
James D. Otvos, Elias J. Jeyarajah, Dennis W. Bennett, and Ronald M. Krauss

We are developing a method for quantifying plasma lipoproteins by proton nuclear magnetic resonance (NMR) spectroscopy that offers advantages over existing clinical methods. We showed that the major lipoproteins have distinct NMR properties sufficient to permit their concentrations to be extracted from a computer lineshape analysis of the plasma lipid methyl resonance envelope (Clin Chem 1991;37:377–86). We have now discovered that the spectra of the subspecies within each lipoprotein class are different enough to influence the composite spectrum of that class and hence the spectrum of whole plasma. By including spectra representative of these subspecies as additional components in the lineshape-fitting analysis, a complete concentration profile of very-low-density, low-density (LDL), and high-density (HDL) lipoproteins plus the subspecies distributions within these classes can be simultaneously generated. A pilot study of 30 plasma samples of widely varied composition demonstrated good agreement between NMR-derived values and lipoprotein lipid concentrations and LDL and HDL subspecies distributions determined by gradient-gel electrophoresis.

Plasma concentrations of low-density (LDL) and high-density (HDL) lipoproteins have well-established value in coronary heart disease (CHD) risk assessment (1,2), and additional predictive discrimination may be provided by the concentration of very-low-density lipoprotein (VLDL) (3). All current methods for quantifying these three major lipoprotein classes require their physical separation by techniques that exploit differences in particle size, density, or chemical composition. Once separated, the individual lipoproteins are quantified by their mass, protein content, or lipid content (usually cholesterol for LDL and HDL and triglyceride for VLDL). Most separation methods, including ultracentrifugation, chromatography, and electrophoresis, are too time consuming and labor intensive to be routinely used clinically and are therefore used mainly in research settings. Procedures involving selective precipitation are simpler but generally less accurate. A method in widespread clinical use that uses selective precipitation to give HDL-cholesterol (HDL-C) concentrations is that of Friedewald et al. (4), in which LDL-C concentrations are determined indirectly by the combined information provided by separate assays for plasma triglyceride, cholesterol, and HDL-C. The main drawback of the procedure, besides being sensitive to measurement errors associated with three different lipid assays, is the requirement for fasting plasma specimens free of chylomicrons (the triglyceride-rich lipoproteins that transport dietary lipids).

Although attention has focused primarily on associations between CHD and the concentrations of the major lipoprotein classes, it is well-known that each class comprises a heterogeneous population of lipoprotein subspecies differing in particle size, density, and chemical composition (5). Chylomicrons are the least-dense species (<0.94 kg/L) and span the largest range of particle diameters (80–500 nm). VLDLs are somewhat smaller (30–80 nm) and are usually separable into large and small VLDL subpopulations by nondenaturing gradient-gel electrophoresis (6). LDL subspecies are distinguishable by several methods, including density-gradient ultracentrifugation and gradient-gel electrophoresis (7). The latter enables the LDL to be fractionated into the following particle-size intervals: LDL-I, 22.0–23.2 nm; LDL-II, 23.3–24.1 nm; LDL-III, 24.2–24.6 nm; LDL-IVA, 24.7–25.5 nm; LDL-IVB, 25.5–26.4 nm; LDL-V, 26.0–28.5 nm; and intermediate-density lipoproteins (IDL), 28.0–30.0 nm (8). Similarly, HDL comprises several distinct subclasses having different mobilities on nondenaturing polyacrylamide gradient gels: HDL-Ca, 7.2–7.8 nm; HDL-Cb, 7.8–8.2 nm; HDL-Ce, 8.2–8.8 nm; HDL-Cf, 8.8–9.7 nm; and HDL-Cg, 9.7–12 nm (9).

