papers on methodology

Quantification of HDL_2 and HDL_3 cholesterol by the Vertical Auto Profile-II (VAP-II) methodology

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Abstract Of the several existing methods for quantification of major subspecies of high density lipoprotein (HDL), HDL_2 and HDL_3, the methods based upon double precipitation are particularly useful for large-scale studies or for routine assay because of their high speed and low cost. The Vertical Auto Profile-II (VAP-II) method developed in our laboratory primarily for the direct single test measurement of cholesterol (C) in all major lipoproteins, including Lp[a] and IDL, is rapid, highly sensitive, and suitable for large-scale studies. Here we describe the modification of this procedure so as to be able to quantify both HDL_2- and HDL_3-C in addition to all major lipoproteins without any additional assay steps, time, or cost. The VAP-II procedure was validated by comparison with four other methods using plasma samples obtained from 35 healthy subjects: *1*) HDL-VAP-II (a variation of the VAP-II procedure designed specifically to separate HDL subspecies); *2*) dextran sulfate (DS)/Mg^{2+} double precipitation method performed at Northwest Lipid Research Laboratories (NWLRL), Seattle, WA; 3) 4–30% polyacrylamide-agarose ($^4/_{30}$ PAA) nondenaturing gradient gel electrophoresis (GGE); and 4) analytical ultracentrifugation (AUC), with both GGE and AUC performed at the Donner Laboratory, University of California at Berkeley. Both HDL_2- and HDL_3-C measurements by VAP-II correlated well with the measurements by all comparison methods (r for HDL_3-C: HDL-VAP-II, 0.948; NWLRL, 0.947; GGE, 0.861; and AUC, 0.706, and r for HDL_2-C: HDL-VAP-II, 0.867; NWLRL, 0.854; GGE, 0.885; and AUC, 0.721). The measurements of HDL_2- and HDL_3-C by the VAP-II method are reproducible, with the long-term between-rotor CV of 5.0% for HDL_3-C and 9.0% for HDL_2-C.—**Kulkarni, K. R., S. M. Marcovina, R. M. Krauss, D. W. Garber, A. M. Glasscock, and J. P. Segrest.** Quantification of HDL_2 and HDL_3 cholesterol by the Vertical Auto Profile-II (VAP-II) methodology. *J. Lipid Res.* 1997. **38:** 2353–2364.

Supplementary key words lipoproteins • HDL subclasses • ultracentrifugation • continuous flow analysis

It is now well established that increased levels of high density lipoprotein cholesterol (HDL-C) provide protection against coronary artery disease (CAD) (1, 2), whereas a low level of HDL-C acts as an independent and powerful CAD risk factor (3). HDL is a highly heterogeneous lipoprotein consisting of several subspecies with varying density, size, and apolipoprotein (apo) composition. Variations in these properties may influence the degree of protection against CAD provided by the HDL subspecies. HDL has been commonly separated into two major subspecies, namely HDL_2 and HDL_3, based on their density, although further heterogeneity among these subspecies has been demonstrated using high resolution techniques such as gradient gel electrophoresis (GGE) (4, 5) and isoelectric focusing (6, 7). In addition, HDL has been immunochemically separated into two populations of particles, LpA-I (lipoprotein containing apoA-I without apoA-II) and LpA-I: A-II (lipoprotein containing apoA-I with apoA-II), based on their apolipoprotein composition (8, 9). These immunochemically separated subspecies may have distinctly different metabolic functions (10) as well as clinical significance (11, 12).

Several studies have shown that the HDL_2 subspecies is more protective against CAD than the HDL_3 subspecies and that inter-individual variations in HDL-C are primarily due to the variations in HDL_2-C, with HDL_3-C remaining relatively constant. For example, as early as in 1966, Gofman, Young, and Tandy (13) found that

Abbreviations: HDL-C, LDL-C, IDL-C, VLDL-C, cholesterol associated with high (HDL), low (LDL), intermediate (IDL), and very low (VLDL) density lipoproteins, respectively; Lp[a]-C, cholesterol associated with Lp[a]; TC, total cholesterol; apo, apolipoprotein; TG, triglycerides; VAP, Vertical Auto Profile; LpA-I, lipoprotein containing apoA-I without apoA-II; LpA-I:A-II, lipoprotein containing both apoA-I and apoA-II.

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the HDL_2 levels were substantially reduced in subjects with CAD compared to the subjects without CAD, while HDL_3 levels were similar in both groups. Reduced levels of HDL_2-C with similar HDL_3-C levels have also been found in survivors of myocardial infarction compared to the control subjects (14). However, HDL_3 has been found to be more protective against CAD risk in some studies (15, 16). In several of the studies that made the measurement of LpA-I and LpA-I:A-II in various populations, including normolipidemic patients with angiographically proven CAD (11, 17), CAD patients with hypertriglyceridemia (18, 19), patients with the history of myocardial infarction (20), and children whose parents had a premature CAD (21), LpA-I has been reported to be significantly reduced compared to control subjects. LpA-I:A-II was also reduced but not in all of these studies (17, 21). Thus LpA-I, in general, was the more powerful discriminating factor among these two subpopulations of particles.

Although inconclusive, the above-mentioned studies suggest that the relationship between HDL and the risk of CAD is actually much more complex than one can predict from the measurement of total HDL-C alone. The measurement of HDL subspecies may, therefore, lead to a better understanding of the association of HDL with the risk of CAD, with a subsequent improvement in assessment and treatment of CAD.

Although various methods, including ultracentrifugation (analytical, sequential flotation, density gradient, and zonal), double precipitation [heparin-Mn^{2+}/dextran sulfate (DS), DS/Mg^{2+}, and polyethylene glycol/DS], and nondenaturing gradient gel electrophoresis, have been developed for the measurement of HDL_2 and HDL_3, only the precipitation methods have proved useful in large-scale studies because of their high speed and low cost compared to other methods. However, precipitation procedures require careful standardization because of their sensitivity to various reaction conditions and hence can be performed satisfactorily only in specialized laboratories. Therefore, the development of additional methods for the measurement of HDL_2 and HDL_3 will be of immense help both in atherosclerosis research as well as in the clinical assessment of CAD risk.

In this report we describe a rapid, direct, and simultaneous quantification procedure for HDL_2- and HDL_3-C using the Vertical Auto Profile-II (VAP-II) method. The VAP-II procedure, which is based upon a short single vertical spin centrifugation, has been developed in our laboratory primarily to quantify cholesterol concentrations of all major lipoproteins, including those of Lp[a] and IDL, directly and simultaneously in a single test, using only 40 μL of plasma (22). However, simultaneous quantification of subspecies of major lipoproteins, such as HDL and LDL, is also possible as lipoprotein subspecies are also partially separated along with the major lipoproteins during centrifugation due to the differences among their densities and flotation rates. Thus, both HDL_2- and HDL_3-C can be quantified in addition to all major lipoproteins by appropriately modifying the previously described cholesterol absorbance curve deconvolution procedure for the VAP-II method (22), without any additional assay steps, time, or cost. The speed of the VAP-II procedure combined with no additional cost and time of analysis makes it a potentially useful technique both in atherosclerosis research and in clinical assessment of CAD risk.

