

Quantification of cholesterol in all lipoprotein classes by the VAP-II method

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Abstract We have developed a high resolution microvolume Vertical Auto Profile (VAP) method for the simultaneous measurement of cholesterol in all lipoprotein classes, including lipoprotein[a] (Lp[a]) and intermediate density lipoprotein (IDL). This method, designated as VAP-II, uses a non-segmented continuous flow (controlled-dispersion flow) analyzer for the enzymatic analysis of cholesterol in lipoprotein classes separated by a short spin (47 min) single vertical ultracentrifugation. Cholesterol concentrations of high (HDL), low (LDL), very low (VLDL), and intermediate (IDL) density lipoproteins, as well as Lp[a], are determined by decomposing the spectrophotometric absorbance curve, obtained from the continuous analysis of the centrifuged sample, into its components using software developed in this laboratory. Analysis by VAP-II is rapid and sensitive (as little as 40 μ l plasma is required per assay). The resolution of lipoprotein peaks is considerably enhanced in the present analyzer compared to the previous analyzer (VAP-I, which used the Technicon AutoAnalyzer); improvement is especially noticeable for Lp[a] and IDL. Total and lipoprotein cholesterol values obtained by VAP-II correlated well with the values obtained by Northwest Lipid Research Laboratories (NWLRL). VAP-II Lp[a] cholesterol values also correlated well with the Lp[a] mass values obtained by an immunoassay technique performed at NWLRL ($r = 0.907$). The reproducibility and accuracy of the method are within the requirements of the CDC-NHLBI (Centers for Disease Control-National Heart, Lung, and Blood Institute) Lipid Standardization Program.—Kulkarni, K. R., D. W. Garber, S. M. Marcovina, and J. P. Segrest. Quantification of cholesterol in all lipoprotein classes by the VAP-II method. *J. Lipid Res.* 1994. 35: 159–168.

Supplementary key words high density lipoprotein • low density lipoprotein • intermediate density lipoprotein • very low density lipoprotein • lipoprotein[a] • continuous-flow analysis • density-gradient ultracentrifugation

Elevated levels of blood total cholesterol (TC), especially low density lipoprotein (LDL) cholesterol, are strongly associated with an increased risk of coronary heart disease (CHD) (1, 2). Reduction in TC and LDL cholesterol levels reduces the incidence of CHD (3). However, a strong inverse relationship exists between high density lipoprotein (HDL) cholesterol and the risk of CHD (4, 5). While increased HDL cholesterol protects

against CHD and increases longevity (6), low HDL cholesterol is an independent and powerful predictor of CHD (7).

In addition to LDL and HDL, several other lipoproteins have also been shown to represent risk factors for CHD. Plasma lipoprotein[a] (Lp[a]), in particular, appears to be a major independent coronary risk factor with an importance approaching that of the level of LDL or HDL cholesterol (8). Increased plasma concentrations of Lp[a], a cholesterol-rich lipoprotein, have been observed in survivors of myocardial infarction (9, 10). Increased levels of intermediate density lipoprotein (IDL) cholesterol have also been found to be associated with CHD (11, 12). Elevation of plasma very low density lipoprotein (VLDL) cholesterol is seen in survivors of myocardial infarction as well (13).

Measurement of TC alone may not be adequate to identify subjects at risk for CHD. An individual with normal or near-normal levels of TC may still be at risk because of low HDL cholesterol (4, 5), elevated Lp[a] (9) or IDL (14) cholesterol. Therefore, measurement of the distribution of cholesterol among all lipoproteins (a lipoprotein cholesterol profile), in addition to the TC and LDL cholesterol, is desirable in order to assess risk for CHD accurately.

Determination of a cholesterol profile in a clinical laboratory usually involves independent cholesterol measurement for each lipoprotein class using different aliquots of the same plasma sample, often using the formula of

Abbreviations: VAP, Vertical Auto Profile; HDL, high density lipoproteins; LDL, low density lipoproteins; IDL, intermediate density lipoproteins; VLDL, very low density lipoproteins; Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; ELISA, enzyme-linked immunosorbent assay; TC, total cholesterol; TG, triglycerides; NWLRL, Northwest Lipid Research Laboratories; CDC, Centers for Disease Control; NHLBI, National Heart, Lung, and Blood Institute; NCEP, National Cholesterol Education Program; CHD, coronary heart disease.

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Friedewald, Levy, and Fredrickson (15). Multiple analyses and the assumptions involved in the Friedewald formula may make this procedure susceptible to error. In addition, the reported LDL cholesterol is not only an indirect value, but also includes Lp[a] cholesterol and IDL cholesterol values. At present, simple methods are not available for the quantification of Lp[a] cholesterol or IDL cholesterol.

The original Vertical Auto Profile (VAP) method (16–18), designated here as VAP-I, provides cholesterol concentrations of all lipoprotein classes using a single aliquot of plasma in a single analysis. The VAP method provides the continuous enzymatic analysis of cholesterol using a Technicon AutoAnalyzer in lipoprotein classes separated by single vertical spin density-gradient ultracentrifugation. However, the VAP-I method has several shortcomings. Most importantly, the Technicon AutoAnalyzer used in this method causes overlapping of adjacent lipoprotein peaks, resulting in a substantial loss of resolution. Therefore, quantification of cholesterol in lipoproteins such as Lp[a] and IDL, which are not well separated from other lipoproteins by density-gradient centrifugation, is somewhat problematical using this method. In addition, the VAP-I method requires a relatively large volume (1.3 ml) of plasma.

Recently, we described a highly sensitive new method (19) referred to as VAP-IIfs [Vertical Auto Profile-II (fingerstick)] and its application to fingerstick blood cholesterol profile analysis using only 18 μ l of plasma. VAP-IIfs was designed to overcome several of the problems associated with VAP-I method. Like VAP-I, the VAP-IIfs method is based on continuous enzymatic analysis of cholesterol in lipoprotein classes separated by single vertical spin centrifugation, but using a different flow analyzer. VAP-IIfs uses a controlled-dispersion flow analyzer, designed and assembled in our laboratory. However, due to unique features of fingerstick analysis (i.e., small plasma volume and increased speed of analysis), resolution in VAP-IIfs was essentially the same as in the VAP-I analyzer.

