New Micromethod for Measuring Cholesterol in Plasma Lipoprotein Fractions

Thomas J. Bronzert and H. Bryan Brewer, Jr.

A method is described for the reliable, fast, and relatively inexpensive fractionation of plasma lipoproteins and quantitation of their cholesterol content. This procedure requires 350 μl of plasma and can be completed within 3 h. Plasma lipoproteins (175 μl of plasma) were pre-stained with Fat Red 7B and centrifuged (Beckman Airfuge) at plasma density \( (d = 1.006 \text{ kg/liter}) \) and at a solvent density of 1.060 kg/liter, adjusted by adding solid KBr. Pre-stained centrifuged samples demonstrated the characteristic elevation of chylomicrons in phenotypes I and V, low-density lipoproteins of phenotype II, very-low-density lipoproteins in phenotype IV and V, and a continuum of pink color throughout the centrifuge tube, diagnostic of the floating beta lipoprotein of type III. Centrifu-ged samples were separated into top and bottom fractions by aspiration. Cholesterol was quantitated with an enzymic oxygen-electrode analyzer (Beckman Cholesterol Analyzer). Correlation coefficients between cholesterol values for plasma from normal and hyperlipidemic individuals obtained with the Beckman Analyzer vs. the Technicon AutoAnalyzer II and SMAC systems were 0.977 and 0.973, respectively.

Additional Keyphrases: centrifugation of density-supplemented plasma · categorization of hyperlipoproteinemia · heart disease · diagnostic aids · intermethod comparison

Hypercholesterolemia is a well-recognized risk factor for the development of atherosclerosis. The above-normal plasma cholesterol associated with premature cardiovascular disease is carried primarily with the low-density lipoproteins (LDL)

\(^{(1)}\) (1). Recently, retrospective analysis of epidemiological data has established an inverse correlation between decreased cholesterol concentrations in HDL and premature heart disease (2–5). It is apparent that if individuals with an increased risk of premature heart disease are to be clearly identified, cholesterol concentrations in both HDL and LDL must be ascertained.

Currently, plasma lipoproteins are measured by methods standardized by the LRC (6). Plasma lipoproteins are fractionated by ultracentrifugation (5 ml of plasma) and precipitated with heparin/manganese chloride (2 ml of plasma). The cholesterol in plasma and lipoprotein fractions is quantitated with a Technicon AutoAnalyzer II with use of the Liebermann–Burchard reagent after zeolite isopropranol extraction. The LRC methodology has been used extensively to identify patients with hyperlipoproteinemia and to classify these patients into phenotypes I–V (7). However, the cost of the equipment and time required for analysis makes this methodology impractical for the clinical laboratory.

Materials and Methods

Plasma Samples

Plasma collected for analysis was obtained by venipuncture from fasting (12–16 h) subjects and anticoagulated with disodium edetate (1 g/liter). Samples were cooled on ice immediately after collection and centrifuged (IEC Model PR-2; International Equipment Co., Needham Heights, Mass. 02194) at 1500 × g for 30 min at 4 °C. Centrifuged plasma was separated from cells with a Pasteur pipette, transferred to 15-ml screw-cap test tubes, and stored in the dark at 4 °C within 3 h of collection. Plasma lipoprotein fractionation was not affected by storage of the sample for as long as three weeks. Patients with hyperlipidemia were classified into lipoprotein phenotypes I–V by using the procedures of electrophoresis and lipoprotein fractionation detailed in the LRC manual (6).