MATERIALS AND METHODS

Study subjects

Blood samples from 62 fasting subjects were collected into EDTA-containing Vacutainer tubes in two batches. Plasma was separated from blood by low speed centrifugation. Plasma samples obtained from the first batch (n = 27) were used for initial studies intended to develop a modified version of the VAP-II cholesterol absorbance curve deconvolution program (22) that would allow simultaneous quantification of HDL_2 and HDL_3 in addition to the major lipoproteins. Both hypertriglyceridemic and hypercholesterolemic subjects in addition to normolipidemic subjects were included in this batch so that the modified deconvolution program can be used for HDL subspecies quantification in a wide range of plasma samples. Plasma samples obtained from the second batch (n = 35, containing primarily normolipidemic subjects) were used to test and validate the modified VAP-II method by comparison with the HDL-VAP-II method (a variation of the VAP-II method that specifically separates HDL subspecies) and three other methods: DS/Mg^{2+} double precipitation, 4–30% polyacrylamide–agarose (4/30 PAA) nondenaturing gradient gel electrophoresis, and analytical ultracentrifugation (AUC). Aliquots from the plasma samples obtained from the second batch (n = 35) were sent to the Northwest Lipid Research Laboratories (NWLRL) for the precipitation analysis and to the Donner Laboratory, University of California at Berkeley, for GGE (n = 35) and AUC (n = 12) analyses.

VAP-II procedure

HDL_2- and HDL_3-C were quantified using the VAP-II procedure described previously (22) for the direct and simultaneous measurement of major lipoproteins. However, the VAP-II cholesterol absorbance curve was de-

convoluted using a newly modified deconvolution program (described below) in order to quantify HDL_2- and HDL_3-C in addition to the major lipoproteins. Briefly, the VAP-II procedure consisted of preparing a density gradient by pipetting 1.42 mL of density-adjusted 40-fold diluted plasma (corresponding to 35.5 μl of undiluted plasma) into a Beckman Quick Seal centrifuge tube (~5 mL volume, catalog No. 342412) and overlayering with 3.90 mL of saline with a density of 1.006 kg/L. The density of plasma was adjusted to 1.21 kg/L by adding a calculated amount of dry KBr. Samples were centrifuged at 80,000 rpm and 20°C for 47 min (including acceleration and deceleration time) in a Beckman Instruments VTi-80 vertical rotor and L8-80M Floor Model Ultracentrifuge (centrifugation settings: time, 31 min; acceleration, 6; and deceleration, 6). The gradient containing the separated lipoproteins and their subspecies was then analyzed for cholesterol distribution using the VAP-II continuous flow analyzer.

VAP-II cholesterol profile deconvolution program

Although the VAP procedure was developed primarily to quantify major lipoproteins, the quantification of lipoprotein subspecies is also possible as subspecies are also simultaneously separated along with the major lipoproteins by this method. The peaks due to subspecies are generally not visible in the cholesterol absorbance curve of the VAP profile because of the small differences in the densities and flotation rates between the subspecies (22). However, the separation of subspecies is evident from the shapes as well as the relative positions of the major lipoprotein peak maxima in the density gradient that vary between VAP profiles.

In the VAP procedure, cholesterol concentrations of major lipoproteins are derived by mathematically deconvoluting the absorbance curve, obtained by the continuous cholesterol analysis of the separated gradient, into five curves, with each curve corresponding to a major lipoprotein (HDL, Lp[a], LDL, IDL, or VLDL) (22). Each lipoprotein curve in turn consists of a predefined number of exponential gaussian subcurves that correspond to subspecies of that major lipoprotein. However, the original deconvolution program was not optimized in terms of the subcurve positions and shape parameters (widths at half height and the exponential parameter) for the purpose of accurate quantification of subspecies. Therefore, the deconvolution program was modified to quantify HDL_2- and HDL_3-C by assigning an appropriate number of subcurves to the HDL curve and adjusting their positions and shape parameters such that an optimal fit for the HDL curve was obtained under most conditions. During a profile deconvolution, peak heights of the predefined subcurves for all lipoprotein classes, including those for HDL subcurves, were simultaneously varied until the sum of the squared deviations between the sum of the subcurves and the parent absorbance curve was minimized using a regression method.

Optimization of the program was also performed using several VAP-II profiles to identify the two sets of HDL subcurves that respectively correspond, in terms of cholesterol values, to HDL_3-C and HDL_2-C obtained from the corresponding HDL-VAP-II profiles (described later in this section); the cholesterol values of the HDL subcurves were calculated by converting the subcurve areas into cholesterol values by assigning the total HDL-C value obtained from the unmodified deconvolution program of VAP-II to the sum of the areas under all HDL subcurves and then calculating cholesterol per unit area. The HDL_3- and HDL_2-C values obtained from the HDL-VAP-II method were used as the standard values for the initial studies, as HDL-VAP-II resolves HDL more effectively into HDL_3 and HDL_2, thus providing more reliable values.

Initial optimization performed using several representative profiles from the initial sample batch indicated that a total of seven HDL subcurves were needed to be defined to achieve an optimal curve fitting, with the first four subcurves together corresponding to HDL_3 and the remaining three subcurves to HDL_2. The optimized program was then used for deconvolution of the remaining profiles from the initial batch while making minor adjustments in the deconvolution parameters in order to arrive at a refined final version of the modified deconvolution program. An example of a VAP-II cholesterol profile obtained by the deconvolution of cholesterol absorbance curve using the optimized program is shown in **Fig. 1.** The profile shows the curves corresponding to HDL_3 (representing the sum of the areas of the first four HDL subcurves) and HDL_2 (representing the sum of the areas of the last three HDL subcurves) in addition to the curves corresponding to all other major lipoproteins. Furthermore, a shoulder that corresponds to HDL_2 subspecies can be observed on the descending portion of the HDL peak in the main cholesterol absorbance curve. All the profiles from the initial studies were deconvoluted using the final version of the program, and the HDL_2- and HDL_3-C values thus obtained were compared to the corresponding values obtained from HDL-VAP-II. The accuracy of the rigorously optimized deconvolution program was then tested using an additional 35 plasma samples obtained from the second batch of subjects.

