We describe a method for measuring high-density lipoprotein cholesterol. MgCl₂ and dextran sulfate are used to precipitate all low-density and very-low-density lipoproteins. The supernate contains only high-density lipoproteins, the cholesterol concentration of which is estimated by an enzymic method, with a discrete analyzer (Abbott Bichromatic Analyzer). Concentration and instrument response are linearly related to 50 mg/liter. The precision of the method is excellent in the range of clinical interest (100 to 1000 mg of cholesterol per liter). The precision and efficiency of the precipitation are shown at various concentrations of high-density lipoprotein cholesterol. The method was compared to that of two laboratories in the Cooperative Lipoprotein Phenotyping Study group by testing a number of split samples, and agreement was good.

The direct correlation between an increased concentration of low-density lipoproteins (LDL, \( d = 1.006-1.063 \)) in serum and the risk of developing clinically obvious atherosclerosis, particularly coronary heart disease, is well known (1). Recently, the concentration of high-density lipoprotein cholesterol (HDL cholesterol; \( d = 1.063-1.25 \)) has been found to correlate strikingly and inversely with coronary heart disease (2, 3). The recently published report (1) of the Cooperative Lipoprotein Phenotyping Study involving 6859 individuals from five different populations demonstrated this very strong association, and another epidemiological study (4) in Tromsø, Norway, of 6596 men 20 to 49 years old strengthens the conclusion that the inverse relationship is significant, more so than the direct correlation with LDL cholesterol (5). Thus a low HDL cholesterol concentration may be a common antecedent of clinically apparent coronary heart disease, and may accelerate the progress of coronary atherosclerosis.

The mechanism of the protective role that HDL cholesterol evidently plays may be that it facilitates the uptake of cholesterol from peripheral tissues, especially the arterial wall, and its transport to the liver, where it undergoes catabolism and excretion (6); it has also been suggested that HDL inhibits the uptake of cholesterol-rich LDL, and partly suppresses the net increment in cell sterol content induced by LDL, particularly in the walls of arteries (7).

We describe a method in which the LDL and VLDL are precipitated from serum with MgCl₂ and dextran sulfate. The HDL remains in the supernate. The cholesterol content of the supernate, and hence the HDL cholesterol, is measured by the enzymic method of Allain et al. (8), with use of the ABA Bichromatic Analyzer. Concentration and instrument response are linearly related up to 50 mg of cholesterol per liter. The efficiency of separation was shown by lipoprotein electrophoresis. Precision studies were undertaken at various concentrations of cholesterol, both in unFractionated and fractionated samples. Also, we measured HDL cholesterol in patients' samples obtained from a laboratory in the Cooperative Lipoprotein Phenotyping Study Group.

Materials and Methods

Equipment

The ABA-100 Bichromatic Analyzer (Abbott Laboratories, Diagnostic Division, South Pasadena, Calif. 91030) and the Beckman J-6 Refrigerated Centrifuge (Beckman Instruments, Inc., Palo Alto, Calif. 94304) were used.

Reagents

Magnesium chloride, 2.0 mol/liter (Mallinckrodt Inc., St. Louis, Mo. 63160). Stable for six months at 4 °C in an amber-colored glass bottle.

Dextran sulfate, M₆₅₀, 000, 20 g/liter (Sigma Chemical Co., St. Louis, Mo. 63178). Stable for six months at 4 °C in an amber-colored glass bottle.

ABA Agent Cholesterol Reagent (Abbott Laboratories, Diagnostic Division, South Pasadena, Calif. 91030).

Cholesterol standard, 500 mg/liter (Boehringer Mannheim Corp., New York, N.Y. 10017; cat. no. 125912).

Procedure

To 0.5 ml of sample, add 25 μl of MgCl₂ (2 mol/liter) and 25 μl of dextran sulfate (20 g/liter).³ Vortex-mix immediately for 3 s. Let tubes set for 5 min at 25 °C. Centrifuge for 30 min at 4 °C and 2500 rpm. Perform a cholesterol analysis on the supernatant fraction, using the ABA Bichromatic Analyzer and ABA Agent Cholesterol Reagent. Multiply the result by 1.1, to correct for dilution.

We modified the routine ABA cholesterol method in order to increase absorbance and consequently the sensitivity. This was done by using the 1:51 syringe plate (instead of the 1:101 plate) and by increasing the sample size from 5 to 10 μl.

