Analysis for Cholesterol in All Lipoprotein Classes by Single Vertical Ultracentrifugation of Fingerstick Blood and Controlled-Dispersion Flow Analysis

Krishnaji R. Kulkarni, David W. Garber, Charles F. Schmidt, Santica M. Marcovina,1 Mat H. Ho, Brian J. Wilhite, Kenneth R. Beaudrie, and Jere P. Segrest2

This new, highly sensitive analytical system, based on controlled dispersion of the flowing sample, gives a rapid, continuous, and direct analysis for cholesterol in all lipoprotein classes, separated by single vertical-spin density-gradient ultracentrifugation. In this Vertical Auto Profile-II fingerstick system, designated VAP-IIFs, a narrow-bore Teflon coil serves as the reactor with no segmentation of the analytical stream by air bubbles, in contrast to the Technicon AutoAnalyzer used in the VAP-I method. Concentrations of high-, low-, intermediate-, and very-low-density lipoprotein cholesterol and lipoprotein(a) cholesterol are determined by decomposing the spectrophotometric absorbance curve for the continuous analysis of the centrifuged sample, with use of software developed in this laboratory. Total cholesterol is determined from the total area under the absorbance curve. For assaying total cholesterol, the CV between aliquots within a rotor ranged from 1.35% to 3.15%; the CV between rotors was 2.45%. Because only 18 μL of sample is required, VAP-IIFs can be readily adapted to analysis for lipoprotein cholesterol profiles in capillary blood samples. Total cholesterol values by VAP-IIFs for fingerstick and venous samples from 23 subjects agreed well: slope = 1.01 (SD 0.03), intercept = −21 (SD 51) mg/L, $S_{y|x}$ = 50 mg/L, and $r = 0.992$. Results by VAP-IIFs also correlated highly with results for duplicate samples analyzed at the Northwest Lipid Research Laboratories.

Additional Keyphrases: continuous-flow analysis · spectrophotometry · density-gradient ultracentrifugation · capillary vs venous blood samples

Although increased concentrations of total cholesterol (TC) in plasma have been strongly correlated with atherosclerotic coronary heart disease (1), individual lipoprotein classes represent additional and independent risk factors.3 Increased concentrations of very-low (VLDL-C) and intermediate-density lipoprotein cholesterol (IDL-C) (2), low-density lipoprotein cholesterol (LDL-C) (3), and lipoprotein(a) [Lp(a)], a cholesterol-rich lipoprotein (4), have all been associated with greater risk of coronary heart disease. Increased high-density lipoprotein cholesterol (HDL-C) is protective against atherosclerotic heart disease (5), such that a decrease in HDL-C is a strong predictor of coronary heart disease (6). Therefore, measurement of the cholesterol content of all plasma lipoproteins—a cholesterol profile—is very useful for accurate assessment of risk for atherosclerosis (7).

Sequential ultracentrifugation can separate lipoproteins into classes according to density, so that their cholesterol content can then be accurately measured. However, this method is time consuming and not well suited to clinical applications. The most common clinical laboratory procedures for measuring cholesterol profiles involve multiple analyses of different aliquots of the same plasma sample. TC is measured in one aliquot, apolipoprotein (apo) B-containing lipoproteins are precipitated from a second aliquot, and the cholesterol measured in the remaining supernate of the second aliquot is HDL-C. VLDL-C either is estimated by using the formula of Friedewald et al. (8) after the triglycerides are measured in a third aliquot or is measured directly after ultracentrifugal isolation of the VLDL

---

1 Nonstandard abbreviations: HDL, LDL, VLDL, high-density, low-density, and very-low-density lipoprotein, respectively; HDL-C, LDL-C, IDL-C, VLDL-C, high-density, low-density, intermediate-density, and very-low-density lipoprotein cholesterol, respectively; TC, total cholesterol; Lp(a), lipoprotein(a); Lp(a)-C, lipoprotein(a) cholesterol; apo, apolipoprotein; VAP, Vertical Auto Profile; NWLRL, Northwest Lipid Research Laboratories; CDC, Centers for Disease Control; NHLBI, National Heart, Lung, and Blood Institute; and NCEP, National Cholesterol Education Program.

CLIN. CHEM. 38/9, 1898–1905 (1992)

Analysis for Cholesterol in All Lipoprotein Classes by Single Vertical Ultracentrifugation of Fingerstick Blood and Controlled-Dispersion Flow Analysis

Krishnaji R. Kulkarni, David W. Garber, Charles F. Schmidt, Santica M. Marcovina,1 Mat H. Ho, Brian J. Wilhite, Kenneth R. Beaudrie, and Jere P. Segrest2