HDL-VAP-II procedure

The HDL-VAP-II procedure is a variation of the VAP-II procedure designed specifically to separate HDL subspecies (23). The densities of the gradient layers are

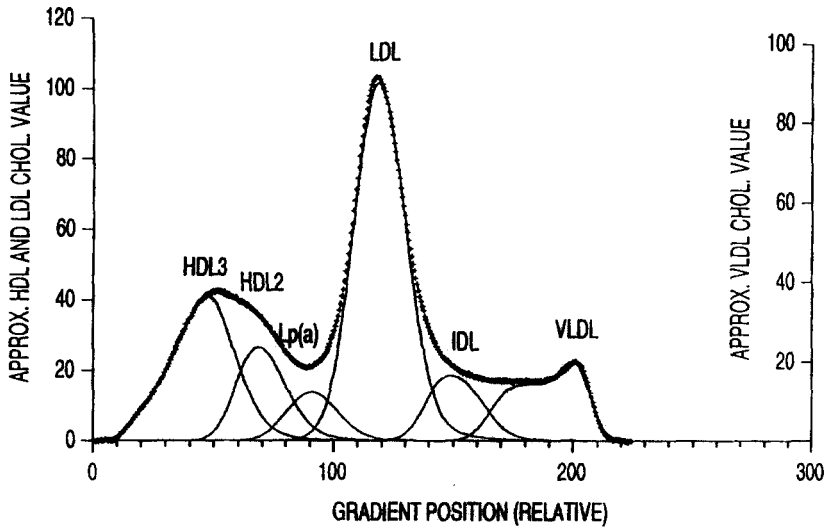


Fig. 1. An example of VAP-II cholesterol profile showing the distribution of HDL_3 and HDL_2 along with the distribution of all major lipoproteins. The broken curve indicates the parent cholesterol absorbance curve obtained by the continuous analysis of the centrifuged sample using the VAP-II analyzer and the solid curves indicate areas under the respective lipoproteins derived from the deconvolution of the parent cholesterol absorbance curve.

chosen such that the HDL spreads into much of the centrifuge tube while forcing all other lipoproteins to float at the top of the tube. This results in a very effective separation of HDL into HDL_3 and HDL_2. The purpose of utilizing HDL-VAP-II in this work was 2-fold. First, because HDL_3 and HDL_2 are well resolved by this procedure, their cholesterol values obtained by this method can be more reliable and thus could be used as standard values for the development of modified VAP-II. Second, the HDL-VAP-II can also serve as a comparison method in the final validation of the VAP-II procedure.

The HDL-VAP-II procedure consisted of preparing a density gradient by pipetting 1.42 mL of density-adjusted 20-fold diluted plasma (corresponding to 71 μl of undiluted plasma) into a Beckman Quick Seal tube (~5 mL volume) and overlaying with 3.90 mL of saline solution with a density of 1.06 kg/L. The density of plasma was adjusted to 1.21 kg/L by adding calculated amount of dry KBr. Samples were centrifuged at 80,000 rpm and 10°C for 90 min including deceleration time (centrifugation settings: time, 71 min; acceleration and deceleration, 6). The cholesterol content of the gradient containing HDL subspecies was then analyzed using the VAP-II continuous flow analyzer. Two reasonably well-resolved density peaks corresponding to HDL_2 and HDL_3 are thus observed in an HDL-VAP-II cholesterol absorbance curve (**Fig. 2, A**). The absorbance curve was deconvoluted using a newly developed deconvolution program (described below) to provide HDL_2- and HDL_3-C values obtained by the areas under the two curves, which correspond to HDL_3 and HDL_2 density peaks (Fig. 2, B). The area under the HDL portion of the HDL-VAP-II profile was calibrated in terms of cholesterol units by assigning the total HDL-C obtained from the unmodified VAP-II procedure to the total area under the HDL curve and calculating cholesterol per unit area.

The deconvolution program for the HDL-VAP-II was developed by defining an appropriate number of exponential gaussian subcurves to the HDL portion of the HDL-VAP-II and then adjusting the position and shape parameters for each subcurve until an optimal fit was achieved for HDL. The optimization process suggested the need to define seven subcurves for HDL portion in order to achieve an optimal curve fit. During profile deconvolution, the peak heights of the subcurves for all lipoprotein classes, including those of HDL subcurves, were simultaneously varied until an optimal fit was obtained i.e., until the sum of the squared deviations between the sum of the subcurves and the parent absorbance curve was minimized, using a regression method. Initial optimization indicated that areas under the first four HDL subcurves correspond to the area under the HDL_3 density peak, while the sum of the areas under the remaining HDL subcurves correspond to HDL_2 density peak. Thus, in an HDL-VAP-II profile, the first four HDL subcurves are summed together to provide a single curve that corresponds to the HDL_3 density peak and similarly, the last three HDL subcurves are summed together to provide a curve that corresponds to the HDL_2 density peak (Fig. 2, B).

In conjunction with 4/30 PAA nondenaturing GGE and immunoaffinity chromatography, we previously showed that the HDL separated by the HDL-VAP procedure consists of nine subspecies, denoted $HDL_{[1]}$–$HDL_{[9]}$, with increasing size and decreasing density; $HDL_{[1]}$, $HDL_{[2]}$, $HDL_{[8]}$, and $HDL_{[9]}$ were found to be minor subspecies, with only trace amounts of concentrations (23). Furthermore, we observed that $HDL_{[7]}$, consisting of three separate subspecies ($_{[7a]}$, $_{[7b]}$, and $_{[7c]}$),