There are at least two reasons for the current interest in determining lipoprotein subspecies distribution within the major lipoprotein classes. First, understanding lipoprotein metabolism and how it is influenced by various disease states will require a better understanding of the precursor–product relationships for lipoprotein subspecies. Second, subspecies may differ significantly in their association with CHD risk. For example, individuals with a preponderance of small, dense LDL particles are at increased risk of myocardial infarction (10); also, high HDL-C concentrations are associated with reduced CHD risk, whereas HDL-C cholesterol appears to be a less reliable marker (11–13). A possible reason for the weaker associations seen with HDL-C is suggested by the recent finding that one HDL-C subcomponent, HDL-Cb, appears to have a correlation with known CHD

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1 Department of Biochemistry, North Carolina State University, Box 7622, Raleigh, NC 27695.
2 Department of Chemistry, University of Wisconsin-Milwaukee, WI.
3 Lawrence Berkeley Laboratory, University of California, Berkeley, CA.
4 Nonstandard abbreviations: LDL, HDL, VLDL, and IDL, low-, high-, very-low-, and intermediate-density lipoproteins; CHD, coronary heart disease; HDL-C, HDL-cholesterol; and NMR, nuclear magnetic resonance.

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risk factors opposite to that of other HDL subspecies (14).

What has hampered more extensive research into the relationships between heart disease and lipoprotein subspecies is the absence of a simple and reliable technique for measuring subspecies distributions. Nondenaturating polyacrylamide gradient-gel electrophoresis is currently the most widely used method for this purpose because of its high resolving power and the relatively inexpensive equipment required. However, several limitations restrict its utility and make it unsuitable for studies of large populations. Among these are low sample throughput, long analysis time, extensive experimental manipulation, and an inability to measure VLDL, LDL, and HDL subspecies simultaneously on a single gel.

Here we describe an alternative method that may provide an inexpensive, automated means of generating complete lipoprotein profiles of nonfasting plasma samples consisting of VLDL, LDL, and HDL concentrations and their subspecies distributions. The method is an extension of a previously described procedure that involves computer decomposition of the proton nuclear magnetic resonance (NMR) spectral fingerprint of plasma lipids (15). Unlike other methods, no physical separation of the lipoproteins is required. Instead, the procedure exploits the observation that the NMR spectral properties of each lipoprotein subclass appear to be sufficiently distinct and reproducible to exert an influence on the overall plasma lipid lineshape. By modifying the original method of analysis by including spectra representative of these subclasses in the plasma lineshape-fitting algorithm, we can obtain more accurate quantification of VLDL, LDL, and HDL and also derive information about LDL and HDL subspecies distributions that closely agrees with that supplied by gradient-gel electrophoresis.

**Materials and Methods**

**Laboratory Methods**

Lipoprotein fractions were isolated from a pooled plasma sample by sequential flotation ultracentrifugation at 4 °C (16). The plasma was obtained from blood samples collected from two fasting healthy donors into Vacutainer Tubes (Becton Dickinson, Rutherford, NJ) containing EDTA (final EDTA concentration, 1 g/L). The following components were isolated: VLDL (<1.006 kg/L), LDL (1.006–1.063 kg/L), large LDL (1.006–1.035 kg/L), small LDL (1.035–1.063 kg/L), HDL (1.063–1.21 kg/L), large HDL (1.063–1.125 kg/L), small HDL (1.125–1.21 kg/L), and protein (>1.21 kg/L: bottom fraction). Each fraction was dialyzed and concentrated as previously described (15) to give a final concentration approximately two- to fivefold greater than in the original plasma. Cholesterol and triglyceride concentrations of the isolated lipoproteins were determined in the laboratory of John Parks at Bowman Gray School of Medicine (Winston-Salem, NC) by enzymatic methods (17, 18).

The 30 frozen plasma samples selected for NMR analysis were obtained from a subset of 105 healthy, nonobese normolipemic men participating in a study of dietary effects on plasma lipoproteins conducted at Lawrence Berkeley Laboratory, University of California. Informed consent was obtained from all participants, and procedures were approved by the Committee for Protection of Human Subjects of the University of California–Berkeley. The subset was selected to represent a range of plasma lipid values and LDL particle size determined by nondenaturing gradient-gel electrophoresis, as described below. Samples were taken after subjects had fasted overnight and after 6 weeks of outpatient consumption of a diet containing 24% fat with a ratio of polyunsaturated to saturated fatty acids of 0.7 and a cholesterol content of 150 mg/1000 kcal.