The controlled-dispersion flow analyzer has been adapted to achieve high resolution analysis of lipoproteins, while retaining several advantages of VAP-IIfs. The technique described here is designated as VAP-II. Quantification of cholesterol in all lipoprotein classes including Lp[a] and IDL by VAP-II and the clinical usage of this technique are evaluated in the present study.

MATERIALS AND METHODS

Blood samples

Blood samples were drawn into tubes containing EDTA by venipuncture from subjects fasted for 12 h. Plasma was separated by low speed centrifugation and stored at 4°C. All samples were analyzed within 5 days of collection.

Plasma samples isolated from blood, obtained from healthy volunteers, were used as calibrators for the conversion of area under the absorbance curve into cholesterol units. TC values for the calibrator plasma samples were assigned by the analysis at Northwest Lipid Research Laboratories (NWLRL) at the University of Washington, Seattle, WA, one of the laboratories of the National Cholesterol Reference Laboratory Network coordinated by the Centers for Disease Control (CDC), Atlanta, GA.

Separation of plasma lipoproteins

Plasma lipoproteins were separated by single vertical spin density-gradient ultracentrifugation as in the VAP-I method (18), except that plasma was diluted due to the increased sensitivity of VAP-II. Briefly, for the initial evaluation of the method, fresh plasma samples (50 μ l) obtained from healthy volunteers were diluted 30-fold with 29 parts of saline/EDTA solution (0.9% NaCl, 1 mmol/l EDTA, pH 7.4, density 1.006 kg/l) and then adjusted to a density of 1.21 kg/l by adding dry KBr. Plasma samples (40 μ l) obtained from hyperlipidemic subjects were diluted at least 40-fold for the clinical evaluation of the method. A density-gradient was prepared in 13 \times 51 mm polyallomer Quick Seal ultracentrifuge tubes (Beckman Instruments, Palo Alto, CA) by first pipetting 1.4 ml of density-adjusted diluted plasma into a Pasteur pipette placed in each tube and then overlaying with 3.9 ml of saline/EDTA (as described above) using a peristaltic pump after removal of the Pasteur pipette. After sealing, the tubes were centrifuged in an L8-80M ultracentrifuge (Beckman Instruments) using a VTi 80 rotor with the following conditions: centrifugation time, 31 min at a speed of 80,000 rpm ($w^2t = 1.31 \times 10^{11}$); temperature, 20°C; acceleration and deceleration settings, 6. The total centrifugation time including deceleration was 47 min. A tube containing calibration plasma with a known TC value was included in each rotor.

Vertical Auto Profile-II (VAP-II) analyzer

After the centrifugation, the cholesterol content in the tube was continuously analyzed using the VAP-II analyzer. The schematic representation and the operation of the VAP-II analyzer (controlled-dispersion flow analyzer) have been previously described (19). However, for the present study, the operating parameters of the analyzer were reoptimized to achieve high resolution analysis of lipoprotein classes separated as described above. The following optimum conditions were selected for routine operation of the analyzer: reagent flow rate, 0.62 ml/min; reaction mixture flow rate, 1.56 ml/min (note that the sample flow rate is calculated as the difference between reaction mixture and reagent flow rates); reagent concentration, 250 mg/ml; and Teflon tubing length, 2.0 m (0.8 mm i.d.). Undiluted density-adjusted plasma sam-

ples were also analyzed using the VAP-I analyzer for comparison of lipoprotein peak resolution between the two analyzers.

Quantification of cholesterol in lipoprotein classes

A Data Translation analog-to-digital conversion board (Data Translation Inc., Marlboro, MA) and software developed in this laboratory were used to continuously collect and digitize the absorbance data as the sample is analyzed. A cholesterol profile (absorbance curve) was obtained by plotting digitized absorbance units (i.e., area units) on the Y-coordinate and the relative gradient position (calculated from the sample drain time) on the X-coordinate. The cholesterol profile was then decomposed into curves corresponding to lipoprotein classes using other software previously developed in this laboratory, but modified for the VAP-II method. TC was determined by adding areas under all subcurves. The algorithm is based on the assumption that the curve shape for individual lipoprotein class is the same in a VAP profile as it is when preisolated lipoprotein classes are analyzed.

Peaks of lipoprotein classes, however, may vary among individuals in a number of ways, including density and subclass distribution. The VAP-II decomposition program was designed to handle variability in lipoprotein peak densities often seen in subjects with lipid disorders. A set of subcurves was empirically defined for each lipoprotein class whose shape parameters (widths at half height, peak height, and exponential parameter) were adjusted to provide optimal fit of the lipoprotein class under most conditions, initially using pre-isolated lipoprotein classes. The numbers of subcurves defined for each lipoprotein class were: HDL, 5; Lp[a], 4; LDL, 4; IDL, 2; and VLDL, 3; a total of 18 subcurves. During a profile decomposition, peak heights for the pre-defined subcurves for all lipoprotein classes were simultaneously varied until the sum of the squared deviations between the sum of the subcurves and the parent profile was minimized using a linear regression method. Thus, if the areas under the subcurves corresponding to the two ends of a parent lipoprotein curve (the curve representing the sum of the subcurves) were much less than in a normal profile, then the parent curve was narrower than in the normal profile. The location of the peak maximum of the parent curve was determined by the subcurve whose peak height is higher than the other subcurves in that class. Thus, when the peak maximum of a particular lipoprotein was shifted to the left in the main profile due to increased contributions from denser subspecies, the peak maximum of the parent lipoprotein curve was also automatically shifted to the corresponding position of lipoprotein peak maximum in the main profile. Similarly, when the peak density is shifted to the right (when lighter subspecies are predominant), the parent lipoprotein curve maximum shifted

towards the right. The original software has been described previously in detail (17).