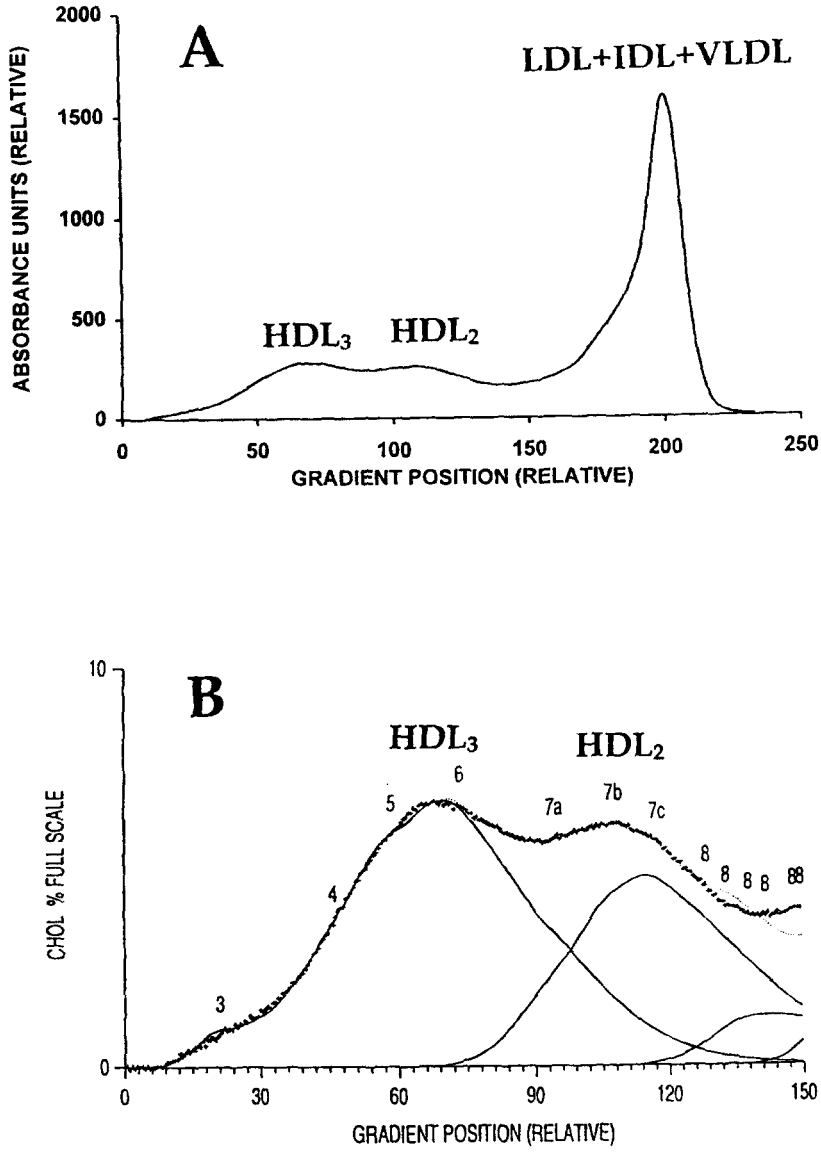


Fig. 2. A: An HDL-VAP-II cholesterol absorbance curve showing the separation of HDL_3 and HDL_2. The gradient conditions (see Materials and Methods) are chosen such that the HDL subspecies distribute into most of the centrifuge tube allowing improved separation, while forcing all other lipoproteins to float at the top of the tube. B: Mathematical deconvolution of the absorbance curve shown in A. The portion of the absorbance curve corresponding to HDL_3 and HDL_2 in A is mathematically deconvoluted into seven subcurves corresponding to $HDL_{[3]}$–$HDL_{[7a]}$, $_{[7b]}$, $_{[7c]}$ (peak maximal position for each subcurve is indicated by its subcurve number (i.e., 3, 4, 5, 6, 7_a, 7_b, or 7_c). Subcurves $HDL_{[3]}$–$HDL_{[6]}$ are summed together and represented by a solid curve underneath the HDL_3 density peak, whereas subcurves $HDL_{[7a]}$–$HDL_{[7c]}$ are summed together and represented as a single solid curve underneath the HDL_2 density peak.

represented $(HDL_{2b})_{gge}$ as defined by Blanche et al. (4) and $HDL_{[6]}$ represented $(HDL_{2a})_{gge}$. Similarly, we observed that the subspecies $HDL_{[3]}$–$HDL_{[5]}$ were within the same size range as that of $(HDL_3)_{gge}$, with $HDL_{[4]}$ and $HDL_{[5]}$ together corresponding to $(HDL_{3a})_{gge}$, $HDL_{[3]}$ corresponding to $(HDL_{3b})_{gge}$, and the minor subspecies $HDL_{[1]}$ and $HDL_{[2]}$ together corresponding to $(HDL_{3c})_{gge}$, which was also found to be a minor subspecies by Blanche et al. (4). As $(HDL_{2a})_{gge}$ was shown both to exhibit polydispersity and overlap considerably with $(HDL_{3a})_{gge}$ (4), we considered $HDL_{[6]}$, which represents $(HDL_{2a})_{gge}$, as one of the HDL_3 components. Furthermore, we have shown that the $HDL_{[6]}$ subspecies contains only LpA-I with A-II particles (23). In addition, the peak location of $HDL_{[6]}$ in HDL-VAP-II profile is also well within the HDL_3 density peak (subcurve 6 in Fig. 2, B). Thus, the four subcurves defined for HDL_3 correspond to $HDL_{[3]}$–$HDL_{[6]}$ and the three subcurves defined for HDL_2 correspond to $HDL_{[7a]}$, $_{[7b]}$, and $_{[7c]}$. In addition, Blanche et al. (4) have shown that the $(HDL_{2b})_{gge}$, which corresponds to $HDL_{[7]}$ of HDL-VAP-II, primarily corresponds to the $(HDL_{2b})_{density(1.063–1.10)}$ obtained by the standard preparative ultracentrifugation procedure which is uncontaminated with HDL_3. Thus, $HDL_{[7]}$ of the HDL-VAP-II, which corresponds to HDL_2 by VAP-II, was considered to be equal to $(HDL_{2b})_{density(1.063–1.10)}$.

Precipitation procedure

Dextran sulfate (50 kDa)/Mg^{2+} double precipitation procedure (24) was used by the Northwest Lipid Research Laboratories to quantify HDL_2- and HDL_3-C lev-

els of the same 35 plasma samples used to test the modified VAP-II method. The procedure essentially consists of two precipitation steps that use two different aliquots of the same plasma sample. In the first step, LDL and VLDL are precipitated out using 10 mg/mL DS and 0.5 mol/L $MgCl_2$, and the total HDL-C is determined by measuring cholesterol content of the supernatant. In the second step, HDL_2, LDL, and VLDL are precipitated out using 19.1 mg/mL DS and 1.95 mol/L $MgCl_2$, and the HDL_3-C is determined by measuring cholesterol content of the supernatant. HDL_2-C is calculated as the difference between the two cholesterol values, i.e., HDL_2-C = HDL-C − HDL_3-C. The reproducibility of this procedure measured as part of the quality control efforts at NWLRL is highly satisfactory. The coefficients of variations (CVs) were 1.4% and 2.4% for high (mean ± SD, 75.0 ± 1.2 mg/dL, n = 169) and low (mean ± SD, 24.4 ± 0.6 mg/dL, n = 171) HDL_3-C, respectively, and 11.4% for low HDL_2-C (mean ± SD, 4.7 ± 0.5 mg/dL, n = 169).