This new, highly sensitive analytical system, based on controlled dispersion of the flowing sample, gives a rapid, continuous, and direct analysis for cholesterol in all lipoprotein classes, separated by single vertical-spin density-gradient ultracentrifugation. In this Vertical Auto Profile-II fingerstick system, designated VAP-IIFs, a narrow-bore Teflon coil serves as the reactor with no segmentation of the analytical stream by air bubbles, in contrast to the Technicon AutoAnalyzer used in the VAP-I method. Concentrations of high-, low-, intermediate-, and very-low-density lipoprotein cholesterol and lipoprotein(a) cholesterol are determined by decomposing the spectrophotometric absorbance curve for the continuous analysis of the centrifuged sample, with use of software developed in this laboratory. Total cholesterol is determined from the total area under the absorbance curve. For assaying total cholesterol, the CV between aliquots within a rotor ranged from 1.35% to 3.15%; the CV between rotors was 2.45%. Because only 18 μL of sample is required, VAP-IIFs can be readily adapted to analysis for lipoprotein cholesterol profiles in capillary blood samples. Total cholesterol values by VAP-IIFs for fingerstick and venous samples from 23 subjects agreed well: slope = 1.01 (SD 0.03), intercept = −21 (SD 51) mg/L, $S_{y|x}$ = 50 mg/L, and $r = 0.992$. Results by VAP-IIFs also correlated highly with results for duplicate samples analyzed at the Northwest Lipid Research Laboratories.

Additional Keyphrases: continuous-flow analysis · spectrophotometry · density-gradient ultracentrifugation · capillary vs venous blood samples

Although increased concentrations of total cholesterol (TC) in plasma have been strongly correlated with atherosclerotic coronary heart disease (1), individual lipoprotein classes represent additional and independent risk factors.3 Increased concentrations of very-low (VLDL-C) and intermediate-density lipoprotein cholesterol (IDL-C) (2), low-density lipoprotein cholesterol (LDL-C) (3), and lipoprotein(a) [Lp(a)], a cholesterol-rich lipoprotein (4), have all been associated with greater risk of coronary heart disease. Increased high-density lipoprotein cholesterol (HDL-C) is protective against atherosclerotic heart disease (5), such that a decrease in HDL-C is a strong predictor of coronary heart disease (6). Therefore, measurement of the cholesterol content of all plasma lipoproteins—a cholesterol profile—is very useful for accurate assessment of risk for atherosclerosis (7).

Sequential ultracentrifugation can separate lipoproteins into classes according to density, so that their cholesterol content can then be accurately measured. However, this method is time consuming and not well suited to clinical applications. The most common clinical laboratory procedures for measuring cholesterol profiles involve multiple analyses of different aliquots of the same plasma sample. TC is measured in one aliquot, apolipoprotein (apo) B-containing lipoproteins are precipitated from a second aliquot, and the cholesterol measured in the remaining supernate of the second aliquot is HDL-C. VLDL-C either is estimated by using the formula of Friedewald et al. (8) after the triglycerides are measured in a third aliquot or is measured directly after ultracentrifugal isolation of the VLDL

---

1 Nonstandard abbreviations: HDL, LDL, VLDL, high-density, low-density, and very-low-density lipoprotein, respectively; HDL-C, LDL-C, IDL-C, VLDL-C, high-density, low-density, intermediate-density, and very-low-density lipoprotein cholesterol, respectively; TC, total cholesterol; Lp(a), lipoprotein(a); Lp(a)-C, lipoprotein(a) cholesterol; apo, apolipoprotein; VAP, Vertical Auto Profile; NWLRL, Northwest Lipid Research Laboratories; CDC, Centers for Disease Control; NHLBI, National Heart, Lung, and Blood Institute; and NCEP, National Cholesterol Education Program.
fraction (9). LDL-C is not measured directly, but is calculated by subtracting the HDL-C and VLDL-C values from the TC concentration; this reported LDL-C value therefore includes Lp(a)-C, LDL-C, and IDL-C. Multiple analyses make this procedure tedious and susceptible to analytical error.

Precipitation of lipoproteins with macromolecular polyanions (such as heparin and dextran sulfate) or with sodium phosphotungstate is commonly used to separate apo B-containing lipoproteins from the HDL fraction (10). Precipitation methods, however, depend on complete and selective precipitation of the target lipoprotein. Incomplete precipitation of apo B-containing lipoproteins can lead to erroneous results.

The Vertical Auto Profile (VAP) method was developed in our laboratory to profile quantitatively the cholesterol in the major plasma lipoprotein fractions (11, 12). The original VAP, designated here as VAP-I, involves short (35 min, excluding deceleration time) density-gradient vertical ultracentrifugation, enzymous enzymatic analysis of cholesterol content in the centrifuge tube by a Technicon AutoAnalyzer (Technicon Instruments, Tarrytown, NY), and computer-assisted digitization and decomposition of the absorbance curve to provide both TC results and the cholesterol values of the lipoprotein classes. VAP-I provides rapid analysis of lipoprotein profiles in a single plasma sample; however, the relatively large amount of sample (1.3 mL) required by VAP-I makes it unsuitable for analysis of small-volume (e.g., fingerstick) blood samples. In addition, the VAP-I instrumentation is cumbersome, making its operation and maintenance somewhat difficult.