Plasma was prepared from venous blood within 2 h of collection, and blood and plasma were kept at 4 °C until processed. Samples (1 mL) of plasma to be analyzed by NMR were stored at −70 °C. Plasma total cholesterol and triglyceride were determined by enzymatic procedures on a Gilford Impact 400E analyzer (Ames Div., Miles Labs., Elkhart, IN). These measurements were consistently in control, as monitored by the Centers for Disease Control standardization program. HDL-C was measured after heparin sulfate and magnesium chloride precipitation of plasma (19) and LDL-C was calculated from the formula of Friedewald et al. (4). Analytical ultracentrifugation was used to measure VLDL, IDL, LDL, and HDL total mass concentrations by the methods of Lindgren et al. (20). LDL subclass distributions in the <1.063 kg/L ultracentrifugal plasma fractions were assessed by nondenaturing gradient-gel electrophoresis on 2–16% gels as previously described (7, 21). Protein distribution of HDL subclasses 2b, 2a, 3a, 3b, and 3c were assessed in the <1.21 kg/L plasma fractions by nondenaturing gradient-gel electrophoresis on Pharmacia (Piscataway, NJ) PAA 4–30% gels as described by Nichols et al. (21).

**NMR Spectroscopy and Data Analysis**

NMR spectra of the isolated lipoproteins were acquired at 250 MHz and 45 °C as described previously (15). Spectra of the frozen plasma samples (thawed at 25 °C for 1 h) were acquired under identical conditions on the same instrument (Model WM-250; Bruker Instruments, Billerica, MA) 9 months later. After zero-filling, Fourier transformation, phasing, and baseline flattening as outlined previously (15), the frequency-domain spectra were transferred to an IBM-compatible personal computer for analysis.

The spectral region used to derive the concentrations of the plasma lipoproteins was that containing the methyl lipid resonance (0.87–0.67 ppm). The digitized data (150 points) from this region of the spectrum of each lipoprotein reference sample were stored in separate arrays in computer memory, as were those of each plasma spectrum. The linear least-squares fits of the plasma methyl resonance lineshapes in terms of the amplitudes (concentrations) of the different lipoprotein constituents were performed as previously described (15) with the following modifications. First, to help

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account for the presence in plasma of lipoprotein sub-species with slightly different NMR spectral properties, the number of lipoprotein components used in the line-shape fits was expanded from four (VLDL, LDL, HDL, and protein) to 19. Because we have not yet isolated a sufficient number of pure lipoprotein sub-classes to serve as reference standards in the lineshape analysis, we used digitally shifted spectra of VLDL, LDL, and HDL to simulate the chemical shifts expected for sub-classes of different diameters. Six artificially shifted VLDL spectra (V1–V6), five shifted LDL spectra (L1–L5), and seven shifted HDL spectra (H1–H7) were used. In common with the convention used in gradient-gel electrophoresis, larger numbers represent particles of smaller diameter. The amount by which spectra were shifted to the left (downfield) to represent larger species or to the right (upfield) to represent smaller species was dictated by the digital resolution of 0.34 Hz/data point. Thus, components V1–V3 were created to represent the expected range of chylomicron diameters and were generated by shifting the methyl resonance of isolated VLDL (V6) to the left by six, four, and two data points, respectively. V4 and V6, representing large and small VLDLs, were created by shifting the spectrum of isolated VLDL to the left and right by one data point, respectively. Similarly, the ranges of LDL and HDL particle sizes were accounted for by inclusion of five LDL and seven HDL spectra generated by shifting the spectrum of isolated LDL (L3) by increments of one data point and that of isolated HDL (H5) by increments of two data points.