Cholesterol Analysis

Equipment. Cholesterol was determined with the Technicon AutoAnalyzer II and SMAC systems (Tech
Fig. 1. Schematic of the adjustable two-level aspiration device used to separate the top plasma fraction from six centrifuged samples

Fig. 2. Visual banding obtained after centrifugation of Fat Red 7B prestained plasma lipoproteins

nicon Instruments Corp., Tarrytown, N. Y. 10591) and a Beckman Cholesterol Analyzer (Beckman Instruments Inc., Fullerton, Calif. 92634). The Beckman Analyzer is an enzymic oxygen-electrode cholesterol analyzer. In this system cholesterol esterase (EC 3.1.1.13) is used to hydrolyze cholesterol esters to free cholesterol; cholesterol is oxidized with cholesterol oxidase (EC 1.1.3.6) to cholest-4-en-3-one and hydrogen peroxide. Unlike other enzymic systems (8–17), this analyzer measures the rate (rather than the endpoint) of oxygen consumption with an oxygen electrode in place of a hydrogen peroxide-coupled reaction (11).

Reagents

Cholesterol oxidase, 60 U/vial, 6.0 × 10^4 U/liter, Beckman Instruments. Distilled water, 600 µl, was added to the lyophilized powder as supplied by the manufacturer.

Cholesterol esterase, 200 U/vial, 2.0 × 10^5 U/liter, Beckman Instruments. The cholesterol esterase was used directly in liquid form as supplied by the manufacturer.

Mixed enzyme reagent. The mixed enzyme solution was prepared by adding 600 µl of the cholesterol oxidase solution to 400 µl of the cholesterol esterase solution.

N indicates a normal plasma while I-V are plasmas which illustrate the lipoprotein distribution of their respective phenotype. The top panel contains plasma centrifuged at plasma density (d = 1.006 kg/liter) while the plasma of the bottom panel was adjusted to 1.060 kg/liter by adding solid KBr. The dotted lines indicate the level of aspiration (26%, top panel; 46%, bottom panel) to separate the fractionated lipoproteins. See the text for the significance of the staining variations illustrated.
The trifuged plasma Fig. 1. Abundant concentrations of lipoproteins obtained by centrifugation is shown below the electrophoretograms. See the text for procedures.

Observations of note for phenotype: (I) chylomicrons at origin of electrophoretogram and on side and top of centrifuge tube; (II) heavy LDL band in both electrophoretogram and tube; (III) the broad band between LDL and VLDL on electrophoretogram and between VLDL and LDL of the centrifuged sample; (IV) the abundant VLDL in both the electrophoretogram and tube; (V) the elevated concentrations of chylomicrons and VLDL seen by both techniques.

Fig. 4. (bottom left) Agarose electrophoresis performed on whole plasma (P) and the top (T) and bottom (B) fractions of the centrifuged samples from a patient with type I phenotype. The dotted lines indicate the removal of 26 and 46% of the samples centrifuged at plasma density and at a density of 1.060 kg/liter, respectively. Note the removal of chylomicrons from the bottom fraction at plasma density and only the HDL remaining in the bottom of the 1.060 kg/liter tube.

Fig. 5. (top right) Agarose electrophoresis performed on whole and fractionated plasma from a patient with type II phenotype. See legend to Figure 4 for abbreviations. Of importance is the removal of the supranormal LDL from the top of the 1.006 kg/liter tube and from the bottom of the 1.060 kg/liter tube. Therefore, the increased concentration of cholesteryl-rich LDL did not affect the measurement of HDL cholesterol.

Fig. 6. (bottom right) Agarose electrophoresis performed on whole and fractionated plasma from a patient with type III phenotype. See legend to Figure 4 for abbreviations. The 1.006 kg/liter top fraction contains a lipoprotein which floated between VLDL and LDL in the centrifuged sample ("floating beta"), but which migrated electrophoretically within the LDL region. This "floating LDL" as well as normal LDL is completely removed by aspiration of the top 46% of the sample centrifuged at density 1.060 kg/liter.
Table 1. Within-Run Precision of Beckman Cholesterol Analyzer

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<tr>
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<th>Beckman SD</th>
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<td>273</td>
<td>5.1</td>
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</table>

Procedure

Cholesterol was determined with the Beckman Cholesterol Analyzer by directly adding 10 µl of the mixed enzyme reagent to the reaction chamber or as 10 µl of mixed enzyme reagent in 1 ml of sodium phosphate buffer (1 mol/liter, pH 6.0). The concentrated mixed enzyme solution was stable for at least one week at 4°C; the diluted enzyme reagent in phosphate buffer was stable for about 4 h at 37°C.