Method of comparison

Determination of total, HDL, LDL, IDL, and VLDL cholesterol. Blood samples were collected from 23 normolipidemic and 20 hyperlipidemic subjects for the comparison study. Each plasma was divided into two portions after separating from blood. One portion was retained for the analysis by VAP-II and the other portion was sent to NWLRL. Analyses in this CDC-standardized laboratory were performed as summarized here. TC in whole plasma was measured using an automated enzymatic system (Abbot Spectrum Multichromatic Analyzer, Abbott Laboratories, North Chicago, IL). HDL cholesterol was enzymatically quantified in the supernatant after chemical precipitation of VLDL and LDL from the whole plasma with dextran sulfate-Mg²⁺ (20). LDL and VLDL were quantified using the Lipid Research Clinics β -quantification method (21). Cholesterol in the IDL fraction was determined as the difference between the cholesterol in $d > 1.006$ kg/l plasma fraction and the cholesterol in the $d > 1.019$ kg/l plasma fraction. To obtain the $d > 1.019$ kg/l fraction, plasma was adjusted to $d = 1.019$ kg/l with KBr and centrifuged for 18 h at 105,000 g and the top $d < 1.019$ kg/l fraction was removed.

Determination of Lp[a]. Fresh plasma samples ($n = 63$) covering a wide range of Lp[a] cholesterol values were obtained from the Lipoprotein Laboratory, University of Alabama at Birmingham, AL which uses the VAP-I method for the cholesterol profile determination. Lp[a] protein concentration was measured at the NWLRL by an in-house-developed enzyme-linked immunosorbent assay (ELISA) method in which two monoclonal antibodies specific for apolipoprotein[a] (apo[a]) with no cross reactivity with plasminogen and directed to different epitopes on the apo[a] molecule are used (22).

RESULTS

Optimization of VAP-II analyzer

In order to establish the operating conditions that provide maximum lipoprotein peak resolution and minimum analysis time (i.e., sample drain-time), effects of the following parameters on resolution and analysis time were studied: reaction mixture flow rate, reagent flow rate, reagent concentration, and Teflon tubing length. (Note that the sample flow rate in the present analyzer is calculated as the difference between the flow rates of reaction mixture and reagent which are controlled by different peristaltic pumps.) Each parameter was varied over a wide range of values (reaction mixture flow rate, 1.30–2.1 ml/min; reagent flow rate, 0.28–0.94 ml/min; reagent con-

centration, 62.5–250.0 mg/ml; and Teflon tubing length, 1.0–3.0 m with 0.8 mm i.d.) while keeping all other parameters constant. The value of the parameter under study that provided optimum resolution and analysis time was then used to study the next parameter, and so on, until all parameters were adjusted to the optimum values. Plasma was diluted 30-fold for the optimization study due to the increased sensitivity of the present method.

The resolution increased with decrease in reaction mixture flow rate, increase in reagent flow rate, increase in reagent concentration, and decrease in Teflon tubing length. However, analysis time increased with flow rates that provided increased resolution. Therefore, the following optimum values were chosen for further work after a compromise between resolution and analysis time: reaction mixture flow rate, 1.56 ml/min; reagent flow rate, 0.62 ml/min; reagent concentration, 250 mg/ml; Teflon tubing length, 2.0 m (0.8 mm i.d.). With the above flow rates, time required to analyze one centrifuged sample was 5 min and 40 sec, thus closely matching analysis time for eight tubes contained in a VTi 80 rotor (55 min including delay time between samples) with centrifugation time (47 min). The above values for reagent concentration and Teflon tubing length also provided optimum sensitivity for the assay in addition to optimum resolution.

As VAP-II is highly sensitive, appropriate dilution of sample is required to bring the cholesterol concentration of each lipoprotein class within the linear range of the analyzer. In order to determine the level of plasma dilution, three plasma samples with increasing LDL cholesterol values (100, 125, and 153 mg/dl) were selected; plasma samples containing lipoproteins other than LDL in the same concentration range could also be used as all lipoproteins react with similar kinetics in this analyzer. Note that the linearity of the assay is limited only by the concentrations of individual lipoproteins, not the TC, because ultracentrifugally separated lipoprotein classes react with the reagent at different time points. The relative sensitivities (area under the profile in arbitrary units \times dilution factor) of the cholesterol profiles obtained using aliquots prepared by diluting the plasma (containing LDL cholesterol concentration of 100 mg/dl) 2-, 5-, 10-, 15-, 20-, 30-, 40-, and 50-fold with saline solution were 11314, 20670, 29860, 36945, 38440, 41220, 42000, and 41350, respectively. The relative sensitivity increased with increasing dilution as the concentration of all lipoproteins approached the linear range and remained constant once they were within the linear range. Thus, relative sensitivities were maximum and similar for profiles obtained with 30-, 40-, and 50-fold diluted aliquots, suggesting that plasma samples containing any or all lipoproteins with cholesterol concentrations of 100 mg/dl or below be diluted at least 30-fold for quantitative analysis.

Similarly, the relative sensitivities for the plasma with

LDL cholesterol of 125 mg/ml were maximum and similar only for aliquots obtained by diluting the plasma 30-, 40-, and 50-fold (47451, 47400, and 47451 respectively). However, for the plasma containing 153 mg/dl LDL cholesterol, relative sensitivities were maximum and similar (50224, 50170) only for aliquots prepared by diluting the plasma 40- and 50-fold, suggesting that plasma be diluted at least 40-fold when any lipoprotein cholesterol value exceeds 150 mg/dl. Plasma samples containing any or all lipoproteins with a maximum cholesterol concentration of 200 mg/dl (upper limit of linear range \times dilution factor; i.e., 5×40) may be analyzed by diluting 40-fold. A 40-fold dilution of plasma is, therefore, recommended for routine analysis, since most clinical samples can be quantified by this dilution. However, the assay should be repeated if any of the lipoprotein cholesterol concentration exceeds 190 mg/dl by further diluting the plasma until two subsequent dilutions result in no change of lipoprotein cholesterol values.