Because of the increased number and close similarity of the spectral components used to deconvolute the plasma lineshapes, several modifications were made to the least-squares fitting program to address the greater mathematical difficulty of the linear problem. First, singular value decomposition (22) was used rather than matrix inversion to solve the sets of linear equations. In addition, a nonnegativity algorithm that used Simplex optimization was incorporated to restrict the solutions to those giving positive lipoprotein concentrations (23). Details of the algorithm will be presented elsewhere.

The NMR method produces fractional concentrations of the lipoprotein constituents of plasma, expressed relative to the concentrations of the isolated lipoproteins whose spectra provide the basis for the lineshape fits (16). The program automatically converts these fractional concentrations to units of lipoprotein lipid concentration by multiplying by the measured lipid concentrations of the reference samples, which here were 3.88 mmol/L (3.44 g/L) for VLDL triglyceride, 12.87 mmol/L (4.98 g/L) for LDL-C, and 3.52 mmol/L (1.36 g/L) for HDL-C. Reported NMR concentrations of VLDL triglyceride, LDL-C, and HDL-C are the sums of the V1–V6, L1–L5, and H1–H7 sub-class concentrations, respectively. The program also calculates the single variables (V2, L2, and H2) for the VLDL, LDL, and HDL particle-size distributions by summing the size-weighted relative contribution made by each sub-class to the total concentration of a given lipoprotein. For example, if NMR analysis of a plasma sample indicated the presence of 4 mmol/L H1-cholesterol (20%), 2 mmol/L H2-cholesterol (10%), and 14 mmol/L H4-cholesterol (70%), the calculated HZ value would be 3.20, or (1 × 0.20) + (2 × 0.10) + (4 × 0.70).

Results and Discussion

We reported (24) a detailed comparison of the proton NMR spectral properties (chemical shift and line width) of the methyl resonance from the lipids in VLDL, LDL, and HDL isolated from the plasma of many individuals. Our results showed small but consistent spectral differences between the signals from the different lipoproteins, and the signals of each of these varied slightly from person to person. On the basis of these findings, we proposed that the concentrations of VLDL, LDL, and HDL in plasma could be derived simultaneously by a linear least-squares fit of the composite lineshape of the plasma methyl resonance envelope (15). A computer program was written to perform the lineshape analysis and preliminary data showed reasonably good agreement between lipoprotein lipid concentrations and NMR-derived values.

The feasibility of the NMR method was recognized from the beginning to rest on two key assumptions. The first is the existence of a linear relationship between the information supplied by NMR (signal amplitude from each lipoprotein) and that provided by methods that quantify lipoprotein concentration in terms of lipid content. There is now good evidence to support the validity of this assumption when the NMR measurements are made at a temperature above the lipid order–disorder transitions (15). The second assumption is fundamental to the appropriateness of using a linear least-squares approach to separate the plasma spectrum into its component parts, namely, that plasma contains a discrete number of different lipoprotein constituents having distinct and invariant spectral properties. In a strict sense, our earlier data (24) indicated that the major lipoproteins (chylomicrons, VLDL, LDL, and HDL) do not meet the criterion of having absolutely invariant methyl resonance lineshapes or chemical shifts, presumably because of underlying particle size heterogeneity and (or) lipid compositional differences. Nevertheless, for convenience we used spectra of these four lipoprotein classes as the reference standards (basis set) for our initial plasma lineshape fits, with the understanding that discrepancies between the spectral properties of the lipoprotein standards and those of the lipoproteins in the plasma would be a direct source of analytical error (15).

To assess the extent to which such analytical error might arise from differences in sub-class distribution within a given lipoprotein class, we fractionated plasma by ultracentrifugation into narrower density ranges and compared the spectra of the fractions. Without exception, we found that the larger, less-dense sub-classes give rise to methyl lipid resonances that are shifted downfield (to the left in the normal frequency spectrum) relative to the smaller, more-dense particles (Figure 1).
In addition, subtle differences in lineshape are observed among LDL and HDL subspecies. Although the chemical shift and lineshape differences between the subspecies are much smaller than those between the major lipoprotein classes, they are completely reproducible. As shown in Figure 1, for example, the signals from chylomicrons and VLDL differ by only 0.007 ppm (1.8 Hz), those from large and small LDL by 0.004 ppm (1 Hz), and those from large HDL (HDLa) and small HDL (HDLb) by 0.008 ppm (2 Hz). At present, the physical basis for these size-related chemical-shift differences is unknown. A hypothesis being investigated is that the lipids in the particle core and surface shell, both of which contribute to the observed methyl resonance, have different magnetic susceptibilities. Because the ratio of core lipids to surface lipids varies continuously with particle diameter (25), so too would the spectral properties of the composite methyl resonance from these particles be expected to vary.