The Beckman Cholesterol Analyzer was calibrated with an aqueous cholesterol standard (2000 mg/liter, Beckman Instruments) and with three plasma pools prepared in our laboratory (950, 2370, and 3150 mg of cholesterol per liter). Five-microliter samples were routinely used for cholesterol determination; however, sample volumes up to 50 µl can be used for measurement of hypolipidemic samples such as those obtained from patients with Tangier disease or abetalipoproteinemia.

Cholesterol was also determined with the Technicon AutoAnalyzer II (6, 18–21) and SMAC systems (18–21). In order to compare cholesterol values from the three instruments, a cholesterol calibration plasma (2960 mg/liter) from the Center for Disease Control, Atlanta, Ga., was used to calibrate them all. Cholesterol standards in organic reagents, routinely used in the AutoAnalyzer II, are not suitable for the aqueous systems used in the Beckman Analyzer and Technicon SMAC.

Plasma Centrifugation and Lipoprotein Fractionation

Equipment. Plasma was centrifuged in an air-driven centrifuge (Airfuge; Beckman Instruments, Palo Alto, Calif. 94304). The rotor holds six cellulose tubes, each containing 175 µl. The centrifuge can achieve 100 000 rpm (160 000 x g) in less than 1 min.

After centrifugation, the lipoproteins were separated into top and bottom tube fractions by aspiration (Beckman Airfuge Tube Fractionator; Beckman Instruments, Palo Alto). A schematic of the aspirator used in these studies is shown in Figure 1. The turret holds six samples. An aspiration rate of 1–1.2 ml/s resulted in a clear separation of aspirated sample from the infranatant fraction. The aspirated sample may be collected for analysis in a 1.5-ml plastic tube (Brinkmann Instruments Inc., Westbury, N. Y. 11590). Two set screws permit adjustment of the aspirator tubing to different levels within the tube for aspiration of various quantities of the centrifuged sample.

Reagents

Prestaining solution. Fat Red 7B (Sigma Chemical Co., St. Louis, Mo. 63178) in dimethylformamide (25 µl, 5 mg/liter) was activated just before use by adding 5 µl of NaOH, 0.1 mol/liter, and one drop of the surfactant Triton X-100 (22, 23). Activated Fat Red 7B is also available from Beckman Instruments.

KBr solution. Potassium bromide in distilled water, 2.32 mol/liter.

KBr tubes. KBr solution (50 µl, 13.8 mg KBr) was pipetted into 175-µl Airfuge tubes (Beckman Instruments), lyophilized, and capped to maintain dryness. The addition of 13.8 mg of KBr adjusts 175 µl of plasma (assume a density of 1.006 kg/liter) to a solvent density of 1.060 kg/liter.

Procedure

Plasma lipoproteins were prestained by adding 1 µl of prestaining solution to 175 µl of plasma. These stained aliquots were centrifuged at plasma density, and at a solvent density of 1.060 kg/liter, to permit fractionation of lipoproteins for cholesterol quantitation (23–27). After mixing by inversion, all samples were centrifuged at 100 000 rpm for 2.5 h at 25°C. After centrifugation, the stained, fractionated lipoproteins were viewed and observations recorded. Samples centrifuged at both densities were transferred to the aspirator for separation of ultracentrifugal top and bottom fractions. Plasma centrifuged at plasma density contains chylomicrons and VLDL in the top, and LDL and HDL in the bottom fraction. Plasma centrifuged at a solvent density of 1.060 kg/liter contains chylomicrons, VLDL, and LDL in the top, and HDL in the bottom fraction. Plasma lipoproteins were quantified by measuring the cholesterol content of whole plasma and the infranatant fraction of samples centrifuged at a density of plasma and 1.060 kg/liter. The cholesterol content in each of the plasma lipoprotein fractions is calculated as follows: HDL cholesterol is quantitated in the infranate of plasma centrifuged at density 1.060 kg/liter; LDL cholesterol equals the value obtained from the infranate of plasma centrifuged at plasma density (LDL plus HDL) minus the value for HDL cholesterol; VLDL cholesterol equals total plasma cholesterol minus the value for LDL plus that for HDL cholesterol. The presence of chylomicrons was ascertained by inspection of whole plasma stored at 4°C overnight, and by the appearance of chylomicrons in the ultracentrifugal top fraction of plasma centrifuged at plasma density (see Results section). The top fractions of centrifuged samples can be collected during aspiration (Figure 1); these fractions may be used for additional cholesterol determinations or lipoprotein electrophoresis.