The above dilution study also indicated that resolution increased with increase in dilution. However, the increase was considerable only up to 10-fold dilution. The resolution remained essentially the same for aliquots obtained by diluting plasma more than 10-fold. The following optimum conditions were selected for routine analysis based on the above results: reaction mixture flow rate, 1.56 ml/min; reagent flow rate, 0.62 ml/min; reagent concentration, 250 mg/ml; Teflon tubing length, 2.0 m (0.8 mm i.d.); and plasma dilution, 40-fold (in the initial stage of the work, characterization and evaluation of VAP-II method was performed by diluting normolipidemic plasma samples 30-fold).

A typical cholesterol profile obtained with the VAP-II analyzer using plasma from a normal subject is shown in Fig. 1A. All three major lipoprotein classes (HDL, LDL, and VLDL) present in this subject are well resolved. Cholesterol in five lipoprotein classes (HDL, Lp[a], LDL, IDL, and VLDL) can be quantified using software developed in this laboratory (see Materials and Methods). The decomposed form of the Fig. 1A profile is shown in Fig. 1B. Five subcurves corresponding to the five lipoproteins mentioned above are derived from the decomposition of the main absorbance curve, providing a complete cholesterol profile.

Characterization of VAP-II analyzer

Linearity of response. Linearity of response was determined using individual lipoprotein classes isolated from plasma samples by fixed-angle preparative ultracentrifugation (18). Aliquots containing cholesterol concentrations in the range of 1–7 mg/dl were prepared by diluting individual lipoprotein classes with saline/EDTA. After adjusting to a density of 1.21 kg/l, aliquots were subjected to the single vertical spin density-gradient ultracentrifugation using the same conditions as for the whole plasma.

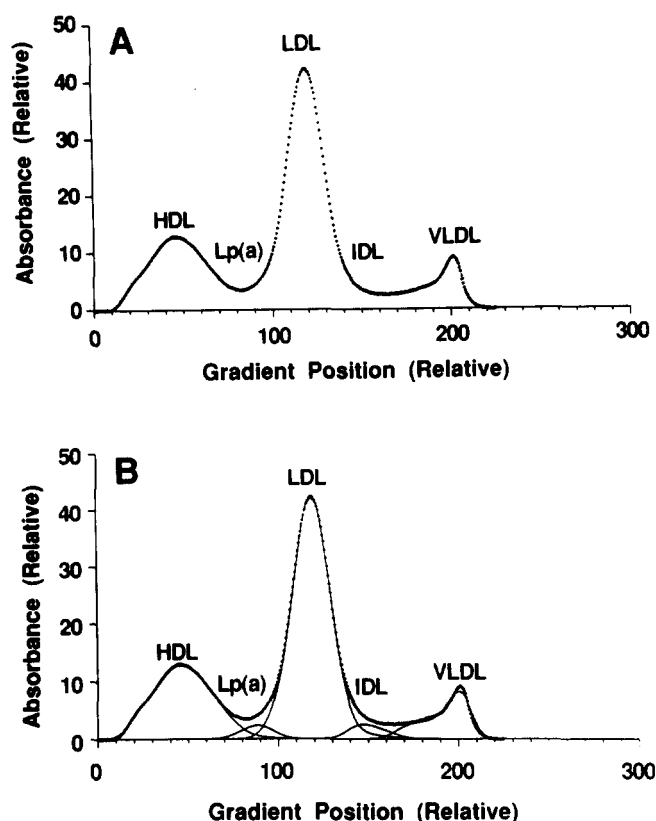


Fig. 1. Original and computer decomposed cholesterol profiles obtained by VAP-II method. **A:** A VAP-II original cholesterol profile (absorbance curve) of a normal subject as obtained by the chart recorder. **B:** Quantification of cholesterol in lipoprotein classes. The output from the spectrophotometric detector was digitized (broken curve), and the cholesterol content of each lipoprotein class was determined using computerized decomposition (solid curves) as described in Materials and Methods.

The response, as measured by the area under the lipoprotein peak, was linear at least up to 5 mg/dl for each lipoprotein class. This corresponds to an upper limit of 200 mg/dl for individual lipoprotein classes in an undiluted plasma, as plasma is diluted 40-fold for VAP-II analysis.

The linear regression characteristics of the curves, obtained by plotting concentration of lipoprotein cholesterol versus peak area, for HDL, Lp[a], LDL, and VLDL, respectively, were: correlation coefficient (r): 0.999, 0.996, 0.995, and 0.989; slope (area units per mg/dl): 83.0, 82.0, 77.0, and 86.0; and intercept (area units per mg/dl): -12.0, -13.0, -6.0, and 9.0. The similar slope values suggest similar reaction kinetics for pre-isolated lipoprotein classes in this analyzer. Similar reaction kinetics allow the measurement of a transient signal (i.e., a signal measured before the reaction reaches completion) with the VAP-II method.

Reproducibility. Within- and between-run reproducibility of measurement for total and lipoprotein cholesterol was studied using 28 aliquots of a 30-fold diluted plasma. Each run, using a VTi 80 rotor, consisted of seven aliquots of the diluted plasma and an aliquot of 30-fold diluted calibrator plasma. CVs obtained from this study are shown in **Table 1**. Both within-run (0.48–1.12%) and between-run (1.34%) CV values for TC are well within the $\pm 3\%$ requirement of the CDC-NHLBI (National Heart, Lung, and Blood Institute) Lipid Standardization Program (23). HDL cholesterol measurements are also highly reproducible; within-run (1.31–2.89%) and between-run (2.47%) CVs are, again, within the requirement of $\pm 6\%$ of the above standardization program (23). The reproducibility of measurement for other lipoproteins including Lp[a] and IDL is also satisfactory.