To overcome these problems, we developed a new, highly sensitive analytical system, VAP-IIIs (fingerstick), based on controlled dispersion of the sample during analysis. Tabletop ultracentrifugation reduces the sample volume needed and decreases analysis time. Here we describe the results obtained from the VAP-IIIs method and its application to the determination of lipoprotein cholesterol distribution in fingerstick blood samples.

Materials and Methods

Single vertical-spin density-gradient ultracentrifugation. Separation of lipoproteins by single vertical-spin density-gradient ultracentrifugation was as previously described (11), except that the plasma samples were much smaller. Fresh plasma samples (18 μL) were diluted 30-fold with saline/EDTA solution (per liter: 9 g of NaCl, 1 mmol of EDTA, pH 7.4, density = 1.006 kg/L) and adjusted to a density of 1.21 kg/L by adding dry KBr. A discontinuous gradient was formed in 2-mL Polyallomer Quick Seal ultracentrifuge tubes (Beckman Instruments, Palo Alto, CA) by pipetting 1.35 mL of the saline/EDTA solution into a glass Pasteur pipette placed in each tube and then underlayering with 0.55 mL of density-adjusted plasma sample. Tubes were sealed, placed immediately in a Beckman TLV-100 rotor (which holds eight tubes), and centrifuged in a Beckman TL-100 tabletop ultracentrifuge at 100,000 rpm for 22 min (excluding deceleration time) at 20 °C, with acceleration and deceleration settings of 5. A tube containing calibration plasma with a known TC concentration [determined by the Northwest Lipid Research Laboratories (NWRL) at the University of Washington, Seattle, WA] was included in each rotor for quantitative analysis.

Controlled-dispersion flow analysis. The VAP-IIIs flow-analysis system, shown schematically in Figure 1, consists of a tube-piercing needle assembly (Beckman Instruments), two peristaltic pumps to control flow rates of sample and reagent, a Plexiglas module with a Y-shaped capillary channel for mixing sample and reagent, a 1.5 m × 0.8 mm (i.d.) Teflon coil enclosed in a temperature-controlled glass chamber (37 °C) that acts as the reactor, a spectrophotometric liquid-chromatographic detector (Pharmacia LKB, Uppsala, Sweden) with a micro-volume flow cell, a strip-chart recorder, and a computer to collect and analyze the absorbance data. The pump placed near the needle assembly controls the flow rate of cholesterol reagent; a pump placed downstream from the detector controls the flow rate of the reaction mixture containing the sample and reagent and can be used to adjust the sample-to-reagent ratio.

Cholesterol reagent ("High performance"; Boehringer Mannheim Diagnostics, Indianapolis, IN) was prepared freshly each day in de-ionized, distilled water (250 g/L). Brij 35 (Sigma Chemical Co., St. Louis, MO) was added to the reagent solution (25 mL/L) to facilitate the breakdown of lipoprotein particles. All liquid solutions, including the cholesterol reagent solution, were degassed under reduced pressure to minimize formation of air bubbles. After centrifugation, the sample tube was placed in the needle assembly, the top was removed, and the tube was carefully punctured at the bottom so as not to disturb the formed gradient. The sample was drawn into the Plexiglas module by the peristaltic pump downstream from the detector and was mixed continuously with a stream of cholesterol reagent. The flow rate of the reaction mixture was maintained at 1.1 mL/min (re-
agent, 0.5 mL/min; sample, 0.6 mL/min). The absorbance of the enzymatic cholesterol reaction product was monitored continuously at 505 nm as it passed through the detector. The system was washed briefly with water between samples by switching the flow from sample to water at the end of sample drain via the valve on the flow module; reagent flow was not interrupted at any time.

**Computer-assisted analysis for lipoprotein classes.** Data were acquired and digitized with a Data Translation (Marlboro, MA) analog-to-digital conversion board and software developed in this laboratory. The digitized absorbance curve was then separated into its components (“decomposed”) to quantify the cholesterol content in each lipoprotein class by use of other software previously developed in this laboratory. The algorithm, described earlier (12), is based on the assumption that the curve shapes for the individual lipoprotein classes in a sample are the same as when isolated individual lipoprotein classes are analyzed with the VAP. The functional form of this curve was assumed to be bicameral gaussian with an exponential tail (12); an originally symmetrical gaussian peak in the centrifuged tube would be convoluted by flow and mixing factors to a form having a larger half-width on the trailing side than on the leading side and an exponential modification to the trailing side. Location in the profile and peak shape parameters (widths at half-height, peak height, and the exponent) for each lipoprotein class were determined by isolating individual lipoproteins by preparative ultracentrifugation and then analyzing the isolated lipoproteins in the VAP-IIIs procedure. After determining the peak times and peak shape parameters for each lipoprotein class, the area under each subcurve was determined. TC concentration was determined by adding the areas under all the subcurves; at each instant, the TC present is the sum of the contributions of the individual lipoprotein classes. A calibration plasma sample, for which the TC value was known accurately, was included in each rotor to calibrate the area in terms of cholesterol concentration. The TC of a sample can also be determined by comparing the total area under the undecomposed curve with that of the calibration plasma.