Electrophoresis

Equipment. For agarose electrophoresis we used
slides, applicators, electrophoresis cell, and power supply from Bio-Rad Laboratories, Richmond, Calif. 94804. Densitometric tracings were obtained with an integrating densitometer (Model ADC-15; Gelman Instrument Co., Ann Arbor, Mich. 48106).

Reagents

Agarose electrophoresis buffer. The “Bio-Gram A” agarose slides were equilibrated for 24 h at 4 °C in 6 g/liter bovine albumin (Cohn Fraction V) in Bio-Rad Barbital Immunoelctrophoresis Buffer III, pH 8.6, which contains, per liter, 50 mmol of sodium barbital, 30 mmol of calcium lactate, 4.0 g of sodium azide, and 150 mmol of tromethamine (2-amino-2-hydroxymethyl-1,3-propanediol).

Agarose electrophoresis buffer. “Bio-Gram A” electrophoresis buffer was diluted twofold with distilled water (final barbital concentration, 25 mmol/liter).

Staining reagents. Sudan Black B stain and fixing and destaining solutions were prepared and used according to the manufacturer’s (Bio-Rad) instructions for lipoprotein electrophoresis.

Procedure

Paper electrophoresis was performed as outlined in the LRC manual (6). For agarose electrophoresis, presoaked agarose slides were drained of excess solution for 25 min and 3 µl of sample was applied with a plastic pronged applicator supplied by the manufacturer. Samples were electrophoresed at 150 V for 20–25 min at 25 °C. Electrophoresis at 4 °C improved the definition of the lipoprotein bands, but extended the duration of electrophoresis to about 40 min. Fixing, staining with Sudan Black B, and destaining were performed as outlined by the manufacturer (Bio-Rad). Densitometric scanning and integration were obtained at 590 nm with a 0.5 mm slit.

Heparin/Manganese Chloride Precipitation of Plasma for the Separation of HDL Cholesterol

Equipment. Airfuge (Beckman Instruments).

Reagents

Manganese chloride solution. Manganese chloride in distilled water, 0.5 mol/liter.

Heparin solution, 1.0 × 10⁻⁶ int. units/liter.

Procedure

Manganese chloride-containing tubes were prepared by adding 15 µl of the manganese chloride solution to an Airfuge tube, drying under reduced pressure, and capping. Cold plasma, 150 µl, was pipetted into these tubes and mixed. Cold heparin solution, 25 µl, was added and the contents of the tube were remixed. The centrifuge tubes were transferred to the rotor and placed on ice for 30 min while the heparin/manganese chloride solution precipitated VLDL and LDL. The samples were then centrifuged at 100 000 rpm for 5 min. HDL cholesterol was measured in the supernate pipetted directly from these tubes. Cholesterol values were multiplied by 1.17 to correct for dilution.