Nondenaturing gradient gel electrophoresis

4/30 PAA nondenaturing GGE (4) was performed at the Donner Laboratory, University of California at Berkeley, using aliquots from the same 35 plasma samples. Plasma separated at a density of 1.21 kg/L was used for the gel electrophoresis and each GGE run was stained with Coomassie blue for protein and then scanned with a laser gel scanner. The areas under five predetermined peak positions for HDL_{3c}, HDL_{3b}, HDL_{3a}, HDL_{2a}, and HDL_{2b} were then calculated as the percent of total HDL area. These percentages were then converted to HDL_2- and HDL_3-C values as follows. *1*) As discussed above, we considered HDL_{2b} of GGE as equivalent to $HDL_{[7]}$ (actually $HDL_{[7a]+[7b]+[7c]}$) of HDL-VAP-II, which in turn is considered equivalent to HDL_2 of the VAP-II. Similarly, the sum of the remaining percentages of GGE peaks were considered to be equivalent to the HDL_3 of HDL-VAP-II (i.e., $HDL_{[3]}$–$HDL_{[6]}$) as well as that of VAP-II. *2*) Using an average of the three known values of cholesterol-to-protein ratio (C/P) for HDL_2 and HDL_3 (25–27), we calculated an average C/P ratio of 0.527 for HDL_2 and 0.309 for HDL_3. *3*) The percent total protein for HDL_{2b} was multiplied by 0.527 to calculate a relative (r) HDL_2-C and the remaining percent total protein multiplied by 0.309 to calculate a relative (r) HDL_3-C. *4*) Finally, $\%HDL_2\text{-C} = 100 \times rHDL_2\text{-C}/(rHDL_2\text{-C} + rHDL_3\text{-C})$ and $HDL_2\text{-C} = \%HDL_2\text{-C} \times$ total HDL-C (measured by NWLRL or VAP-II).

Analytical ultracentrifugation procedure

HDL_2 and HDL_3 were quantified using 12 of the 35 samples by the analytical ultracentrifugation method (28) at the Donner Laboratory. HDL_3 was measured as the lipoprotein mass in the fraction with flotation rate corresponding to S_f0–3.5 and HDL_2 as the fraction with $S_f > 3.5$–9.0. HDL_2 and HDL_3 masses were then converted to their respective cholesterol values by using an average of the three known percentage C values (25–27) for HDL_2 and HDL_3 (the same C percentage values used in the calculation of C/P ratios for GGE work were used).

Statistical methods

Cholesterol values are expressed as mean (mg/dL) ± SEM. Simple linear regression was used to compare the measurements made by any two methods. Tests of significance were performed using Student's paired *t* test.

RESULTS

Optimization of the VAP-II cholesterol profile deconvolution program

Initial studies were performed using 27 plasma samples obtained from the first batch of subjects (which included both normolipidemic and hyperlipidemic subjects) to modify the VAP-II cholesterol absorbance curve deconvolution program, so as to be able to quantify both HDL_2- and HDL_3-C along with all major lipoproteins in a single test. As described in Materials and Methods, the HDL_2- and HDL_3-C values obtained from the HDL-VAP-II were used as the standard values to optimize the VAP-II deconvolution program. The optimization was first performed using several representative profiles and then the optimized program was used to deconvolute the remaining profiles, while simultaneously refining the program when necessary. The HDL_2- and HDL_3-C values were then obtained using the final version of the deconvolution program for all 27 samples and compared with the corresponding values obtained from the HDL-VAP-II using linear regression method. Both HDL_2- and HDL_3-C values obtained from the VAP-II method correlated well with the corresponding HDL-VAP-II values, with a correlation coefficient of 0.956 for HDL_3-C and 0.919 for HDL_2-C. The slope and intercept (mg/dL) were, respectively, 0.81 and 7.0 for HDL_3-C, and 1.09 and −2.2 for HDL_2-C. The mean HDL_3-C value obtained from VAP-II (33.4 ± 1.2, mg/dL) was also close to the corresponding HDL-VAP-II mean value (32.5 ± 1.41) ($P > 0.05$). Similarly the VAP-II mean value for HDL_2-C (11.6 ± 1.07) did not significantly differ from the mean HDL-VAP-II HDL_2-C value (12.6 ± 0.9) ($P > 0.05$). These data suggest a

TABLE 1. Reproducibility of HDL_3 and HDL_2 cholesterol measurements by the VAP-II method

Rotor		Coefficient of Variation (%)				
		1	2	3	4	Between-Rotor
Plasma 1	HDL_3	2.2	2.9	2.4	4.6	4.4
	HDL_2	2.7	4.0	3.1	5.1	4.7
Plasma 2	HDL_3	2.2	0.9	1.5	1.8	2.6
	HDL_2	6.1	4.4	5.5	4.3	5
Plasma 3	HDL_3	1.5	1.7	1.2	1.2	2.3
	HDL_2	0	3.5	3.9	3.2	7.9
Plasma 4	HDL_3	2.1	2.4	4.0	2.0	4.1
	HDL_2	13.0	11.3	8.5	9.2	16.4

Twenty-four aliquots from each plasma sample were analyzed in four rotors as described in Materials and Methods. Each rotor contained six aliquots of the plasma and two aliquots of calibration plasma which was used to convert the area under the cholesterol profile into cholesterol units. The HDL_3-C concentrations (mg/dL) of plasma samples 1, 2, 3, and 4 were 62, 55, 33, and 28, respectively, and HDL_2-C concentrations (mg/dL) of plasma samples 1, 2, 3, and 4 were 43, 22, 14, and 4, respectively.

good agreement between the VAP-II and HDL-VAP-II, and thus the use of HDL-VAP-II values for the optimization process of VAP-II cholesterol absorbance curve deconvolution program is justifiable.

Reproducibility

Reproducibility of HDL_3- and HDL_2-C measurements by the VAP-II procedure was assessed by analyzing four plasma samples representing a wide range of HDL_2- and HDL_3-C values. Twenty four aliquots from each plasma sample were analyzed in four rotors to determine within-rotor and between-rotor reproducibility; each rotor contained six aliquots of plasma under study and two aliquots of calibration plasma. The calibration plasma whose total cholesterol was determined at NWLRL was used to convert the area under the VAP-II cholesterol profile to its cholesterol value (22). The results obtained from reproducibility study are presented in **Table 1.** Within-rotor coefficient of variation (CV) ranged from 0.9 to 4.6% for HDL_3-C and from 0 to 6.1% for HDL_2-C for plasma samples 1–3, which contained high to normal levels of HDL_3- and HDL_2-C. The between-rotor CV for these three plasma samples ranged from 2.3 to 4.4% for HDL_3-C and from 4.7 to 7.9% for HDL_2-C. The plasma sample 4, which contained low HDL_3-C (27.5 ± 1.1 mg/dL) and HDL_2-C (4.3 ± 0.7 mg/dL), yielded a highly satisfactory within-rotor CV (2.0–4.0%) and between-rotor CV of 4.1% for HDL_3-C. However, within-rotor CV (8.5–13%) and between-rotor CV (16.4%) for HDL_2-C were somewhat higher for this plasma sample. The long-term reproducibility was assessed by analyzing 24 aliquots from a single plasma sample in 24 different rotors over a period of 9 weeks (the mean [±SD] HDL_2-C for all aliquots of this plasma sample was 20.6 ± 1.9 mg/dL). The between-rotor CV was 5.0% for HDL_3-C and 9.0% for HDL_2-C. In addition, plasma sample 1 also contained an Lp[a]-cholesterol concentration of 32 mg/dL. The highly satisfactory CVs obtained using this plasma sample suggest that high Lp[a] levels do not affect the reproducibility of HDL subclass quantification by the VAP-II method.