**Cholesterol profiles.** Venous and capillary blood samples (obtained via fingerstick) from 23 fasting subjects were collected simultaneously. Venous samples were collected into EDTA; fingerstick samples were collected into heparinized hematocrit tubes. Plasma was separated from the fingerstick blood samples by using a microhematocrit centrifuge. Venous samples were divided, and half were sent to the NWLRL for analysis as described below. Venous and fingerstick sample pairs were subjected to single vertical-spin density-gradient ultracentrifugation in the same rotor, according to the conditions described above, and cholesterol distribution was determined by the VAP-IIIs method. All samples were analyzed within 5 days of collection.

**Comparison of VAP-IIIs analysis with NWLRL method.** Venous plasma samples from the 23 subjects were sent to the NWLRL for comparison of analysis. Analyses in this Centers for Disease Control (CDC)-standardized laboratory were performed as follows: cholesterol was assayed in whole plasma by an automated enzymatic system (Spectrum Multichromatic Analyzer; Abbott Labs., North Chicago, IL 60064). The HDL fraction was obtained after chemical precipitation of VLDL and LDL with dextran sulfate–magnesium (Selected Method) (13); the cholesterol in this fraction was quantified enzymatically by the Abbott Spectrum, with use of a separate calibration appropriate for low cholesterol values.

VLDL-C and LDL-C were quantified by using the Lipid Research Clinics Beta Quantification method (9), which involves ultracentrifugation of plasma to separate VLDL (in the density <1.006 kg/L; supernate) from LDL and HDL (in the density >1.006 kg/L infranate). The infranatant and supernatant fractions are assayed enzymatically for cholesterol content; LDL-C is determined as the difference between the infranatant cholesterol value and the HDL-C value determined previously. The VLDL-C reported is determined as the difference between the whole-plasma and the infranatant cholesterol values.

Assay performance of the Beta Quantification procedure was monitored by including normal and above-normal quality-control sera (Q17, Q19; obtained from the CDC, Atlanta, GA) with each plasma assay run, including quality-control sera (aQ, from CDC; and DSC, from Solomon Park Research Labs., Kirkland, WA) in each precipitation run, and including a low-cholesterol quality-control serum (mQ; CDC) in each HDL-C assay run. If, in addition, performance in “blind” assay of split duplicates was monitored monthly; the CVs for TC ranged between 1% and 3%; for HDL-C, between 0.5% and 2%; and for LDL-C, between 1.5% and 4%.

**Statistical analysis.** Total and lipoprotein cholesterol values are reported as mean ± SE. For comparisons between methods of analysis, we used two-tailed paired t-tests; differences were considered statistically significant at P <0.05. Mean paired differences are reported as mean ± SE. Correlations between the VAP-IIIs and the NWLRL Beta Quantification methods were assessed by linear-regression analysis.

**Results**

**Assay optimization.** In a preliminary study, we determined the effect of the flow rate of the reaction mixture (containing approximately equal volumes of reagent and sample), the length and inner diameter of the Teflon coil, and the concentration of the enzymatic reagent on the resolution of the lipoprotein peaks. The resolution improved with decreased flow rate, decreased dimensions of the coil, and increased concentration of reagent. However, analysis time (sample drain time) also increased with the decrease in flow rate. To match more closely the analysis time required for the eight samples in a TLV-100 rotor with the centrifugation time (28 min including deceleration), we compromised between resolution and flow rate of reaction mixture, selecting the operating conditions stated above: flow rate of reaction...
mixture, 1.1 mL/min (reagent = 0.5 mL/min, sample = 0.6 mL/min); 1.5 m × 0.8 mm (i.d.) for the Teflon coil; and 250 g/L concentration of reagent. The drain time for each sample at this flow rate was 3 min.

The preliminary study involved undiluted plasma from a single pool. However, the high sensitivity of the flow-analysis system required that plasma be diluted for quantitative determination. To determine the degree of sample dilution required for quantification with optimum sensitivity, we analyzed plasma diluted 2-, 3-, 5-, 10-, 15-, 20-, and 30-fold with saline/EDTA solution. The sensitivity of the VAP-IIIs system was adequate for quantitative analysis even with plasma diluted 30-fold, corresponding to 18 µL of sample per assay.

VAP-IIIs cholesterol profile. A VAP-IIIs cholesterol profile of a fingerstick blood sample obtained under the conditions described above is shown in Figure 2A: HDL, LDL, and VLDL peaks are well resolved. A shoulder peak from Lp(a) was also observed in some samples. In the example shown in Figure 2A, the time for the HDL, LDL, and VLDL peaks to reach their maxima was 40, 110, and 180 s, respectively, from the beginning of the HDL peak.