Results

Cholesterol Determinations

The within-run precision of the Beckman Cholesterol Analyzer was determined by repetitive analyses of three pooled plasma controls prepared in our laboratory. The mean values (n = 35) with one standard deviation and the coefficients of variation (CV) for the three pools were as follows: 950 ± 20 mg/liter, CV 2.1%; 2370 ± 50 mg/liter, CV 2.1%; and 3150 ± 70 mg/liter, CV 2.2%. Day-to-day precision was determined by daily analyses of one of these controls. For n = 35, the mean and SD was 2371 ± 85 mg/liter, with a CV of 3.6%. The concentration and instrumental response were linearly related from 200 to 4000 mg of cholesterol per liter (1 to 80 µg per 5 µl of sample) and cholesterol values were not altered with changes in sample volume from 1 to 50 µl. The ability to use a wide range of sample volumes permitted the re-analysis of samples with abnormally high or low cholesterol values without the need for dilution or concentration of the original sample. The precision of the Beckman Analyzer was also determined by using samples from patients with Tangier disease and lipoprotein phenotypes I–V. Measurements of these samples would indicate whether precision is affected by above-normal concentrations of chylomicrons (type I and V), LDL (type II), floating beta (type III), or VLDL (types IV and V). Table 1 shows results of analysis for cholesterol in samples from these patients.

We compared the Beckman Analyzer with the Liebermann-Burchard reaction assays used in the Technicon AutoAnalyzer II (isopropanol extracted plasma) and SMAC (unextracted plasma) systems. All instruments were calibrated with a cholesterol calibration plasma supplied by the Center for Disease Control, having a stated value of 2960 mg of cholesterol per liter. Results of the Beckman Analyzer total-cholesterol method correlated well with those by the AutoAnalyzer II method (total cholesterol, range 1380–2840 mg/liter, n = 44, yextr = 1.028xAA-11 + 45; r = 0.977), and with those results by the SMAC system (total cholesterol, range 1380–2840 mg/liter, n = 44, yextr = 1.039xSMAC + 12; r = 0.973). For comparison, the AutoAnalyzer II method correlated with the SMAC system (total cholesterol, range 1380–2840 mg/liter, n = 44, yAA-II = 1.015xSMAC - 41; r = 0.993).

These results indicate that the Beckman Cholesterol Analyzer is an acceptable substitute for the AutoAnalyzer II used in the LRC method for cholesterol quantitation. The response of the Beckman Cholesterol Analyzer is linear from 200–4000 mg of cholesterol per liter, it can use a sample volume of 1–50 µl, and it has good precision with samples of various lipid composition, including lipoprotein phenotypes I–V.

Plasma Centrifugation and Lipoprotein Fractionation

Samples were centrifuged for 1–18 h. An optimal time of 2.5 h was ascertained by viewing the separation of the
prestained lipoproteins, and by electrophoresis and cholesterol quantitation of the separated fractions. Prestained plasma from a normal individual (N) and from patients with lipoprotein phenotypes I–V, centrifuged for 2.5 h at 100,000 rpm and 25 °C, are illustrated in Figure 2. Prestained plasma (normal subject) centrifuged at plasma density (d = 1.006 kg/liter) contained a trace of VLDL at the top of the tube and an intensely stained band containing LDL and HDL at the bottom of the tube (Figure 2). The plasma sample centrifuged at a solvent density of 1.060 kg/liter had a dense band at the top, containing VLDL plus LDL, and a faintly stained band of HDL in the lower section of the tube. Each centrifuged plasma sample of phenotypes I–V demonstrated an intensely stained band characteristic of the increase in the specific lipoprotein fraction pathognomonic of the individual phenotype. Samples from patients with phenotypes I and V (fasting hypercholesterolemia) showed an intensely stained band at the top of the tubes centrifuged at both densities. Patients with phenotype II had intensely stained bands containing LDL; prestained samples, centrifuged at plasma density, from patients with type III phenotype revealed a unique diffuse staining between VLDL and LDL due to the presence of floating beta lipoprotein, the hallmark of the type III phenotype. This diffusely stained band between VLDL and LDL was only seen in those patients with hypertriglyceridemia, was used in conjunction with electrophoresis (Figure 3) to classify patients into phenotypes I–V.

