A rapid and sensitive micro-assay for the enzymatic determination of plasma and lipoprotein cholesterol

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Summary A rapid and inexpensive micro-assay for determining cholesterol in plasma and isolated lipoprotein fractions has been established which utilizes a commercially available enzymatic reagent with semi-automated instruments and microtiter plates. The assay is sensitive, precise, and easy to perform. The color development is linear from 0.4 to 20 μg cholesterol/well, with sample volumes of 2 to 100 μl. Inter- and intra-assay variability yielded coefficients of variation (CV) of 2.75% (n = 51) and 1.09% (n = 32), respectively. The concentrations of total plasma and lipoprotein cholesterol (d > 1.006 g/ml) obtained with this method were compared with those analyzed in a lipid laboratory standardized to the Centers for Disease Control. The correlation coefficients between the two methods were 0.976 and 0.964, respectively. For total high density lipoprotein (HDL) and the HDL_3 subfraction, inter-assay variability was 4.12% and 6.33% (n = 27), respectively; the intra-assay variability was 2.79% and 4.19% (n = 12). —**Auerbach, B. J., J. S. Parks, and D. Applebaum-Bowden.** A rapid and sensitive micro-assay for the enzymatic determination of plasma and lipoprotein cholesterol. *J. Lipid Res.* 1990. **31:** 738–742.

Supplementary key words HDL • HDL_3

Abbreviations: HDL, high density lipoproteins; CDC, Centers for Disease Control.

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In the past decade, there has been an increasing awareness of cardiovascular problems associated with high plasma cholesterol levels. The desire to measure cholesterol in various lipoprotein fractions has stimulated the development and availability of reagent kits. The most notable improvement in the determination of cholesterol has been the shift from the chemically based Liebermann-Burchard method of analysis to a system based on an enzymatic procedure developed by Allain et al. (1). The enzymatic system utilizes cholesterol esterase to cleave any esters present in the sample followed by cholesterol oxidase to oxidize the free cholesterol. The peroxide liberated in the latter reaction reacts with peroxidase and 4-aminophenazone to produce a colored product in proportion to the amount of total cholesterol present.

The enzymatic cholesterol method is now the system of choice for most laboratories. In clinical laboratories a large number of similar samples are easily measured using automated instruments. Lipoprotein research laboratories, however, may generate many fractions from a single sample through ultracentrifugation and column chromatography. The cholesterol mass in these isolated fractions can vary more than 100-fold across the separation. Autoanalyzers, though efficient for plasma samples, would require much reprogramming to accommodate this wide range of concentrations. Even if a clinical laboratory would perform these assays, sending such a large number of samples to an outside laboratory for analysis would be time-consuming and expensive. Thus, there is a need for a flexible quick method for cholesterol analysis in the research laboratory. This paper describes a semi-automated precise procedure utilizing the enzymatic cholesterol reagents that meets these criteria and greatly reduces the amount of reagent, sample, and technician time required to determine the cholesterol concentration in a variety of samples.

MATERIALS AND METHODS

Reagents

Stock cholesterol solutions of 500, 1000, 1500, 2000, 3000, and 4000 μg/ml (Preciset Cholesterol Calibrators, Boehringer Mannheim Diagnostics) were used to standardize the assay. Each stock solution was diluted 1:5, 1:10, 1:25, and 1:50 with 0.9% NaCl, to be used with sample volumes of 10, 20, 50, and 100 μl, respectively. These solutions were stored at room temperature, and used until there was a 5% change in their absorbance. The enzymatic cholesterol reagent kit chosen was the Autoflow Cholesterol High Performance, purchased from Boehringer Mannheim Diagnostics (Indianapolis, IN), and was reconstituted and stored as recommended by the manufacturer.

Quality control

Inter-assay variability was monitored using the Omega Lipid Fraction Control Serum (lot # 46101001, Cooper Biomedical, Malvern, PA). Vials containing the dry serum were reconstituted with 3 ml of the supplied diluent plus 12 ml of 0.9% NaCl, and 100-μl aliquots were frozen for future use.

Sample preparation

Plasma samples and lipoproteins isolated at a density greater than 1.006 g/ml by ultracentrifugation (2), were diluted 1:5 with 0.9% NaCl using a Hamilton Microlab M Diluter/Dispenser (Bonaduz, Switzerland). Differential isolation of HDL and HDL_3, from 500 μl of plasma, was accomplished using the dextran sulfate–$MgCl_2$ precipitation method (3, 4). HDL, HDL_3, and other samples with concentrations less than 100 mg/dl were not diluted. As a control for the differential isolation of high density lipoprotein (HDL) and HDL_3, ten bottles of the Omega serum (lot # 7V0957) were reconstituted, each with 3 ml of diluent, as instructed by the supplier, and 0.5-ml aliquots were frozen for future use.