³ Preliminary experiments show that the MgCl₂ and dextran sulfate may be mixed in equal volumes and stored at 25 °C. The mixture is added to serum for the precipitation step (add 50 μl of the mixture). Such a stored mixture appears to be suitable for use in the test for as long as two months.
Results

Precision

The intra- and inter-assay precision of the 500 mg/liter cholesterol standard in absorbance units (±SD) was, respectively: 0.131 ± 0.0018 (CV, 1.37%; n = 30), and 0.131 ± 0.0021 (CV, 1.6%; n = 35). We examined the precision of a control pool at various concentrations of cholesterol. Ortho Normal serum (cholesterol concentration of 1500 mg/liter) was diluted to give several lower concentrations. Inter-assay variances (±SD) were as follows (mg/liter): 1501 ± 31.5 (CV, 2.1%), 748 ± 14.2 (CV, 1.9%), 376 ± 7.9 (CV, 2.1%), 186 ± 3.6 (CV, 1.95%), 94 ± 2.1 (CV, 2.2%), and 51 ± 1.6 (CV, 3.14%). Thirty replicates over 30 testing days were assayed. The enzymic cholesterol method is apparently linear to at least 50 mg/liter.

The reproducibility of the precipitation technique was studied by assaying a pooled sample of normal sera, and the same pool diluted two- and fourfold with 0.155 mol/liter NaCl. Replicates of the undiluted and diluted pools were subjected to precipitation and measurement of supernatant cholesterol. Intra-assay (n = 30) and inter-assay (n = 30) over 30 testing days) precision showed, respectively (HDL cholesterol, mg/liter): 395 ± 6.7 (CV, 1.7%), 196 ± 3.8 (CV, 1.9%), 101 ± 2.5 (CV, 2.5%), and 396 ± 6.9 (CV, 1.7%), 197 ± 3.9 (CV, 2.0%), 93 ± 2.7 (CV, 2.9%).

Accuracy

The predictive studies of the relation between HDL cholesterol and coronary heart disease are largely the result of the work of Castelli et al. (1). We, therefore, desired to check the validity of our method with samples that had been assayed by that group. Dr. Castelli kindly supplied us with 20 samples of serum that he had tested in his laboratory. The concentrations of HDL cholesterol ranged from 230 to 680 mg/liter. Comparison with our method gave the following least squares regression: y = 1.01x + 6.2 (mg/liter); x = Castelli samples, y = dextran sulfate-Mg²⁺ method, Sₓ = 14.2 mg/liter; r = .981.

We sent 30 patients' samples for HDL cholesterol analysis to Bio-Science Laboratories, Van Nuys, Calif. 91405, which has standardized their procedure with the Cooperative Lipoprotein Phenotyping Study laboratories. (Bio-Science measures HDL cholesterol on the iso-propanol extract of a manganese-heparin supernate.) The range of concentrations was 140 to 890 mg/liter. A regression analysis of the data obtained using our method (y) compared to those of Bio-Science Laboratories (x) showed: y = 0.9887x + 8.6 (mg/liter); Sₓ = 16.2 mg/liter; r = .9783.

Completeness of precipitation by Mg²⁺-dextran sulfate was demonstrated by the absence of the beta and pre-beta bands and the presence of only the alpha band, when the supernatant was subjected to lipoprotein electrophoresis. When the precipitate was redissolved and subjected to electrophoresis, only the beta and pre-beta bands were seen.

Ten patients' samples were fractionated; analysis of the supernatant (HDL) cholesterol and the redissolved precipitate (VLDL, LDL) cholesterol showed the combined cholesterol content of both fractions approximated the cholesterol concentration measured in the unfractionated serum (range: 96 to 102%).

Stability

Samples may be kept at 4 °C for two weeks, and at -20 °C for three months with no alteration of HDL cholesterol. Also, the supernate may be analyzed after two weeks at 4 °C or three months at -20 °C. Both the native serum and the supernate may be stored for one week at 25 °C with no change in HDL concentration.

Discussion

The method of precipitation recommended by the Lipid Research Clinics Program (Center for Disease Control, Atlanta, Ga.) is that in which MnCl₂ and heparin are used (9, 10); the cholesterol in the supernate is measured by using a Liebermann-Burchard reaction after extraction with isopropanol. Bachorik et al. (11) used MnCl₂ and heparin, and measured the HDL cholesterol by a Liebermann-Burchard reaction. Comparison with patients' samples measured by ultracentrifugation was satisfactory. The use of Mn⁴⁺ has been criticized because it produces complexes of varying insolubility between HDL and heparin in isolated fractions (12), and the degree of insoluble complex formation is determined by the relative proportion of the HDL subclases. This effect is not seen with Mg²⁺ and Ca²⁺ (12). However, Bachorik et al. (11) have shown that Mn²⁺-heparin precipitation of native, unfractionated plasma does effectively precipitate LDL and VLDL, but not HDL. Further studies by Bachorik et al. (13) confirm a close correspondence between results by the Mn²⁺-heparin and ultracentrifugation procedures, which led them to conclude that there is no significant loss of HDL when unfractionated plasma is used. The observation that isolated HDL appears to behave differently from that in unfractionated plasma, in the presence of Mn²⁺-heparin, remains unexplained (11-13).