Plasma lipoproteins were quantitated by measuring the cholesterol content of whole plasma, and the bottom fraction of samples centrifuged at a density of plasma and 1.060 kg/liter. Centrifuged samples were separated into top and bottom fractions by aspiration, since tube slicing was difficult to perform without disturbing the infranate. Samples were aspirated at 5% increments throughout the centrifuged sample in order to determine the correct depth of aspiration. The sample was then inspected for the presence of prestained lipoproteins, or alternatively, a sample not prestained with Fat Red 7B was analyzed by agarose electrophoresis. Aspiration of 26% of the top fraction of the sample centrifuged at plasma density completely removed the chylomicrons and VLDL; aspiration of the top 46% of the sample centrifuged at a density of 1.060 kg/liter removed chylomicrons, VLDL, and LDL, leaving only HDL in the infranatant fraction (in Figure 2, the level of aspiration for each centrifuged sample is indicated by the dashed line).

The precision of the aspiration technique was determined by gravimetric analysis of the top and bottom fractions (Table 2). Mean analytical recovery was >98% with a CV of <0.5%. The small percentage of sample lost is due to the plasma remaining in the transfer tubing.

This loss did not affect the cholesterol quantitation since only the bottom fractions were analyzed. Because centrifugation concentrates the lipoprotein fractions, any measurement of cholesterol within these fractions must be corrected to the whole plasma concentration. Removing 26% from the top of the sample centrifuged at plasma density will concentrate the cholesterol of the VLDL fraction into 26% of the original volume. Therefore, the cholesterol concentration measured must be multiplied by 0.26 in order to obtain the VLDL cholesterol at plasma concentration. Similar calculations for the other top and bottom fractions obtained after aspiration would include multiplying by: 0.54 to correct the HDL cholesterol found in the bottom of the tube centrifuged at 1.060 kg/liter; 0.74 to correct the LDL plus HDL cholesterol found in the bottom of the plasma-density centrifuged tube; and 0.46 to correct the VLDL plus LDL cholesterol found in the top of the tube centrifuged at 1.060 kg/liter.

The effectiveness of the lipoprotein fractionation by the Airfuge/aspiration technique was evaluated by analyzing plasma containing above-normal concentrations of chylomicrons (type I phenotype), LDL (type II phenotype), and floating beta lipoprotein (type III phenotype). Figures 4–6 illustrate the results obtained on samples from patients with phenotypes I, II, and III, which were centrifuged at the density of plasma and 1.060 kg/liter, and also the electrophoretograms of whole plasma (P) and top (T) and bottom (B) fractions of each centrifuged sample. The depth of aspiration for the samples centrifuged at a density of plasma (26%) and 1.060 kg/liter (46%) is indicated by the dashed lines. Agarose electrophoresis of the bottom fraction of a sample centrifuged at plasma density from a patient with type I phenotype (total cholesterol, 2.661 g/liter; triglyceride, 15.295 g/liter) revealed no chylomicrons, indicating that aspiration of the sample had effectively removed the greater-than-normal concentration of chylomicrons characteristic of this disorder (Figure 4). No significant contamination by LDL was detected in the electrophoretogram of the bottom fraction of a sample centrifuged at a density of 1.060 kg/liter from a patient with phenotype II (total cholesterol, 4.166 g/liter; triglyceride, 1.680 g/liter) (Figure 5). Centrifugation of prestained plasma from a patient with type III phenotype (total cholesterol, 2.070 g/liter; triglyceride, 2.054 g/liter; LDL cholesterol to plasma triglyceride ratio, 0.38) showed diffuse staining between VLDL and LDL (Figure 6). Upon electrophoresis of the top fraction, this sample showed the "floating beta lipoprotein"

| Table 2. Gravimetric Analysis of Sample Recovery by Aspiration (n = 6) |
|------------------------|--------|--------|--------|
| Aspirator setting, %    | 20     | 30     | 40     |
| Top fraction, %         | 21.2   | 31.5   | 43.5   |
| Bottom fraction, %      | 77.2   | 66.6   | 54.5   |
| Mean anal. recovery, %  | 98.5   | 98.2   | 98.0   |
| SD                     | 0.4    | 0.2    | 0.1    |
| CV, %                  | 0.5    | 0.2    | 0.1    |
A. Type III plasma

\[
\beta \quad \text{pre-} \beta \quad \alpha
\]