Cholesterol determination

The assay was semi-automated using the Microlab M diluter/dispenser with a 1000-μl syringe which accurately measures 10–1000 μl. Ten microliters of either the diluted samples or standards were picked up with the diluter, and dispensed with 200 μl of the enzymatic reagent into a well in a 96 "U"-bottom well microtiter plate (Costar, Camridge, MA). More dilute samples were assayed by increasing the sample volume while decreasing the volume of reagent by the same amount, such that the total volume was always 210 μl. When using sample volumes of 50 μl and 100 μl, the Preciset cholesterol standards were diluted 1:25 and 1:50, respectively, to control for any effects of reagent dilution. The mixtures were incubated at room temperature for 35 min. Absorbance was read at 490 nm, using a MicroELISA Reader (Dynatech Diagnostics, South Windham, ME). Saline (0.9% NaCl), instead of sample, was always dispensed with the reagent into the first well of the plate, and its absorbance was automatically subtracted from each of the samples. A standard curve, calculated from the absorbance values versus μg cholesterol plot of the diluted Preciset standards, was used to determine the sample cholesterol concentration. (Note: dilutions would not be necessary if a diluter capable of accurately measuring 2 μl was used.)

Centers for Disease Control (CDC) standardized lipid laboratory

Cholesterol in the plasma and lipoprotein samples was also measured in the CDC standarized lipid laboratory at the Bowman Gray School of Medicine. The assay was

conducted on a Technicon RA500, using the Boehringer Mannheim Autoflow Cholesterol High Performance enzymatic reagent, and 7 μl of sample.

RESULTS

The color development was linear within the tested range of 0.4 to 20.0 μg cholesterol regardless of the sample volume (10–100 μl, r = 0.9998) (**Fig. 1**) and was stable for up to 90 min. The Omega control serum was used for a measure of both inter- and intra-assay precision. The accumulated mean obtained for 51 assays over a 4-month period was 187 mg/dl with a coefficient of variation between assays of 2.75%. In a separate experiment, the Omega control was assayed in 32 wells of one microtiter plate, yielding a mean cholesterol value of 188 mg/dl with a coefficient of variation of 1.09%. The reference laboratory value for the Omega serum total cholesterol, using the Liebermann-Burchard reagent on a Technicon AA11, was 184 mg/dl (Omega Lipid Fraction Control Serum, Lot #4610100 reference sheet).

The mean cholesterol values obtained for the plasma and the lipoproteins isolated at a density greater than 1.006 g/ml were 228 ± 25.2 mg/dl (mean ± SD; n = 111) and 199 ± 26.6 mg/dl (n = 97), respectively. All of these samples were also assayed in the CDC standardized lipid laboratory at the Bowman Gray School of Medicine. The values obtained in that laboratory were 228 ± 25.0 mg/dl and 202 ± 27.1 mg/dl, respectively. The values with the new micromethod were highly correlated with the results from the CDC standardized lipid laboratory (Whole plasma, r = 0.976, **Fig. 2A;** lipoproteins d > 1.006 g/ml, r = 0.964, Fig. 2B).

The Omega serum (lot #7V0957) HDL and HDL_3 cholesterol concentrations were determined 27 times over a period of 12 months. The mean value of the HDL cholesterol concentration was 44.6 mg/dl with a coefficient of variation of 4.12%. The mean cholesterol concentration of the HDL_3 was 24.3 mg/dl with a coefficient of variation of 6.33%. The reported Omega serum reference laboratory value for the HDL cholesterol, using heparin-manganese precipitation (5), was 49 mg/dl. There is no reference laboratory value for the HDL_3. To determine intra-assay variability, HDL and HDL_3 were measured in twelve 500-μl aliquots taken from a 12-ml pool of reconstituted Omega serum (lot #46101001, HDL reference value = 40 mg/dl). The mean values for the HDL and HDL_3 were 37.6 and 22.5 mg/dl, respectively. The intra-assay coefficients of variation were 2.79% and 4.19% and for HDL and HDL_3, respectively.