Cholesterol in five lipoprotein classes (HDL, Lp(a), LDL, IDL, and VLDL) was quantified by decomposing the digitized output of the spectrophotometric detector. Figure 2B shows the decomposed form of the Figure 2A profile. The time required to analyze the eight centrifuged samples (including a calibration plasma sample) contained in TLV-100 ultracentrifuge rotors was ~30 min (including a delay time between samples). The lipoprotein peak locations in the profiles obtained from replicates of a single plasma sample were highly reproducible. For example, the location of the LDL peak, calculated as the time from the beginning of the HDL peak to the LDL peak maximum divided by the time from the beginning of the HDL peak to the VLDL peak maximum, was 0.62 (SD 0.01), with a CV of 1.8% (n = 35). These data indicate high reproducibility of the flow system and stability of the gradient during analysis.

Characterization of VAP-IIIs. The VAP-IIIs system was characterized for linearity of response and reproducibility by using venous plasma samples. To determine the linearity of response to the cholesterol concentration of each lipoprotein class, we isolated lipoprotein classes from whole plasma by preparative fixed-angle density-gradient ultracentrifugation (14). After determining the cholesterol concentration of each isolated lipoprotein class, we prepared aliquots of each class to contain cholesterol in the range 10.0–70.0 mg/L by diluting the isolated class with saline/EDTA solution and adjusted the aliquots to a density of 1.21 kg/L with subsequent correction of concentration to account for volume changes. We measured cholesterol concentrations in the isolated lipoproteins by the VAP-IIIs procedure, using the same conditions as for whole plasma. The integrated peak areas varied linearly with cholesterol concentration at least in the range 10.0–60.0 mg/L (Figure 3). Given the 30-fold dilution before analysis,

*To convert cholesterol mg/L to mmol/L, multiply by 0.002586.*
Table 1. Reproducibility of Cholesterol Analysis by VAP-Ilfs

<table>
<thead>
<tr>
<th>Rotor</th>
<th>TC</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>LDL-C(NCEP)</th>
<th>VLDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.85</td>
<td>2.50</td>
<td>1.60</td>
<td>2.50</td>
<td>6.20</td>
</tr>
<tr>
<td>2</td>
<td>1.65</td>
<td>2.80</td>
<td>1.90</td>
<td>1.80</td>
<td>6.50</td>
</tr>
<tr>
<td>3</td>
<td>3.15</td>
<td>3.00</td>
<td>2.20</td>
<td>2.50</td>
<td>11.20</td>
</tr>
<tr>
<td>4</td>
<td>1.35</td>
<td>2.00</td>
<td>2.60</td>
<td>1.00</td>
<td>5.00</td>
</tr>
<tr>
<td>5</td>
<td>1.85</td>
<td>1.90</td>
<td>1.40</td>
<td>1.60</td>
<td>9.80</td>
</tr>
<tr>
<td>Inter-rotor</td>
<td>2.45</td>
<td>3.40</td>
<td>2.75</td>
<td>2.00</td>
<td>8.60</td>
</tr>
</tbody>
</table>

CV, %

LDL-C(NCEP) = [LDL-C(90)] + [LDL-C(1800)], the "LDL-C" value referred to by the NCEP and the value usually reported by clinical laboratories. The cholesterol values in the plasma used for the reproducibility study as determined by the VAP-Ilfs method were as follows (in mg/L): TC, 1510; HDL-C, 430; LDL-C, 800; LDL-C(NCEP), 980; and VLDL-C, 90.

this corresponds to an upper limit of 1800 mg/L for cholesterol in each lipoprotein class. Linear least-square characteristics of the calibration curves for HDL-C, Lp(a)-C, LDL-C, and VLDL-C are also described in Figure 3. Slopes (area units per mg/L concentration) of all curves were very similar, indicating similar reaction kinetics for each lipoprotein class. As discussed later, the reaction is not carried to completion in this system.

To study the reproducibility of analysis, we analyzed aliquots of the same plasma (diluted 30-fold) in five rotors (seven aliquots per rotor plus an aliquot of a calibration plasma with known cholesterol concentration to calibrate the total area under the profile curve in terms of cholesterol concentration). Reproducibility (CV) of TC determinations by VAP-Ilfs for aliquots within a rotor ranged from 1.35% to 3.15%; among all aliquots from five rotors, the CV was 2.45% (Table 1). The cholesterol content of individual lipoprotein class was quantified after decomposing the cholesterol profile; the reproducibility of the quantification of lipoprotein classes by this system is also indicated in Table 1. The CV values for VLDL-C were somewhat higher than for the other lipoprotein classes, because of the low VLDL-C concentration (90 mg/L) in the plasma sample used in this experiment.

Comparison of VAP-Ilfs with NWLRL method. We analyzed venous samples from 23 fasting subjects, using both the VAP-Ilfs method and the NWLRL Beta Quantification method. TC values obtained from VAP-Ilfs and from the NWLRL method agreed very well (Table 2). The VAP method separates Lp(a)-C and IDL-C from true LDL-C; thus, to be consistent with the usual reported values for LDL-C, as described by the National Cholesterol Education Program (NCEP; I5) and measured by the Beta Quantification method, we summed the results for Lp(a)-C, LDL-C, and IDL-C measured by the VAP-Ilfs method and henceforth referred to this value as LDL-C(NCEP). Comparisons of HDL-C, LDL-C(NCEP), and VLDL-C are summarized in Table 2.