In addition, the reproducibility of VAP-II measurement of HDL subclasses compared very well with the reproducibility of HDL subclass measurements by the double precipitation method. A plasma sample that contained low HDL_3-C (27.5 ± 1.1 mg/dL,plasma sample 4) yielded within-rotor CV (%) of 2.1, 2.4, 4.0, and 2.0 and between-rotor CV of 4.1% by the VAP-II compared to the CV of 2.4% obtained by the double precipitation at NWLRL for a plasma sample that contained similar HDL_3-C (24.4 ± 0.6 mg/dL, n = 171). Similarly, a plasma sample that contained high HDL_3-C (61.9 ± 2.7 mg/dL, plasma sample 1) yielded within-rotor CV (%) of 2.2, 2.9, 2.4, and 4.6 and between-rotor CV of 4.4% by the VAP-II compared to the CV of 1.2% obtained by the double precipitation for a plasma sample that contained 75.0 ± 1.2 mg/dL (n = 169) of HDL_3-C. The CVs for samples containing low HDL_2-C were somewhat higher for both methods. The plasma sample 4, which contained 4.2 ± 0.7 mg/dL of HDL_2-C, yielded within-rotor CV (%) of 13.0, 11.3, 8.5, and 9.2, and between-rotor CV of 16.4% by the VAP-II compared to the CV of 11.4% obtained by the double precipitation for a plasma sample that contained 4.7 ± 0.5 mg/dL (n = 169) of HDL_2-C. The higher CVs for samples containing low HDL_2-C by both methods appear to be typical of the analyses involving very low concentrations of test analyte.

Comparison of VAP-II with other methods

HDL_2- and HDL_3-C measurements by the VAP-II method were validated by analyzing an additional 35 plasma samples using the VAP-II, HDL-VAP-II, and three other methods: *1*) DS/Mg^{2+} double precipitation

method performed at NWLRL; *2*) 4/30 PAA nondenaturing GGE performed at the Donner Laboratory; and *3*) analytical ultracentrifugation method also performed at the Donner laboratory (n = 12).

Comparison with the HDL-VAP-II. As in the initial studies, both the HDL_2- and HDL_3-C measurements by the VAP-II compared very well with the measurements made by the HDL-VAP-II, with correlation coefficients of 0.867 for HDL_2-C and 0.948 for HDL_3-C. The slope and intercept values, respectively, were 0.837 and 2.7 (mg/dL) for HDL_2-C, and 0.835 and 4.6 (mg/dL) for HDL_3-C. Although the differences among the mean HDL_2-C and HDL_3-C were statistically significant ($P < 0.01$ for HDL_2-C and $P = 0.02$ for HDL_3-C), the actual mean values were close to each other (HDL_2-C: VAP-II, 11.9 ± 0.65; and HDL-VAP-II, 11.0 ± 0.67, and HDL_3-C: VAP-II, 32.2 ± 0.99; and HDL-VAP-II, 33.1 ± 1.12).

Comparison with the DS/Mg^{2+} double precipitation method. The results of the comparison of VAP-II measurements with the double precipitation method (performed at NWLRL) obtained by linear regression method are summarized in **Fig. 3** using scatter plots. HDL_3-C values obtained by the VAP-II procedure correlated very well with the values obtained by the precipitation method (*r:* 0.947). Similarly, the VAP-II HDL_2-C values correlated very well with the precipitation values (*r:* 0.854). However, the HDL_2-C values obtained by the VAP-II procedure were higher compared to the values obtained by the precipitation procedure; the mean values were: NWLRL, 6.1 ± 0.49; and VAP-II 11.9 ± 0.65. The precipitation mean HDL_3-C value, on the other hand, was higher than the corresponding VAP-II value; the mean values were: NWLRL, 42.5 ± 1.51; and VAP-II, 32.2 ± 0.99. The difference in the mean HDL_3-C between NWLRL and VAP-II (10.3 mg/dL) was higher than the corresponding difference in the total HDL-C (4.5 mg/dL). Thus, the differences in HDL_3-C between the VAP-II and precipitation method could not be completely accounted by the differences observed in the corresponding HDL-C values between the two methods.

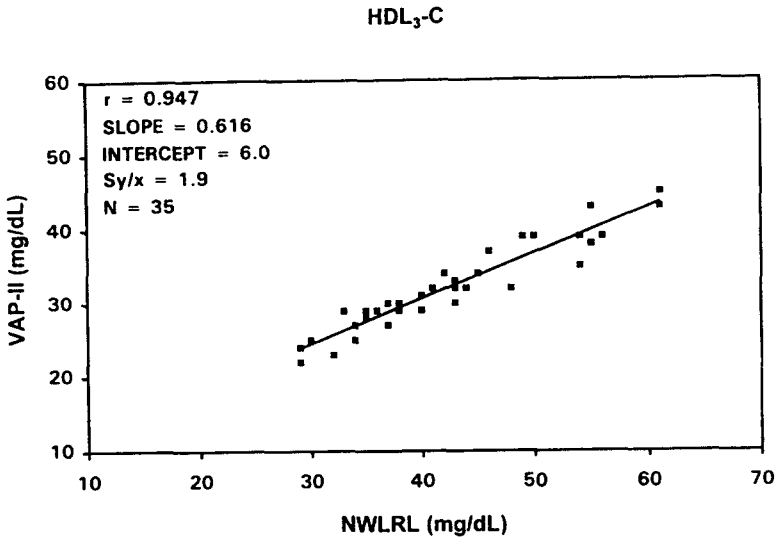
Comparison with the gradient gel electrophoresis method. As the gradient gel electrophoresis method provides only the relative protein concentrations of HDL_2 and HDL_3 (percent of total HDL protein), a more clinically meaningful approach for comparison of VAP-II (which quantifies HDL subclasses in terms of their cholesterol values) would be to derive HDL_2-C and HDL_3-C for GGE from their relative protein concentrations (as described in the Materials and Methods section) and then compare HDL subclass cholesterol concentrations obtained by the two methods. In deriving the absolute HDL_2- and HDL_3-C values for GGE, the total HDL-C value of the comparison method (VAP-II or NWLRL) needs to be assigned to the total HDL-C value of the GGE in the calculations. As the total HDL-C values obtained from different comparison methods varied somewhat from one another, the absolute HDL_2- and HDL_3-C values calculated for GGE also varied slightly, depending upon the total HDL-C obtained by a particular comparison method. However, the HDL_3-C/HDL_2-C ratio for GGE was unchanged irrespective of the method from which the HDL-C value was used in the calculation (the ratios were 3.12 and 3.08 when the mean HDL-C obtained, respectively, from VAP-II and NWLRL were used).