DISCUSSION

The purpose of this study was to develop a rapid and inexpensive microassay for determining plasma and lipoprotein cholesterol concentrations with available enzymatic reagents. Using our modification of a standard enzymatic cholesterol assay, one-tenth of the sample and reagent volume was needed as compared to the recommended manual test tube method for this and other enzymatic test

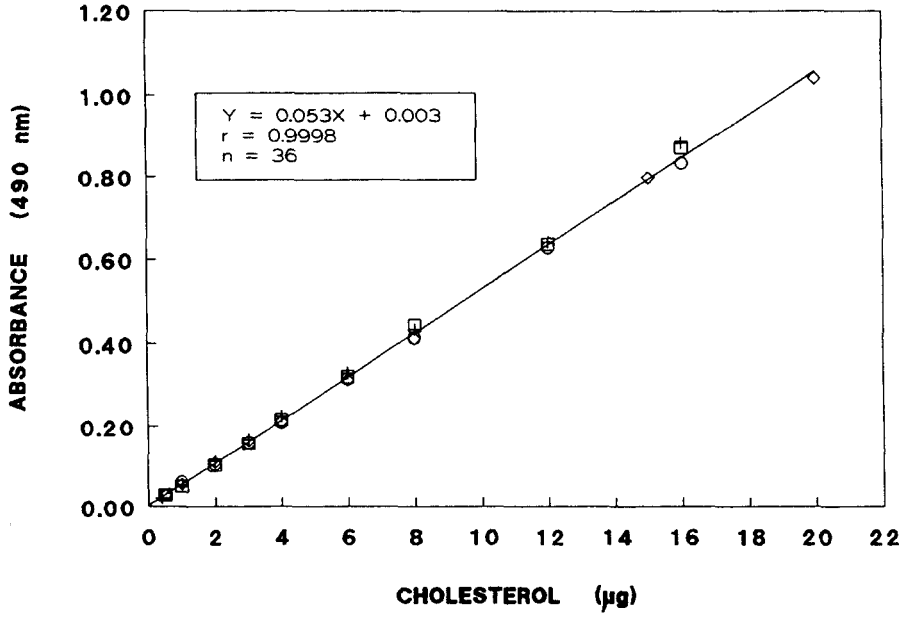

Fig. 1. Absorbance versus μg cholesterol, using 10 (□), 20 (+), 50 (◇), and 100 (○) μl of standard cholesterol solutions, and 200, 190, 160, 110 μl reagent, respectively. The solid line represents line of best fit found by regression analysis.