B. \( \rho = 1.006 \) Top

C. \( \rho = 1.006 \) Bottom

Fig. 7. Densitometric scan of the three electrophoretic patterns obtained with whole and fractionated plasma of Figure 6 (4) Whole plasma from a patient with type III phenotype showing an almost normal lipoprotein profile. (5) The same plasma showing "beta lipoprotein" in the top fraction of the sample centrifuged at plasma density (\( \rho = 1.006 \) kg/liter) along with the expected pre-beta (VLDL) peak. (C) The infranant fraction of the sample centrifuged at 1.006 kg/liter containing the normal pattern of beta (LDL) and alpha (HDL) lipoproteins.

Comparison of the Lipid Clinics and Airfuge Aspirator Methods

The development of the Airfuge aspirator method led to the comparison of this microtechnique with the procedure routinely used by the LRC (6). The LRC methodology requires 5 ml of plasma centrifuged for 18 h at 39 000 rpm, and quantitation of the cholesterol within the LDL plus HDL infranate after tube slicing. HDL cholesterol is measured in the supernate obtained after heparin/manganese chloride precipitation of 2 ml of plasma. The cholesterol content of individual lipoproteins is calculated as previously stated, except that HDL cholesterol is determined directly in the supernantant fraction of the heparin/manganese chloride precipitate. Plasma and centrifuged samples are extracted with isopropanol and zeolite before cholesterol analysis with the Technicon AutoAnalyzer II. The Airfuge aspirator micromethod we describe in this report requires two 175-µl aliquots of plasma, 2.5 h of centrifugation, fractionation of lipoproteins by aspiration, and direct analysis of cholesterol on the Beckman Cholesterol Analyzer.

The precision of the micromethod and the LRC method were compared after quantifying the cholesterol in 10 separate lipoprotein fractionations of a single sample. All fractionated plasma samples were first quantified by using the Beckman Cholesterol Analyzer, in order to rule out variations between two instruments (Table 3). Analyzer variations, as they affect the precision of the two methods, are included in the data of Table 4. The precision, as assessed by the coefficient of variation, was better for whole plasma, HDL, and VLDL with the micro Airfuge aspirator technique (Tables 3 and 4). The CV is greater for VLDL than LDL or HDL in both methods, at least in part due to the fact that the VLDL value is obtained by subtraction, and reflects the combined imprecision of two separate cholesterol analyses. The precision of the VLDL cholesterol quantitation with the new Airfuge aspirator method is a significant improvement when compared to the LRC method, whether a single or both cholesterol analyzers are employed (Tables 3 and 4). The measurement of VLDL cholesterol can be further improved (for \( n = 10 \), CV = 7.8%) by direct analysis of the top fraction of the plasma sample centrifuged at 1.006 kg/liter.

As a final comparison, we analyzed 15 samples obtained from normal individuals and from patients with hyperlipoproteinemia (cholesterol range, 1.700–5.120 g/liter) by both methods. The measurement of cholesterol within each lipoprotein fraction was correlated by linear regression analysis. Again, to avoid errors introduced by the measurement of cholesterol with two different analyzers, we used the Beckman Cholesterol Analyzer alone (upper panel, Table 5). Data obtained by analysis for cholesterol with both the Beckman Analyzer and Technicon AutoAnalyzer II are shown in the lower panel. The correlation coefficients for LDL by both methods were excellent (\( r > .995 \)). The correlation coefficient for HDL is, however, lower in both procedures; this is due, at least in part, to the low concentrations of cholesterol within HDL that often are encountered in patients with hyperlipoproteinemia...
Table 3. Plasma Lipoprotein Fractionation

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<th>LDL</th>
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<td>CV, %</td>
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10 repetitive determinations
* A. Airfuge/aspirator method (0.35 ml plasma).
* B. LRC method (7.0 ml plasma).