Results of the VAP-Ilfs method for cholesterol concentrations in these lipoproteins were highly correlated with those of the NWLRL method. Assessments of the methods by comparing paired differences showed that HDL-C values were consistently lower (-8.6%) for the VAP-Ilfs method than for the NWLRL method (mean ± SE paired difference = -43.5 ± 4.6 mg/L; P <0.001), and that LDL-C(NCEP) values were consistently higher (7.2%) for the VAP-Ilfs method than for the NWLRL method (mean paired difference = 79.3 ± 11.2 mg/L; P <0.001). VLDL-C values were also lower (-11.8%) for the VAP-Ilfs method than for the NWLRL method (mean paired difference = -22.0 ± 6.1 mg/L; P <0.002), but this decrease was not consistent for all samples. Paired differences for TC between the methods were not significant (mean paired difference = 3.5 ± 11.1 mg/L).

Evaluation of fingerstick blood sampling. Table 3 compares the TC values obtained by VAP-Ilfs for fingerstick and venous blood samples in all 23 subjects sampled; the regression data show that TC values in fingerstick samples highly correlated with the values in the corresponding venous samples. Paired differences for TC were not significant (mean paired difference = 2.2 ± 10.3 mg/L). Mean paired differences for cholesterol in all lipoprotein classes in fingerstick and venous blood samples were not statistically significant except for LDL-C(NCEP), for which the mean paired difference was 23.0 ± 10.2 mg/L (P <0.05), the venous values being higher by 1.9%. The regression data in Table 3 also show good correlation between fingerstick and venous cholesterol values in these lipoprotein classes.

Cholesterol profile analysis of fingerstick plasma by the VAP-Ilfs method was also compared with profiles of venous blood analyzed by the NWLRL Beta Quantification method (Table 4). Results were highly correlated between the two methods. Once again, paired comparisons showed that HDL-C values were consistently lower (-7.6%) for the VAP-Ilfs method (mean paired difference = -38.3 ± 6.9 mg/L; P <0.001), and LDL-C(NCEP) values were consistently higher (5.1%) (mean paired difference = 56.3 ± 11.7 mg/L; P <0.001). The mean paired difference (-17.9 ± 7.2 mg/L) for VLDL-C was less significant (P <0.05). TC differences were not significant (mean paired difference = 5.7 ± 10.6 mg/L).

Discussion

Both research and clinical medicine require analysis for cholesterol in lipoprotein classes, in addition to analysis for TC concentrations. The VAP-Ilfs method directly analyzes all lipoprotein classes, in contrast with methods used in most clinical laboratories. The most common methods for determining the cholesterol profile involve multiple analyses with several aliquots of the same sample. Therefore, the accuracy of the determination strongly depends on the accuracy of the individual analyses. For instance, the Friedewald equation (LDL-C = TC - HDL-C - TG/5, where TG = triglycerides) involves not only multiple analyses, but multiple assumptions as well (8); i.e., that the precipitation separation of apo B-containing lipoproteins from HDL is complete, and that VLDL-C is accurately estimated by the factor TG/5. The Beta Quantification method is better in that VLDL-C is measured directly, but this requires an additional analytical step. The method de-
Table 2. Linear-Regression Comparison of Lipoprotein Cholesterol Concentrations Measured by VAP-IIfs (y) and the NWLRL (x) in Venous Samples

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Slope</th>
<th>Intercept, mg/L</th>
<th>r</th>
<th>S_yx, mg/L</th>
<th>Mean (SD)</th>
<th>Mean (SE)</th>
<th>Difference, %&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.95</td>
<td>94 (49)</td>
<td>0.992</td>
<td>50</td>
<td>1802 (84)</td>
<td>1805 (81)</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL</td>
<td>0.91</td>
<td>4 (19)</td>
<td>0.983</td>
<td>20</td>
<td>498 (23)</td>
<td>455 (21)</td>
<td>-8.6</td>
</tr>
<tr>
<td>LDL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.93</td>
<td>157 (36)</td>
<td>0.986</td>
<td>50</td>
<td>1110 (70)</td>
<td>1190 (96)</td>
<td>7.2</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.93</td>
<td>-8 (10)</td>
<td>0.978</td>
<td>28</td>
<td>187 (29)</td>
<td>165 (28)</td>
<td>-11.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ranges of cholesterol values (mg/L) obtained by NWLRL method: TC, 1110-3010; HDL-C, 320-790; LDL-C(NCEP), 480-2070; and VLDL-C, 20-540 (n = 23).