Comparison of lipoprotein peak resolution between VAP-I and VAP-II

Fig. 2 shows cholesterol profiles obtained by VAP-I (A, C, E) and VAP-II (B, D, F) using three plasma samples. The shoulder due to Lp[a] in the VAP-I profile, A, appears as a well-resolved distinct peak in the corresponding VAP-II profile, B. Similarly, a peak due to IDL is seen in the VAP-II profile, D, while it is obscured by the broadening of the LDL peak in the corresponding VAP-I profile, C. Major lipoprotein peaks (HDL, LDL, and VLDL) are also more highly resolved by VAP-II than by VAP-I. The

TABLE 1. Reproducibility of cholesterol measurement by VAP-II method

Run	Coefficient of Variation (%)					
	Total	HDL	Lp[a]	LDL	IDL	VLDL
1	0.72	1.31	3.44	1.03	5.97	4.0
2	0.48	1.98	2.99	1.29	7.21	5.08
3	1.12	2.66	4.06	1.51	5.31	5.96
4	0.57	2.89	5.49	2.86	5.31	3.95
Between-run	1.34	2.47	4.26	1.91	5.89	5.79

Twenty eight aliquots of 30-fold diluted plasma were subjected to density-gradient ultracentrifugation in four rotors (runs) along with an aliquot of 30-fold diluted calibration plasma in each rotor. Total and lipoprotein cholesterol were determined by computer-assisted decomposition of absorbance curves obtained by analyzing centrifuged aliquots using VAP-II. The cholesterol values (mg/dl) of the plasma were: total, 172; HDL, 31; Lp[a], 27; LDL, 80; IDL, 14; and VLDL, 20.

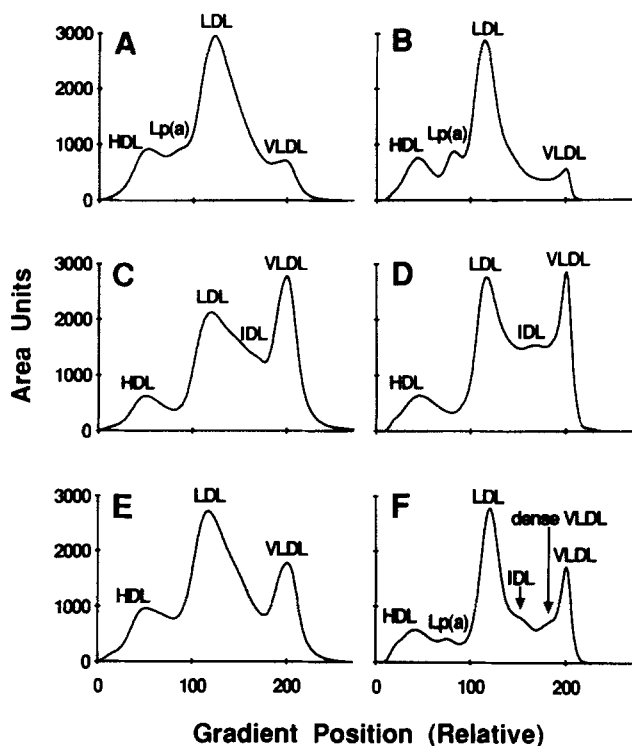


Fig. 2. Comparison of lipoprotein peak resolution between VAP-I and VAP-II analyzers. Cholesterol profiles A, C, and E obtained by VAP-I, respectively, correspond to cholesterol profiles B, D, and F obtained by VAP-II. Plasma samples were diluted 30-fold for VAP-II analysis, whereas undiluted plasma samples were used for VAP-I analysis.

high resolution capability of the VAP-II analyzer is well demonstrated in profile F; in this profile, at least four distinct peaks corresponding to HDL, Lp[a], LDL, and VLDL, and a well-developed shoulder due to IDL can be seen; a shoulder on the ascending portion of the VLDL peak is due to the dense VLDL subclass.

Comparison of results from VAP-II and NWLRL methods

Total, HDL, LDL, IDL and VLDL cholesterol. In the absence of satisfactory reference methods, lipoprotein cholesterol measurements by the VAP-II method were compared with the conventional Lipid Research Clinics methods performed at NWLRL. Plasma samples obtained from 23 normolipidemic and 20 hyperlipidemic subjects were used for comparison. The VAP-II method separates Lp[a] and IDL from true LDL, in contrast to the β -quantification method used for LDL cholesterol determination at NWLRL. Therefore, in order to be consistent with the LDL cholesterol values as measured by the β -quantification method and also as described by the National Cholesterol Education Program (NCEP), LDL cholesterol comparisons were made by summing VAP-II cholesterol values for Lp[a], IDL, and true LDL.

Results of the lipoprotein cholesterol measurements by the two methods are compared in **Fig. 3**. HDL and LDL cholesterol values correlated well. The VLDL cholesterol values obtained from the two methods also correlated satisfactorily. Comparisons made using Student's two-tailed paired *t* test showed that VAP-II HDL cholesterol values were lower than the corresponding NWLRL values (mean paired difference = 3.6%), and that VAP-II LDL cholesterol values were higher than NWLRL values (mean paired difference = 3.3%), both the differences being statistically significant ($P < 0.001$). The mean paired difference for VLDL cholesterol values (7.0%, VAP-II measuring lower) from the two methods was less significant ($P < 0.01$). Comparison of IDL cholesterol values obtained by the two methods using the same 20 hyperlipidemic plasma samples used for the comparison of other lipoprotein cholesterol measurements yielded a correlation coefficient of 0.93 with a slope value of 0.77. The mean values obtained by the two methods differed only by 3.7% and this difference was not statistically significant ($P > 0.2$). Total cholesterol values were in excellent agreement as shown by the correlation data (slope, 0.98; intercept, 5.2 mg/dl; r , 0.996; n , 43); the difference (0.2%) between the two mean values (NWLRL, 220.5 ± 9.0 mg/dl; VAP-II, 220.9 ± 8.9 mg/dl) was statistically nonsignificant ($P > 0.6$).