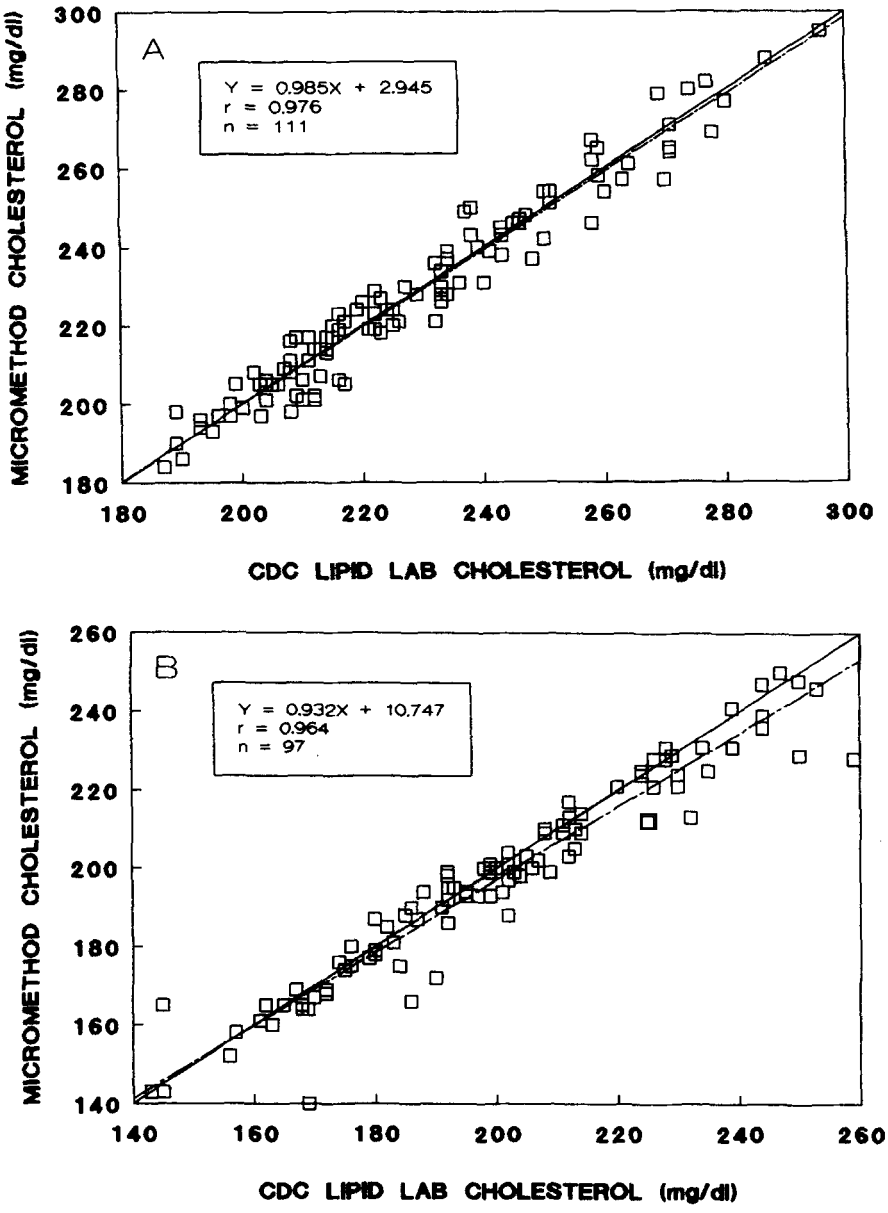

Fig. 2. Paired comparisons between the cholesterol values obtained using the micromethod versus the values determined by the CDC standardized lipid laboratory. The solid line represents identity between the two methods. The broken line demonstrates their relationship by linear regression. A: plasma cholesterol values; B: cholesterol values for d > 1.006 g/ml lipoproteins isolated by ultracentrifugation.

kits (Gilford Single Vial Reagent, Wako Cholesterol CII). Using the Microlab M diluter/dispenser, which has a hand-held dispensing probe attached to a programmable diluting syringe, greatly reduced both assay variability and technician handling. The technician need only press the button on the probe to pick up the correct volume for the sample tube, then place the tip of the probe to the appropriate microtiter well and depress the button again to simultaneously dispense reagent and sample. This instrument also decreases the time needed to aliquot the samples and reagent as compared to the manual pipetting method. The use of the microtiter plates eliminates the need to label and organize assay tubes, and greatly reduces the time needed to read the absorbance of the finished assay, since most MicroELISA readers can scan an entire plate in less than 1 min. Because of these modifications, one technician could easily run more than 100 samples (in duplicate) in less than an hour.

The Omega Lipid Fraction Control Serum was used as a measure of accuracy for this micromethod. The mean value obtained for the Omega serum was within 2% of the published reference value. Precision of this method was also determined with the Omega serum, which had coefficients of variation of 2.75% from day to day, and

1.09% within assay. These coefficients of variation were within the range observed during a multicenter study of the precision and accuracy of the Boehringer Mannheim reagent on fully automated systems (6). Additionally, the accuracy of this micromethod was demonstrated by the comparison of values obtained using this method and the values from a CDC standardized laboratory.

The flexibility of this assay was also demonstrated in the determination of the cholesterol in HDL and its subfractions using the dextran sulfate-$MgCl_2$ precipitation method. The inter- and intra-assay coefficients of variation were <5% and <7% for total HDL and HDL_3, respectively. This level of variation is comparable to that found by Warnick, Benderson, and Albers (7) (among-day variation; HDL: 2.9%; HDL_3: 9.1%). Our calculated mean for total HDL (44 mg/ml), although lower than the reference value (49 mg/dl) determined using the heparin-$MnCl_2$ precipitation method (5), is well within the published range for this lot of Omega serum (36-49 mg/dl).

A major focus in our laboratory is the compositional analysis of apolipoprotein B containing lipoproteins fractionated by density gradient ultracentrifugation. This procedure yields approximately 43 fractions of 0.45 ml each. Cumulative cholesterol recovery (corrected back to whole plasma – HDL cholesterol) across the gradient is routinely greater than 93% (data not shown). By adapting the micromethod to enzyme kits for phospholipid, triglyceride, and free cholesterol we have been able to perform complete lipid analysis on these individual density fractions despite their small volume and mass.

The value of a new assay can be measured by its accuracy, precision, sensitivity, ease of sample processing, and cost effectiveness. The assay procedure described in this report has been demonstrated to be highly accurate with a low coefficient of variation within a range of 0.4 to 20 μg cholesterol, with sample volumes from 10 to 100 μl. The use of a semi-automatic diluter and the MicroELISA plate reader enables many samples to be processed quickly with a minimum of technician handling and with less reagent usage. The micromethod can easily be used to assay phospholipid, triglyceride, and free cholesterol with the respective specific enzymatic kits. Thus, this method is ideally suited for research laboratories needing an accurate and inexpensive, high through-put assay for lipid analysis of plasma and isolated lipoprotein fractions.

This study was supported in part by National Institutes of Health Grants HL-38222, HL-38624, and HL-07115.

Manuscript received 20 August 1989 and in revised form 4 December 1989.

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