The data in Figure 1 indicate that spectral differences among lipoprotein subspecies are large enough to influence the overall plasma methyl resonance lineshape. The program for linear least-squares fitting should therefore include the spectra of the individual subspecies as reference spectra. Such an approach would be expected to produce more accurate quantification of VLDL, LDL, and HDL, and as a byproduct also provide information about particle-size distribution within each of the major lipoprotein classes that may have additional clinical utility. We are currently preparing a number of pure subspecies samples of narrow particle-size range to serve as reference standards that satisfy the dual criteria of spectral distinctness and invariance.

Before using these new components to analyze plasma methyl lineshapes, we sought preliminary evidence of the feasibility of extracting reliable information about subspecies concentrations from analysis of a signal composed of so many closely similar and severely overlapping spectral components. We conducted a pilot study on 30 frozen plasma samples that had been analyzed for lipoprotein lipid concentrations and LDL and HDL subspecies distributions by gradient-gel electrophoresis. The samples were selected to represent the full range of LDL and HDL subspecies distributions. NMR spectra were acquired in duplicate at 45 °C, and the methyl resonance lineshapes were analyzed by using a linear least-squares fitting program modified to accommodate up to 20 lipoprotein components.

In place of reference spectra of real lipoprotein subspecies, 18 artificial subspecies spectra were created by shifting the methyl signal of VLDL, LDL, and HDL by different amounts to represent larger or smaller subspecies. As shown in Figure 2, the 18 reference spectra consist of six VLDL spectra (V1–V6) spanning a total frequency offset range of 2.4 Hz, five LDL spectra (L1–L5) offset by a total of 1.4 Hz, and seven HDL spectra (H1–H7) spanning 4.1 Hz. The use of such digitally shifted component spectra was intended to approximate the chemical shift differences expected for the full range of particle sizes within each lipoprotein class; however, not enough spectral data on pure subspecies are yet available to permit evaluation of the approximations. The methyl resonances of real subspecies differ not only in chemical shift but also in lineshape (Figure 1). Lineshape differences are completely unaccounted for in this pilot study, which makes the linear least-squares fitting problem more difficult and subject to greater error than it will be when real subspecies reference spectra are used.

In Figure 3 is shown the output from a representative

![Graph showing lineshape analysis](image)
plasma methyl resonance lineshape fit, which required <30 s to perform on a Model 386 PC. The computer-derived amplitudes of the reference spectra that added together gave the best fit to the experimental plasma spectrum are shown at the bottom and the lipoprotein lipid concentrations calculated from these amplitudes are given on the right. The printout includes total VLDL triglyceride, LDL-C, and HDL-C concentrations and VZ, LZ, and HZ.

The relationships between the lipoprotein lipid concentrations of the 30 plasma samples determined by NMR and chemical analysis are shown in Figure 4. Strong correlations were observed for all three lipoprotein classes (r = 0.98 for VLDL, r = 0.91 for LDL, and r = 0.93 for HDL). When the same comparison was made with NMR concentrations obtained by lineshape analysis with single VLDL, LDL, and HDL reference standards (data not shown), weaker correlations were observed (r = 0.97 for VLDL, r = 0.84 for LDL, and r = 0.52 for HDL). Thus, the accuracy of NMR lipoprotein quantification is significantly improved by including in the analysis a means of dealing with subspecies-induced lineshape variation. Once we substitute reference spectra of actual subspecies for the artificial reference standards used here, we expect greater improvements in accuracy.