Lp[a] cholesterol. In the 63 plasma samples used for the comparison, Lp[a] total mass ranged from 1.0 to 156.0 mg/dl and Lp[a] cholesterol from 2.0 to 42.0 mg/dl with the mean being 53.7 ± 5.0 and 13.4 ± 1.1 mg/dl, respectively. Linear regression analysis of the comparison between Lp[a] mass measured by ELISA and Lp[a] cholesterol measured by VAP-II yielded a correlation coefficient of 0.907 with an intercept of 3.1 mg/dl and a slope of 0.19 (**Fig. 4**).

VAP-II Cholesterol profiles of dyslipoproteinemic patients

Several types of dyslipoproteinemia are commonly associated with patients who have established CHD or are from families with premature CHD. Many of these types of dyslipoproteinemia have important distinguishing features that are not assessed by standard lipoprotein profiles. **Fig. 5** shows VAP-II cholesterol profiles obtained from the plasma samples of several patients with various forms of dyslipoproteinemia along with a profile from a normal subject. TC and lipoprotein cholesterol values obtained from VAP-II and the measured plasma triglyceride (TG) values of these patients are shown in **Table 2**.

Pure hypercholesterolemia is associated with a marked elevation of LDL cholesterol but with normal levels of triglycerides. It is often caused by an LDL receptor abnormality termed familial hypercholesterolemia. A VAP-II cholesterol profile of a patient with this dyslipidemia is shown in **Fig. 5B**.

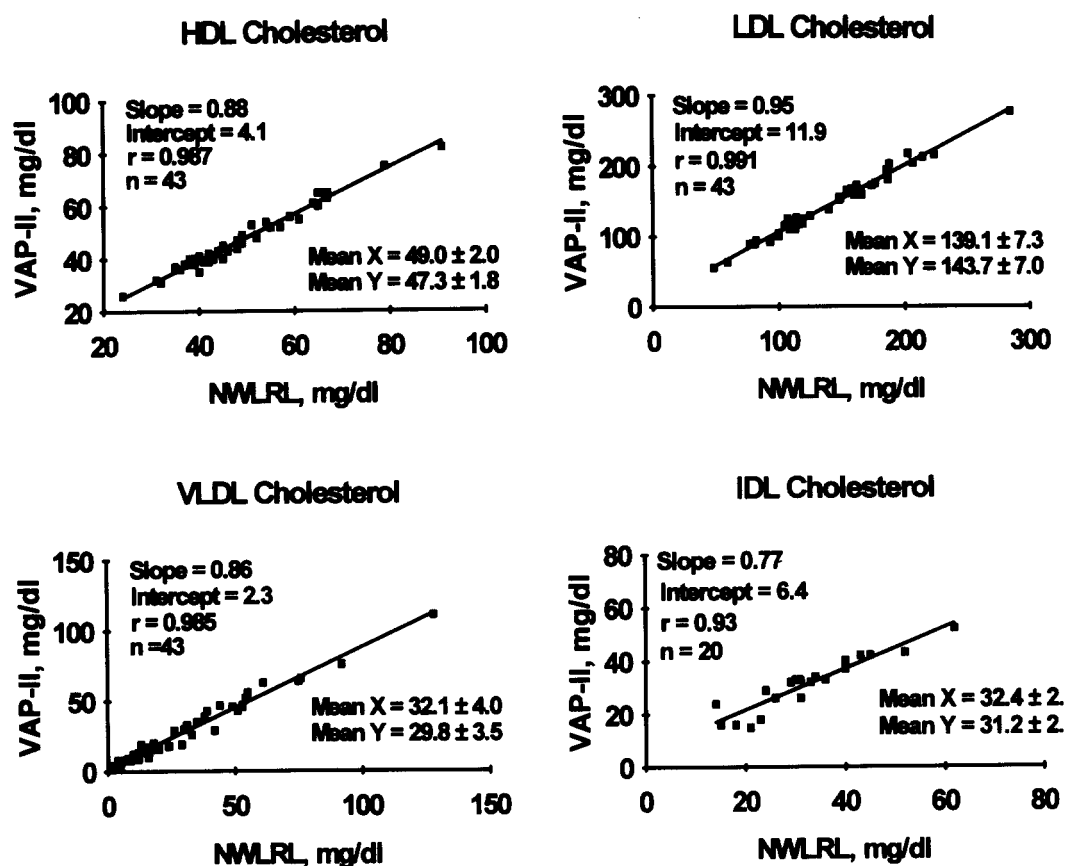


Fig. 3. Comparison of lipoprotein cholesterol measurement by VAP-II and NWLRL methods. Plasma separated from blood obtained from 23 normolipidemic and 20 hyperlipidemic subjects was used for the comparison of methods. Mean cholesterol values are reported as mean \pm SEM. Ranges of cholesterol values (mg/dl) as obtained by NWLRL method: HDL, 24–91; LDL, 48–285; VLDL, 4–128; and IDL, 14–62. Standard deviation from regression (\bar{y} , x) for the curves comparing HDL, LDL, VLDL, and IDL cholesterol were 2.0, 6.1, 4.0, and 3.8, respectively. VAP-II LDL cholesterol values were obtained by summing cholesterol values of Lp[a], IDL, and true LDL for the purpose of comparison. Only plasma from hyperlipidemic subjects was used for the comparison of IDL cholesterol.