If an enzymic technique is used to measure the HDL cholesterol, the Mn²⁺ forms a visible precipitate with enzymic reagents, and also contributes a variable and significant blank (14). Variation in the strength of various lots of heparin has also been put forward as a disadvantage. Steele et al. (15), using ethylenediaminetetraacetate (4 mmol/liter) to make up the reagent (Agent Cholesterol), eliminated the formation of the interfering precipitate. Lopes-Virella et al. (14) reported on the use of sodium phosphotungstate and MgCl₂ as the precipitating agent.

Dextran sulfate, as the polyanion, has been used in the past (16, 17), together with Mn²⁺, Mg²⁺, or Ca²⁺. Kostner (18) described precipitation with dextran sulfate and MgCl₂; the HDL cholesterol was measured enzymically (using Boehringer-Mannheim Reagents) on a two-channel AutoAnalyzer (Technicon).

None of the reports presents data concerning reproducibility and linearity of the cholesterol methods and the HDL cholesterol precipitation at very low concentrations. The range of HDL cholesterol concentrations encountered in clinical samples is from 100 to 1000 mg/liter, and it is important to know the accuracy and precision of the technique, especially in the highly significant lower range, from 100 to 300 mg/liter.

The MgCl₂-dextran sulfate technique with subsequent enzymic cholesterol assay is highly reproducible, and is not affected by a turbid supernate, as may result with the Mn²⁺-heparin or Mg²⁺-phosphotungstate methods (15). Also, the Mn²⁺-heparin requires that the sample, after the precipitating reagents are added, be placed in an ice-bath for 30 min. The precipitation technique proposed is highly efficient. Sera with triglyceride concentrations as high as 140 g/liter have been successfully fractionated. If Mn²⁺-heparin is used, sera with triglyceride concentrations of more than 4 g/liter may require prior dilution before precipitation (11, 15).

Excellent linearity and precision were demonstrated over the range of clinical interest, both in reference to the cholesterol method itself and to the supernatant HDL cholesterol. The reagents used (Mg²⁺-dextran sulfate) produce no discernible blank effect. Good correlation of HDL cholesterol concentration in patients' samples tested by us and two laboratories in the Lipid Research Clinics Program was obtained. The
the proposed method may therefore be used confidently in prediction of coronary heart disease, according to the criteria of the Cooperative Lipoprotein Phenotyping Study Group (1).

References

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Increased Renin Concentration in Plasma and Amniotic Fluid During Storage

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Renin concentration was determined in plasma and amniotic fluid samples immediately after collection, after storage at −18 °C for up to 48 h, and after storage at 5 °C for as long as 48 h. Renin concentration increased, both in plasma (P < 0.05) and amniotic fluid (P < 0.02), after the storage at 5 °C, but no significant alteration occurred on storage at −18 °C. Partial activation of inactive renin could explain the observed increase in renin concentration at 5 °C.

Renin concentration is determined indirectly by measuring angiotensin I generated from exogenous substrate in the presence of angiotensinase inhibitors. There are variations among methods with regard to the pH and duration of incubation, selection of angiotensinase (EC 3.4.99.1) inhibitors, types of substrate used, and the possible inclusion of one or more periods of dialysis. Dialysis to low pH to remove exogenous substrate and angiotensinase activity before assay at pH 7.5 has been used (1) but was later shown to increase the rate of angiotensin I generation, possibly due to the irreversible activation of an inactive form of renin (2). Inactive and active renin have been determined both in plasma (3–5) and amniotic fluid (6). The physiological importance of the inactive form and the mechanism of conversion have still to be elucidated.

Increased rates of angiotensin I generation after storage of plasma at −20 and 4 °C have been reported (6, 7), respectively, although conflicting reports suggest that samples can be stored at −20 °C for short periods before assay (8, 9).

We have assessed the effect of time and temperature on the short-term storage of samples of both plasma and amniotic fluid before assay of renin concentration.

Methods

Blood samples were collected, with disodium ethylenediaminetetraacetate as anticoagulant, into iced tubes from 20