Table 3. Linear-Regression Comparison of Lipoprotein Cholesterol Concentrations in Fingerstick (y) and Venous (x) Blood Samples Measured by VAP-IIfs

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Slope</th>
<th>Intercept, mg/L</th>
<th>r</th>
<th>S_yx, mg/L</th>
<th>Mean (SD)</th>
<th>Mean (SE)</th>
<th>Difference, %&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1.01</td>
<td>-21 (51)</td>
<td>0.992</td>
<td>50</td>
<td>1805 (81)</td>
<td>1807 (83)</td>
<td>0.1</td>
</tr>
<tr>
<td>HDL</td>
<td>0.95</td>
<td>30 (20)</td>
<td>0.978</td>
<td>21</td>
<td>455 (21)</td>
<td>460 (21)</td>
<td>1.1</td>
</tr>
<tr>
<td>LDL</td>
<td>0.96</td>
<td>25 (38)</td>
<td>0.986</td>
<td>50</td>
<td>1055 (60)</td>
<td>1034 (58)</td>
<td>-2.0</td>
</tr>
<tr>
<td>LDL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.98</td>
<td>7 (41)</td>
<td>0.988</td>
<td>49</td>
<td>1190 (66)</td>
<td>1167 (65)</td>
<td>-1.9</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.94</td>
<td>14 (8)</td>
<td>0.989</td>
<td>19</td>
<td>165 (28)</td>
<td>170 (28)</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ranges of VAP-IIfs cholesterol values (mg/L) for venous samples: TC, 1180-2980; HDL-C, 310-740; LDL-C, 470-1910; LDL-C(NCEP), 590-2150; and VLDL-C, 20-577 (n = 23).

<sup>b,c</sup> As in Table 2.

Table 4. Linear-Regression Comparison of Lipoprotein Cholesterol Concentrations Obtained by VAP-IIfs in Fingerstick Samples (y) and by the NWLRL Method in Paired Venous Samples (x)<sup>a</sup>

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Slope</th>
<th>Intercept, mg/L</th>
<th>r</th>
<th>S_yx, mg/L</th>
<th>Mean (SD)</th>
<th>Mean (SE)</th>
<th>Difference, %&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.97</td>
<td>60 (49)</td>
<td>0.992</td>
<td>50</td>
<td>1802 (64)</td>
<td>1807 (83)</td>
<td>0.3</td>
</tr>
<tr>
<td>HDL</td>
<td>0.85</td>
<td>35 (28)</td>
<td>0.958</td>
<td>29</td>
<td>498 (23)</td>
<td>460 (21)</td>
<td>-7.6</td>
</tr>
<tr>
<td>LDL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.92</td>
<td>148 (37)</td>
<td>0.988</td>
<td>50</td>
<td>1110 (70)</td>
<td>1167 (65)</td>
<td>5.1</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.87</td>
<td>61 (11)</td>
<td>0.972</td>
<td>30</td>
<td>187 (29)</td>
<td>170 (28)</td>
<td>-9.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Footnotes as in Table 2.

veloped previously in our laboratory (VAP-I) largely overcomes these problems. It is rapid—eight samples including a calibration plasma sample can be analyzed in ~45 min, excluding centrifugation time—and can determine the cholesterol concentrations in all lipoprotein classes in a single aliquot of sample in a single analysis. However, the VAP-I method, which uses an air-segmented continuous-flow system (Technicon AutoAnalyzer) to assay cholesterol, requires 1.3 mL of plasma and is thus unsuitable for analyzing fingerstick or other low-volume samples. In contrast, the present system, VAP-IIfs, based on a nonsegmented continuous-flow system with a narrow-bore Teflon coil reactor, requires only 18 μL of plasma.

In VAP-IIfs, the sample is continuously mixed with a nonsegmented stream of cholesterol reagent in a flow module containing a Y-shaped capillary channel. Dispersion, which causes overlapping of the lipoprotein peaks in other systems, is controlled by minimizing convection and diffusion of the sample. To do this, we adjusted the flow rates and reduced the length and bore size of the tubing, thereby minimizing the total volume of the tubing. At any given time, the total volume of reagent and sample in the coil is only 0.75 mL. Dispersion is also minimized by placing downstream from the detector the peristaltic pump used to draw the reaction mixture into the Teflon coil; the larger diameter of the pump tubing and the pump rollers could increase the dispersion if the sample were to pass through the pump before passing through the detector.

Because the sample inside the VAP-IIfs flow system is less diluted than in the VAP-I, in which the sample is diluted 10-fold with Brij solution, and because the tightly coiled Teflon tubing promotes efficient mixing of sample with reagent, the sensitivity of the VAP-IIfs exceeds that of the VAP-I. This increased sensitivity, along with reproducible flow rates and linearity of response in the desired concentration range, permits use of a much smaller plasma sample volume than does the VAP-I. Because the reaction kinetics of lipoprotein classes are essentially identical (Figure 3), the increased sensitivity of the VAP-IIfs allows measurement of a transient signal, i.e., a signal measured before the reaction reaches completion. Measurement of a tran-
sient signal means a shorter sample residence (reaction) time is needed, so a shorter Teflon coil can be used. The dispersion of the lipoprotein peaks is reduced with the use of shorter coil.