Familial combined hyperlipidemia is associated with small dense LDL, small dense VLDL (VLDL₃), elevated IDL, and decreased HDL₂ (pattern B as defined by

Austin et al., 24). Patients with familial combined hyperlipidemia often have simultaneous elevation of VLDL and LDL, such that LDL cannot be accurately calculated by the Friedewald's equation. Examples of VAP-II analyses of three patients with this disorder are shown in Figs. 2D, 2F, and 5C.

Elevated Lp[a] carries a high risk for development of CHD. When elevated Lp[a] is associated with other forms of dyslipoproteinemia, e.g., familial hypercholesterolemia (as shown in Fig. 5B) or familial combined hyperlipidemia (as shown in Fig. 2F), the risk for development of premature CHD is increased significantly.

Dysbetalipoproteinemia is associated with premature CHD and an abnormal lipoprotein particle called β -VLDL. The result of this abnormality is that true LDL is never formed, but rather an LDL-like particle is present in the IDL density range. An example of a VAP-II analysis of a patient with dysbetalipoproteinemia is shown in Fig. 5D. Examples of other forms of dyslipoproteinemia are also shown in Fig. 5.

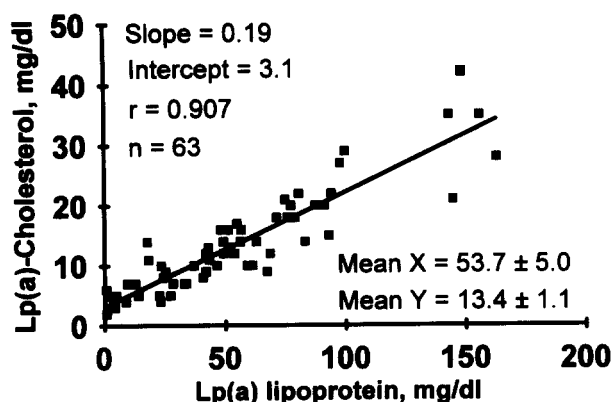


Fig. 4. Comparison between the measurement of Lp[a] cholesterol and total Lp[a] mass. Lp[a] cholesterol was measured by VAP-II method and Lp[a] mass was determined by a monoclonal antibody-based ELISA.

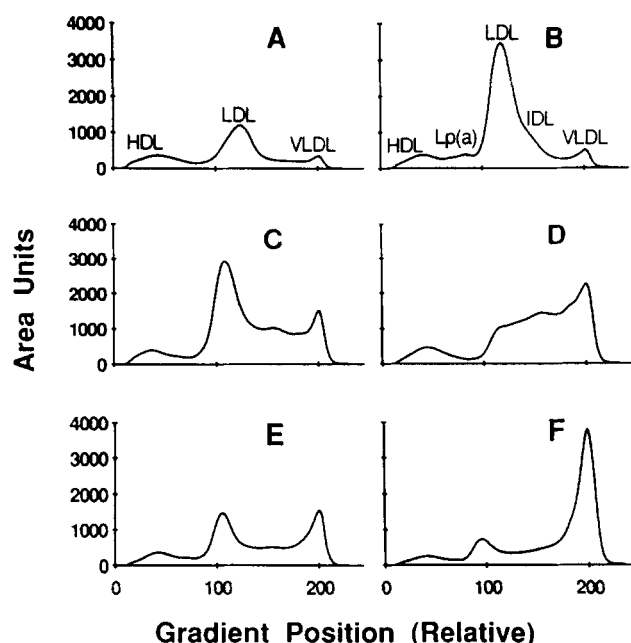


Fig. 5. VAP-II Cholesterol profiles of a normal individual and of dyslipoproteinemic patients. Cholesterol profiles were obtained by subjecting plasma samples obtained from a normal individual (A) and from dyslipoproteinemic patients (B, familial hypercholesterolemia; C, familial combined hyperlipidemia; D, dysbetalipoproteinemia; E, mild hypertriglyceridemia; and F, severe hypertriglyceridemia) to VAP-II analysis. Plasma samples were diluted for analysis as follows: A, 30-fold; B, C, D, and F, 50-fold; and E, 40-fold. Total and lipoprotein cholesterol values of the plasma from each patient were obtained by computer decomposition of the respective cholesterol profiles (Table 2).

DISCUSSION

The need for cholesterol measurement in major lipoproteins such as LDL and HDL, in addition to the measurement of TC, has recently received much attention because of the strong association of these lipoproteins with the development of CHD. However, recent studies have

shown that other lipoproteins, such as Lp[a] and IDL, are also involved in the development of CHD. Clinical interest in Lp[a] has been stimulated by numerous reports linking increased concentrations of plasma Lp[a] with an increased incidence of CHD (8-10). The importance of IDL cholesterol in the development of CHD has been demonstrated by the Type II Coronary Intervention Study of the NHLBI (25). Increased CHD risk may be also related to increased concentrations of IDL in familial dysbetalipoproteinemia (26) and familial combined hyperlipidemia patients (27). Thus, measurement of cholesterol concentrations in lipoproteins such as Lp[a] and IDL, in addition to LDL and HDL, may assist in the identification of a greater number of subjects at risk of CHD. In clinical laboratories, the cholesterol profile is determined by measuring TG, TC, and HDL cholesterol using three different aliquots of the same plasma, and VLDL cholesterol is calculated using the formula: VLDL cholesterol = TG/5. The LDL cholesterol is estimated from the above three measurements using the Friedewald formula (15). This is not only an indirect value but also includes cholesterol values of Lp[a] and IDL. The multiple analyses involved in this procedure, along with the indirect estimations made, may result in inaccurate lipoprotein cholesterol values. In addition, clinical laboratories lack the availability of procedures for the measurements of Lp[a] cholesterol and IDL cholesterol. More accurate measurements may be made using sequential flotation ultracentrifugation, as used in the β -quantification method, but this approach is time consuming for clinical use.