The results of the comparisons of HDL_2- and HDL_3-C measurements by the VAP-II and the precipitation methods with the measurement by GGE are summarized in **Table 2.** The HDL_2- and HDL_3-C values obtained by the VAP-II procedure as well as by the precipitation method correlated very well with their corresponding GGE values (with *r* values ranging from 0.84 to 0.89). The mean HDL_3-C values calculated for GGE using the HDL-C obtained from different comparison methods were similar to the mean value obtained from the VAP-II but higher than the mean value obtained from the precipitation method. These data (good correlation coefficients with significantly different mean values) are suggestive of the methodological differences.

Comparison with the analytical ultracentrifuge method. **Table 3** summarizes the results of comparison of VAP-II and precipitation procedures with the analytical ultracentrifuge procedure using 12 of the 35 samples. The HDL_2-C values measured by the VAP-II procedure as well as by the precipitation procedure correlated equally well with the values obtained by the analytical ultracentrifuge (*r* ranged from 0.72 to 0.76). However, the mean analytical ultracentrifuge HDL_2-C value, like the mean VAP-II HDL_2-C value, was higher than the corresponding precipitation value and tended more towards the VAP-II mean value (NWLRL, 6.3 ± 0.59; AUC, 8.8 ± 1.23; VAP-II, 11.3 ± 0.63). The comparisons of HDL_3-C values obtained from all methods suggest that VAP-II correlated better with the analytical ultracentrifuge than with the precipitation procedure. The mean analytical ultracentrifuge HDL_3-C value was similar to the mean VAP-II HDL_3-C value but lower compared to the precipitation value (NWLRL, 40.6 ± 1.71; AUC, 29.9 ± 2.05; VAP-II, 31.8 ± 1.18).

VAP-II quantification of HDL subclasses for plasma samples containing extreme values of HDL subclasses and other lipoproteins

In order to determine the accuracy of HDL subspecies quantification by the VAP-II when plasma samples contain extreme values of HDL-C, HDL_3-C, HDL_2-C, Lp[a]-C, and VLDL-C, two plasma samples, one with



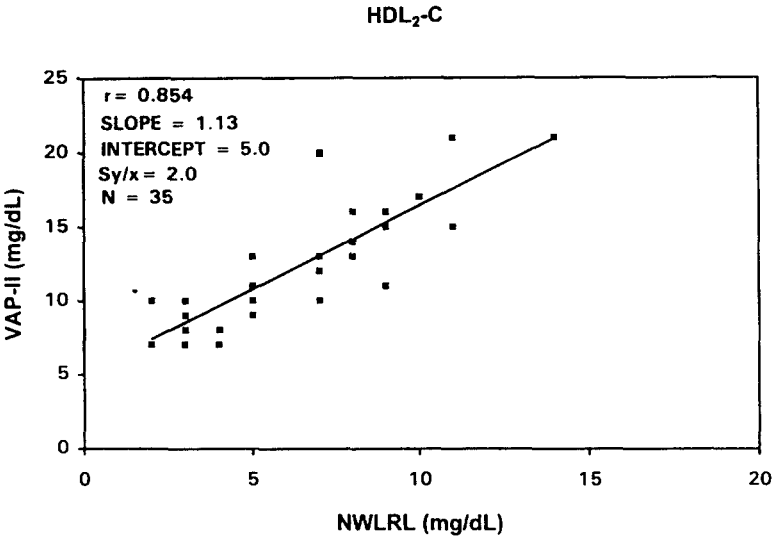


Fig. 3. Scatter plots showing the comparisons of HDL_3- and HDL_2-C measurements made by the VAP-II and by the DS/Mg^{2+} double precipitation method. The double precipitation measurements were made at Northwest Lipid Research Laboratories (NWLRL), Seattle, WA. The mean (mg/dL) (± SEM) values were: HDL_3-C: NWLRL, 42.5 ± 1.51; VAP-II, 32.2 ± 0.99; and HDL_2-C: NWLRL, 6.1 ± 0.49; VAP-II, 11.9 ± 0.65.

very high HDL-C and the other with high triglycerides (TG) (a hypertriglyceridemic plasma with TG of 795 mg/dL), high VLDL-C and very low HDL-C, were chosen for the VAP-II analysis. The first plasma sample yielded (mg/dL) HDL-C, 105; HDL_3-C, 62; HDL_2-C, 43; and Lp[a]-C, 32 and the second plasma yielded HDL-C, 20; HDL_3-C, 13; HDL_2-C, 7; Lp[a]-C, 17; and VLDL-C, 103 by the VAP-II. The accuracy of these analyses by the VAP-II was verified by analyzing the same two samples by the HDL-VAP-II which separates HDL_3 and HDL_2 very effectively and thus provides more reliable values. The corresponding HDL_3- and HDL_2-C obtained by the HDL-VAP-II, respectively, were: 62 and 43 for the first sample (compared to 62 and 43 by VAP-II) and 12 and 8 for the second sample (compared to 13 and 7 by VAP-II). These data suggest a very satisfactory accuracy of VAP-II procedure even under extremely low and high HDL subclass concentrations as well as in the presence of high Lp[a]-C and high TG. In the presence of low Lp[a], HDL_3 and HDL_2 are even more effectively separated from LDL, thereby further improving the accuracy. As VAP-II is a ultracentrifugation procedure, both HDL subclasses are completely separated from IDL and VLDL which contain most of the TG. LDL and Lp[a] (the latter if present) occupy the volume between the HDL subclasses and IDL and VLDL in the centrifu-

TABLE 2. Comparison of HDL_3 and HDL_2 cholesterol measured by VAP-II and double precipitation methods with the gradient gel electrophoresis method

Methods	HDL_3 Cholesterol						
	r	Slope	Intercept	$S_{y/x}$	Mean ± SEM		P^b
			mg/dL		*mg/dL*		
GGE(x) vs. VAP-II(y)	0.861	1.11	−5.0	3.0	GGE[a]	33.4 ± 0.76(VAP-II) 36.7 ± 1.0(NWLRL)	<0.05
GGE(x) vs. NWLRL(y)	0.886	1.38	−8.0	4.2	VAP-II NWLRL	32.2 ± 0.99 42.5 ± 1.51	<0.0001
	HDL_2 Cholesterol						
GGE(x) vs. VAP-II(y)	0.885	0.583	5.7	1.8	GGE[a]	10.7 ± 0.98(VAP-II) 11.9 ± 1.2(NWLRL)	<0.05
GGE(x) vs. NWLRL(y)	0.843	0.356	1.9	1.6	VAP-II NWLRL	11.9 ± 0.65 6.1 ± 0.49	<0.0001

[a]As the gradient gel electrophoresis method provides only the relative protein concentrations of HDL_2 and HDL_3, the absolute HDL_2- and HDL_3-C values for GGE can only be derived by assigning the total HDL-C value of the comparison method (VAP-II or NWLRL) to the total HDL-C value of the GGE in the calculations. As the total HDL-C values obtained from different comparison methods varied from one another, the absolute HDL_2- and HDL_3-C values calculated for GGE also varied slightly, depending upon the total HDL-C obtained by a particular comparison method; n = 35.