Despite the deficiencies of the fitting model, we were interested in knowing how closely related the relative subspecies concentrations derived by NMR lineshape analysis are to those determined by gradient-gel electrophoresis. In Figures 5 and 6 are shown side-by-side comparisons of subspecies distributions determined by the two methods. The densitometry traces of the gradient-gel separations of HDL subspecies from two pairs of plasma samples are shown on the right side of Figure 5. The top trace of each pair identifies the samples as containing predominantly small HDL particles (HDL$_{a}$ and HDL$_{b}$), whereas the bottom traces identify samples that contain substantial amounts of large particles (HDL$_{2a}$ and HDL$_{2b}$). The bar graph representations of the NMR-determined subspecies distributions of the same samples show a close qualitative correspondence to the electrophoresis results. The samples with a preponderance of either small HDL$_{a}$ or large HDL$_{2}$ particles are readily identified by the NMR distributions that show relatively large proportions of the H6 and H7 subspecies for small HDL$_{a}$ and H1 and H2 subspecies for
large HDL₂. Our data seem to indicate that NMR is particularly sensitive to the concentration of HDL₂₀, the subclass that appears most strongly related to decreased CHD risk (11–13). The sum of H1 and H2 concentrations for the 30 samples was found to correlate closely (r = 0.78) with HDL₂₀ concentrations determined by gradient-gel electrophoresis (data not shown). Direct quantitative relationships between the other NMR and gradient-gel subspecies are much weaker, particularly those between the intermediate-size particles (Figure 5). This may be due to inherent limitations in the resolving power of the NMR method or to our use of artificial reference spectra that do not truly reflect the spectral properties of the real subspecies.

A convenient numerical basis for comparing the overall HDL subspecies distributions of the 30 samples is provided by the weighted-average HDL particle size, calculated from the areas under the different regions of the gradient-gel densitometry traces, and HZ, calculated from the relative concentrations of subspecies H₁–H₇. As shown in Figure 7, a very strong correlation (r = 0.88) was found between these two independent measures of HDL particle-size distribution. Thus, if this type of information is desired, the NMR method even at this early stage of development is more advantageous than gradient-gel electrophoresis and all other methods of lipoprotein subclass characterization because it re-

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**Fig. 5.** Comparison of HDL subspecies concentrations determined by plasma NMR lineshape analysis (bar graph) and particle-size distributions determined by gradient-gel electrophoresis (densitometry scans) for two pairs of plasma samples.

**Fig. 6.** Comparison of LDL subspecies concentrations determined by plasma NMR lineshape analysis (bar graph) and particle-size distributions determined by gradient-gel electrophoresis (densitometry scans) for two pairs of plasma samples.

**Fig. 7.** Correlation between the NMR HDL size variable (HZ) and the weighted-average HDL particle size determined by integration of the gradient-gel densitometry scans of the 30 plasma samples.
quires far less time and effort. There is no additional cost associated with obtaining this information, because the subspecies distributions are a byproduct of the procedure used to optimize the accuracy of NMR lipoprotein quantification.

LDL subspecies distributions derived by NMR also correlate remarkably well, with those determined by gradient-gel electrophoresis (Figure 6). The extent of correlation was surprising because of the very small spectral differences between large and small LDL subspecies (Figures 1 and 2). Although it is premature to attempt to draw any firm conclusions from these limited results, it appears that NMR should easily and accurately be able to determine whether an individual has predominantly small, dense LDL or large, less-dense LDL. Such categorization may aid in assessing CHD risk (5, 10).

In conclusion, we have obtained convincing evidence for the feasibility of an optimized version of our original method of NMR lipoprotein analysis (15) that exploits the subtle spectral differences found to exist between lipoprotein subspecies. The ultimate accuracy and resolving power of the method will not be known until more-extensive studies involving pure lipoprotein subspecies standards are completed. Nevertheless, a potential exists for obtaining a complete plasma lipoprotein profile, including subspecies concentrations, automatically by NMR in ~1 min, which otherwise would require several days of analysis.

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