The Vertical Auto Profile-I (VAP-I) method (16, 17), largely overcomes these problems. However, lipoprotein peaks are not optimally resolved by this method, which uses an air-segmented Technicon AutoAnalyzer. In contrast, the present method uses a non-segmented continuous flow (controlled-dispersion flow) analyzer in which a short narrow bore Teflon tubing is used as the reactor to reduce the sample dispersion. Reduced dispersion results

TABLE 2. Cholesterol and triglyceride values in a normal individual and in dyslipoproteinemic patients

Patient	Cholesterol						Triglyceride ^b
	Total	HDL	Lp(a)	IDL	LDL ^a	VLDL	
Normal	166	45	4	5	103	17	84
FH	349	37	16	42	278	34	87
FCHL	392	37	6	57	261	94	237
Dysbetalipoproteinemia	366	41	6	82	164	161	235
Mild HTG	209	28	4	21	100	81	494
Severe HTG	291	24	18	25	80	186	1428

FH, familial hypercholesterolemia; FCHL, familial combined hyperlipidemia; HTG, hypertriglyceridemia. Cholesterol and triglyceride values are expressed in mg/dl. Cholesterol values were obtained from the VAP-II method.

^aThe LDL cholesterol value referred to by the NCEP (National Cholesterol Education Program) and normally reported by clinical laboratories, and is the sum of Lp[a], true LDL, and IDL cholesterol.

^bTriglyceride values were obtained by performing manual assay using triglyceride [GPO-Trinder] reagent (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions.

in enhanced resolution of lipoprotein peaks. The improvement of resolution in VAP-II compared to VAP-I is demonstrated in Fig. 2. The resolution of Lp[a] and IDL peaks is especially important, as these tend to be obscured by adjacent peaks in VAP-I. The resolution in VAP-II is also better than in VAP-IIfs. VAP-IIfs uses a tabletop ultracentrifuge to minimize sample volume and reduce analysis time (19). However, the tube geometry in tabletop ultracentrifugation reduces the resolution of lipoprotein species and the resolution in VAP-IIfs is essentially the same as in VAP-I. The VAP-II method described here uses a floor ultracentrifuge, allowing separations to be performed in tubes with a greater height-to-width ratio, improving resolution.

VAP-II is a reliable method, as demonstrated by its accuracy (Fig. 3) and reproducibility (Table 1). Total cholesterol values obtained from the VAP-II and NWLRL methods agreed very well; the difference between the two mean values (0.2%) is well within the requirements ($\pm 3\%$) of the CDC-NHLBI Lipid Standardization Program (23). Although statistically significant differences were observed between the values of the two methods for the lipoprotein classes (HDL, LDL, and VLDL), the results from the two methods correlated well. In addition, the difference between the two mean HDL cholesterol values (3.6%) is within the $\pm 10\%$ accuracy limit required by the CDC-NHLBI Lipid Standardization Program (23).

Although the correlation between IDL cholesterol values obtained by VAP-II and by the sequential flotation method was somewhat lower ($r = 0.93$, slope = 0.77) than that obtained for total, HDL, LDL, and VLDL cholesterol, it should be noted that IDL is not a well-defined lipoprotein class and is difficult to separate from other classes. The correlation coefficient of 0.93 appears to arise from the differences in the two measurement techniques. IDL cholesterol is measured by the sequential flotation technique as the difference between the cholesterol values of the two infranatant fractions obtained by the ultracentrifugation of two aliquots from the same plasma adjusted to the densities of 1.006 and 1.019 kg/l, which are operationally defined lower and upper limits of density range for IDL. As actual density range for IDL may vary from the operationally defined range and may overlap with the densities of VLDL and LDL in subjects with lipid disorders (28), sequential flotation may overestimate or underestimate the IDL cholesterol. On the other hand, IDL cholesterol measurement by VAP-II is based on the cholesterol profile decomposition program that utilizes fixed subcurve peak parameters (shape and location of the subcurve in the profile) for the individual lipoproteins, making it susceptible to measurement errors in subjects with very abnormal lipoprotein density distribution. Linear regression analysis performed by combining IDL and VLDL cholesterol values, indeed, showed an improvement in slope (0.90 for combined IDL and VLDL

values as opposed to 0.77 for IDL alone or 0.84 for VLDL alone) as well as an improvement in correlation coefficient (0.985 for combined IDL and VLDL values as opposed to 0.93 for IDL alone or 0.98 for VLDL alone).

The correlation between Lp[a] cholesterol values as measured by VAP-II and total Lp[a] mass as measured by an immunochemical approach can be considered very satisfactory if we take into account the differences between the two methods. In fact, while the amount of cholesterol is relatively constant in most Lp[a] particles, the mass of apo[a] per Lp[a] particle varies widely. Therefore, a one-to-one relationship between Lp[a] cholesterol and Lp[a] mass would not be expected. While numerous studies have reported that elevated levels of Lp[a], determined immunochemically by measuring the protein component of Lp[a], are associated with an increased risk of CHD, no data are available on the association of Lp[a] cholesterol and CHD due to the lack of methods for measuring Lp[a] cholesterol in plasma. Considering that the LDL cholesterol values, as determined by the β -quantification method, include IDL and Lp[a] cholesterol, the VAP-II method makes it possible to evaluate the relative contribution of LDL, IDL, and Lp[a] cholesterol to the risk of CHD.

Although the present method is based on a decomposition program that uses fixed subcurve peak parameters in order to quantify individual lipoproteins, a drawback analogous to the use of fixed density ranges for the separation of each lipoprotein class in the β -quantification method, it works quite well for most samples. Use of fixed multiple subcurves for each lipoprotein class does allow for variation in density of the lipoproteins.

In summary, VAP-II is a high resolution method that requires only 40 μ l of plasma for analysis. In contrast to other methods, VAP-II measures cholesterol in all lipoprotein classes (HDL, LDL, VLDL, Lp[a], and IDL) directly. The small sample volume requirement of this method also makes it a useful technique in studies involving small animals. ■

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