Table 4. Plasma Lipoprotein Fractionation

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10 repetitive determinations
* A. Airfuge/aspirator method, Beckman Analyzer (0.35 ml plasma).
* B. LRC method, Technicon AA II (7.0 ml plasma).

(80–150 mg/liter), which are outside the linear response of both cholesterol analyzers (Table 5). The precision with which each cholesterol analyzer measures the low cholesterol concentrations in HDL is therefore less than is the case for the other lipoprotein fractions. This decreased precision was present even when all samples were measured with the Beckman Cholesterol Analyzer alone (upper panel, Table 5). Measurements of low HDL cholesterol with two separate instruments further reduced the correlation (lower panel, Table 5). A solution to this problem is readily apparent, because larger sample volumes (up to 50 μl) can be used for cholesterol quantitation by the Beckman Cholesterol Analyzer. (The correlation coefficient for HDL cholesterol with use of 50-μl sample aliquots was 0.942.) Thus the Airfuge aspirator methodology appears to be reliable, fast, and precise for measuring the cholesterol in plasma lipoproteins. This method gives comparable or more precise results for the cholesterol content of lipoprotein fractions than is obtained with the LRC method.

Table 5. Correlation between Results by the Lipid Clinic and Airfuge/Aspirator Methods

Beckman Analyzer—both methods:

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Technicon AutoAnalyzer II and Beckman Analyzer—both methods:

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n = 15 normal and hyperlipoproteinemic plasmas.

Discussion

Various recent reports describe the use of enzymic reactions for measuring total and free plasma cholesterol (9–12). The cholesterol esterase/cholesterol oxidase system was shown to be accurate and precise (8–12), and the use of an oxygen electrode to monitor the rate of the reaction has been documented (9, 28–30). A customer-modified Beckman glucose analyzer has been used to quantitate cholesterol (9). This instrument was extensively studied with respect to precision of the measurements of free and total cholesterol, and as to the linearity of response with samples from individuals and patients with hyperlipoproteinemia. The present Beckman Cholesterol Analyzer differs from the customer-modified glucose analyzer in that it monitors the reaction rate rather than endpoint, and the results are digitally displayed. The Beckman Cholesterol Analyzer gave precise, well-correlated results with a variety of hyperlipidemic samples, including patients with type I phenotype. This is in contrast to the modified glucose analyzer, which gave a nonlinear response with rat-plasma samples containing supranormal concentrations of chylomicrons.

Correlations between enzymic and colorimetric methods for cholesterol quantitation have been recently reported by several investigators (8–13). Direct correlation between instruments is hampered by the lack of a calibration sample that is compatible with direct analysis by all methods. In the present report, a cholesterol calibration plasma (obtained from the Center for Disease Control) was used to standardize all instruments. The slightly elevated cholesterol values (V_{enz}
In conclusion, recent retrospective analysis of epidemiological data emphasizing the importance of cholesterol within various lipoprotein fractions prompted our search for a rapid, reliable, and inexpensive method for cholesterol quantification comparable to those methods standardized by the Lipid Research Clinics. Our data demonstrate that this new micromethod is precise for the measurement of cholesterol in lipoprotein density fractions, and our results correlate well with the cholesterol values obtained with the LRC method. In addition, centrifugation of prestained plasma provides a technique for the visual inspection of the lipoprotein fractions and is particularly useful for the diagnosis of patients with type III phenotype. The small sample size and high sensitivity of the Beckman Cholesterol Analyzer make it useful for cord blood and neonate blood samples and for compositional analyses, including free and esterified cholesterol of samples obtained from ultracentrifugation, column chromatography, and metabolic studies. Fractionation of plasma lipoproteins by the Airfuge aspirator technique will facilitate the preparation of samples for analyses of apolipoprotein composition by radioimmunoassay and other immunological techniques. In addition, the lower cost of the new micromethod will enable clinical laboratories to quantitate cholesterol in the major density classes of plasma lipoproteins.

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