Large-scale population screening for TC in fingerstick blood is now possible with portable cholesterol analyzers. Differences between fingerstick and venous blood cholesterol values have been reported, with fingerstick blood cholesterol values being either higher (16, 17) or lower (18) than venous blood cholesterol values. One report, which compared lipoprotein profiles obtained by a screening method with those obtained by a laboratory method, found fingerstick blood TC values to average 5% more than the screening venous blood values, whereas the screening venous HDL-C values were too variable to be used to establish HDL-C results reliably (19). These differences have been suggested to be due to sampling techniques (16), insufficient training of operators (20), poor analytical performance of analyzers (21), or true physiological differences (17, 18).

Because only a few microliters of plasma are required for each analysis by VAP-IIs, this method can be used to analyze fingerstick blood samples. However, proper collection of fingerstick blood is essential for accurate and reproducible results.

Although no Reference Methods are as yet available for quantifying cholesterol in individual lipoprotein classes, TC results from VAP-IIs agreed well with the results from the NWRLL method (Table 2). Moreover, reproducibility (Table 1) and accuracy (Tables 2 and 4) for the VAP-IIs measurement of TC are well within the requirements (±3%) of the CDC–National Heart, Lung, and Blood Institute (NHLBI) Lipid Standardization Program (22). Although paired differences in the cholesterol values of some of the individual lipoprotein fractions were statistically significant between the two methods, the results of both methods correlated very well. The differences in HDL-C and LDL-C(NCEP) between the two methods were consistent, the HDL-C values being lower and the LDL-C(NCEP) values higher by the VAP-IIs method. However, the negative differences observed for venous (−6.6%, Table 2) and fingerstick (−7.6%, Table 4) HDL-C values were within the maximum bias of ±10% required by the CDC-NHLBI Lipid Standardization Program (22). Although widely used to isolate HDL because of their simplicity, the accuracy and precision of the precipitation methods depend strongly on complete removal of apo-B-containing lipoproteins. Accurate adjustments of pH, reagent concentration, ionic strength, and temperature are critical to the complete removal of apo-B-containing lipoproteins. Traces of LDL or VLDL remaining in the supernate can lead to overestimation of HDL-C (23). Significant constant differences among various precipitation methods (24, 25) and between precipitation methods and other methods of HDL-C determination have been reported (25, 26). The constant differences in HDL-C values observed between the present two methods are due to the use of two methodologies (i.e., precipitation and ultracentrifugation) that separate HDL by different mechanisms, the former based on formation of a complex with apo-B-containing lipoproteins and the latter based on differences in hydrated densities of lipoproteins.

The CDC-NHLBI Lipid Standardization Program has not yet established Reference Methods for measuring LDL-C(NCEP) or VLDL-C. Even though the Beta Quantification method is generally accepted as the method of choice for LDL-C(NCEP) measurement, its accuracy strongly depends on the accuracy of the HDL-C determination. In the Beta Quantification method, LDL-C(NCEP) is calculated by subtracting the HDL-C value from the cholesterol value measured in the infranate after plasma ultracentrifugation. Therefore, any positive error in HDL-C determination by the Beta Quantification method will result in a negative error for LDL-C(NCEP) values. Thus, systematic positive differences in LDL-C(NCEP) values of the VAP-IIs method with the Beta Quantification values (7.2% for venous, Table 2; and 5.1% for fingerstick, Table 4) are due largely to the corresponding negative differences in HDL-C values between the two methods. Measuring LDL-C(NCEP) by the Beta Quantification method is time consuming and labor intensive compared with the VAP-IIs method, and is also an indirect procedure. The need for a reliable and direct method for measuring LDL-C(NCEP) suitable for routine use in the clinical laboratory has been emphasized (27). Although the percentage differences in VLDL-C between the two methods were somewhat high (−11.8% for venous, Table 2; and −9.1% for fingerstick, Table 4), the absolute differences in the mean values were small (22 mg/L for venous blood, 17 mg/L for fingerstick blood). These higher percentage differences in VLDL-C are probably attributable to the high CVs for this analyte in the VAP-IIs method and the relatively small concentrations of VLDL-C in this set of subjects. CVs as great as 14% for VLDL-C were reported in a similar comparison study (28).

In summary, this highly sensitive new method (VAP-IIs) simultaneously measures cholesterol in all lipoprotein classes in only 18 μL of plasma. The method can be adapted for assaying lipoprotein cholesterol in fingerstick blood. The VAP-IIs method is reliable, as indicated by reproducibility (Table 1) and accuracy (Tables 2 and 4) data that meet the requirements of the CDC-NHLBI Lipid Standardization Program (22). It is rapid and less labor intensive than the Beta Quantification method and measures LDL-C directly. Low sample volume (and hence utility in, e.g., research studies involving animals), graphical output, and much simpler instrumentation (compared with VAP-I) are all significant advantages of the VAP-IIs method. The continuous profile provided by VAP-IIs allows more precise determination of the nature of lipid abnormalities than can conventional methods and allows direct comparisons of two profiles.

We gratefully acknowledge the efforts of Margaret Moore and Pearle Smith for providing isolated lipoproteins, Hong Chung for
previous efforts in the development of the VAP procedure, and Thomas Woolley for assistance in the analysis of data. This work was supported by a grant from the National Heart, Lung, and Blood Institute (HL 34343).

References