cholesterol in high-density lipoprotein. *Clin. Chem.* **24**, 853–856 (1978).

6. Finley, P. R., Schifman, R. B., Williams, R. J., and Lichti, D. A., Cholesterol in high-density lipoprotein: use of Mg^{2+}/dextran sulfate in its enzymic measurement. *Clin. Chem.* **24**, 931–933 (1978).

7. Liedtke, R. J., Busby, B., and Batjer, J. D., Use of the Du Pont *aca* to measure cholesterol in high-density lipoprotein fractions prepared by the heparin/Mn^{2+} precipitation method. *Clin. Chem.* **24**, 161–165 (1978).

8. Röschlau, P., Bernt, E., and Gruber, W., Enzymic determination of total cholesterol in serum. *Abstracts 9th International Congress on Clin. Chem.*, Toronto, Canada, Abstr. no. 1, 1975.

9. Trinder, P., Determination of glucose in blood using glucose-oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.* **6**, 24–26 (1969).

10. Weeke, B., Rocket immunoelectrophoresis. In *A Manual of Quantitative Immunoelectrophoresis Methods and Applications*, N. H. Axelsen, J. Krøll, and B. Weeks, Eds., Universitetsforlaget, Oslo, Norway, 1973, pp 37–46.

11. Enger, S. C., Herbjøensen, K., Erikssen, J., and Fretland, A., High-density lipoproteins (HDL) and physical activity: The influence of physical exercise, age, and smoking on HDL-cholesterol and the HDL/total cholesterol ratio. *Scand. J. Clin. Lab. Invest.* **37**, 251–255 (1977).

12. Tonks, D. B., A study of accuracy and precision of clinical chemistry determinations in 170 laboratories. *Clin. Chem.* **9**, 217–233 (1963).