[b]*P* values are calculated using paired *t* test by comparing the mean values obtained by the respective methods shown in column 1.

gate. Therefore, hypertriglyceridemic samples do not present a problem in the HDL subspecies analysis by VAP-II. In addition, the VAP-II analysis system is designed so as to minimize the loss of resolution between the adjacent lipoprotein peaks.

DISCUSSION

Of the several methods currently used for the measurement of HDL subspecies, only the methods based upon double precipitation are suitable for large-scale studies because of their high speed and low cost. However, precipitation methods require careful standardization because of their sensitivity to reaction conditions and hence can be satisfactorily performed only in reference laboratories. Other methods such as sequential flotation and preparative density gradient methods are time consuming and expensive for routine use.

The VAP-II procedure described here for the simultaneous measurements of HDL_2- and HDL_3-C is rapid, reliable, and reproducible. The reproducibility of VAP-II is highly comparable with the reproducibility of double precipitation method for the HDL subclasses. In addition, the VAP-II method, which requires less than 40 μL of plasma because of its extremely high sensitivity, quantifies HDL_2- and HDL_3-C merely as a small part of a single comprehensive test intended for the measurement of all major lipoproteins (HDL, LDL, VLDL, IDL, and Lp[a]). Thus, additional assay steps, time, or cost are not required. Simultaneous quantification of lipoprotein subspecies using the VAP-II method is possible as subspecies are also separated along with the major

TABLE 3. Comparison of HDL_3 and HDL_2 cholesterol measured by VAP-II and double precipitation methods with the analytical ultracentrifuge method

Methods	HDL_3 Cholesterol						
	r	Slope	Intercept	$S_{y/x}$	Mean ± SEM		P^a
			mg/dL		*mg/dL*		
AUC(x) vs. VAP-II(y)	0.706	0.407	19.6	3.0	AUC[b]	29.9 ± 2.05	>0.02
AUC(x) vs. NWLRL(y)	0.549	0.457	26.9	5.2	VAP-II NWLRL	31.8 ± 1.18 40.6 ± 1.71	<0.001
	HDL_2 Cholesterol						
AUC(x) vs. VAP-II(y)	0.721	0.370	8.0	1.6	AUC[b]	8.8 ± 1.23	<0.02
AUC(x) vs. NWLRL(y)	0.727	0.351	3.2	1.5	VAP-II NWLRL	11.3 ± 0.63 6.3 ± 0.59	<0.02

[a]*P* values are calculated using paired *t* test by comparing the mean values obtained by the two respective methods in column 1.

[b]See text for details on how cholesterol values of HDL_2- and HDL_3-C are calculated for AUC method; n = 12.

TABLE 4. Mean HDL_3/HDL_2 cholesterol ratios obtained by the various methods using 35 plasma samples

	VAP-II	HDL-VAP-II	AUC (n = 12)	GGE	NWLRL
HDL_3/HDL_2	2.7	3.0	3.4	3.1	7.0

lipoproteins. Although peaks due to subspecies are not visible in the VAP profile because of the small differences in the densities and flotation rates between the subspecies, the subspecies can be quantified along with the major lipoproteins by using the modified VAP-II cholesterol profile deconvolution program described here.

The measurements of HDL_2- and HDL_3-C by the VAP-II procedure were validated by comparing them with other commonly used methods. Both HDL_2- and HDL_3-C values obtained from the VAP-II procedure correlated satisfactorily with the values obtained by the DS/Mg^{2+} double precipitation method (performed at NWLRL). However, the mean HDL_2-C value obtained by NWLRL was lower than that obtained from the VAP-II method, and the difference in the mean HDL_3-C values between the two methods was higher than could be accounted for by the difference in the mean total HDL-C values observed between the two methods. The measurements of HDL_2- and HDL_3-C by VAP-II correlated well with both GGE and AUC. The correlation coefficients obtained by comparing both HDL_2-C and HDL_3-C from all methods with GGE ranged between 0.84 and 0.89 (Table 2). Considering the assumptions that went into calculating HDL_2- and HDL_3-C by GGE, the correlations were particularly good. Further, the mean HDL_2-C value obtained from the VAP-II procedure was similar to the GGE mean (Table 2). Both HDL_2- and HDL_3-C values obtained from the precipitation procedure also correlated well with the values obtained from GGE, and the correlation coefficients were in the same range as that obtained by comparing the VAP-II with GGE. However, similar to the comparison of precipitation values with the VAP values, HDL_2-C values obtained from the precipitation were lower than GGE values. The results of comparison of VAP-II and precipitation procedures with AUC are similar to the results obtained by similar comparisons with GGE, thus suggesting that HDL_2- and HDL_3-C measurements by the VAP-II procedure are more comparable with the GGE and AUC methods. The HDL_3-C/HDL_2-C ratios obtained from VAP-II, HDL-VAP-II, GGE, and AUC were also similar to each other, while being significantly different from that of the precipitation value (**Table 4**). The differences caused by the differences in HDL-C values obtained from different methods are eliminated when the HDL_3-C/HDL_2-C ratios are considered. However, both HDL_2- and HDL_3-C values obtained from VAP-II correlated highly with the corresponding precipitation values.

These results suggest that the differences observed among the various methods compared here may primarily be due to the methodological differences, as each method is essentially based on a different principle of lipoprotein separation (VAP-II is based on the hydrated density of lipoprotein; the GGE used here primarily depends upon the size of lipoprotein; AUC depends upon the flotation rate of lipoprotien, which is a function of hydrated density and size of the lipoprotein; and the precipitation method depends mainly upon the charge of lipoprotein). However, the close agreement of VAP-II with GGE and AUC is primarily due to the close relationship between the properties used by these methods for subspecies separation i.e., density versus size. On the other hand, the precipitation method depends upon the charge of lipoprotein, which is not as closely related to density or size of lipoprotein as the density is related to size. Therefore, the differences among the VAP-II, GGE, and AUC with the precipitation values appear to be due to the differences caused primarily by the methodological differences. Such differences among the methods have been previously observed while measuring major lipoproteins, particularly in the case of HDL, due to the structural and compositional complexities of lipoproteins. Although differences in subspecies values are observed among various methods studied here, the results obtained from each method should be valid as long as any one particular method is consistently used throughout an entire study.

In conclusion, VAP-II is a reliable method for the quantification of HDL_2- and HDL_3-C that requires no additional assay procedures. Further, VAP-II can be a potentially useful tool for the determination of HDL_2- and HDL_3-C in large-scale studies because of its speed and other advantages. In addition, microvolume requirement of plasma (less than 40 μl of sample) by this method also makes it a very valuable tool when sample is scarce, such as in transgenic animal studies or studies involving newborn infants.

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