papers on methodology

^{1}H NMR-based absolute quantitation of human lipoproteins and their lipid contents directly from plasma

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Abstract A new method is presented for absolute quantitation of lipid and protein contents of human lipoproteins directly from plasma. The method enables complete lipoprotein lipid profiles to be obtained in a total time of less than one hour. Absolute concentrations of triglycerides, phospholipids, total cholesterol, free cholesterol, esterified cholesterol, total proteins, and total masses can be estimated for the very low density (VLDL) and low density (LDL) lipoprotein fractions. For the high density lipoprotein (HDL) fraction all components except triglycerides can be quantitated. The method is a combination of ^{1}H NMR spectroscopy and a sophisticated lineshape fitting analysis technique. In this paper we present the calibration of the method using 15 plasma samples followed by a double-blind test of 51 plasma samples from 43 individuals. In total, 66 plasma samples were analyzed. Comparison of the ^{1}H NMR-based results with the data of the biochemical assays showed excellent agreement; the correlation coefficient for VLDL triglycerides was 0.98, for LDL cholesterol 0.88, and for HDL cholesterol 0.93. This method can be directly integrated to many kinds of biomedical NMR studies to offer additional biochemically important quantitative lipoprotein information, the measurement of which is usually too laborious by conventional biochemical methods and too high-priced to be adapted into the study protocols. Moreover, the method also has considerable potential to be developed for a routine clinical assay.—**Ala-Korpela, M., A. Korhonen, J. Keisala, S. Hörkkö, P. Korpi, L. P. Ingman, J. Jokisaari, M. J. Savolainen, and Y. A. Kesäniemi.** ^{1}H NMR-based absolute quantitation of human lipoproteins and their lipid contents directly from plasma. *J. Lipid Res.* 1994. **35:** 2292–2304.

Supplementary key words VLDL • LDL • HDL • lipoprotein lipids

Recently two different outlines for ^{1}H NMR-based lipoprotein quantitation have been independently published by our group (1–3) and Otvos et al. (4–6). Advantages of these NMR approaches are that quantitation can be done directly from a ^{1}H NMR spectrum of a plasma sample in a time scale of minutes and no sample pretreatment or any physical or chemical decomposition of the lipoproteins is needed. The sample can thus be used in further analyses after NMR measurements if necessary. Furthermore, there is evidence that the NMR-based estimates can be at least as accurate as biochemical estimates (5, 7).

In this paper we present a new method, based on ^{1}H NMR spectroscopy and a sophisticated lineshape fitting analysis technique, to permit quantitation of lipid and protein contents of human lipoproteins directly from plasma in a total time of less than 1 h. Absolute concentrations of phospholipids, total cholesterol, free cholesterol, esterified cholesterol, total proteins, and total masses can be estimated for VLDL, LDL, and HDL fractions. VLDL and LDL triglycerides can also be quantified.

The method is based on mathematical model lineshapes for the lipid methyl resonances of the VLDL, LDL, and HDL fractions. They were developed on the basis of 20 ^{1}H NMR measurements and lineshape fitting analyses for each ultracentrifuged lipoprotein fraction. These model lineshapes were used to analyze 15 ^{1}H NMR spectra of different plasma samples, and comparison with biochemical lipid and protein analyses of the same samples was used to calibrate the method. A double-blind test of 51 plasma samples from 43 persons was then performed to assess the capabilities of the new method.

Abbreviations: NMR, nuclear magnetic resonance; FID, free induction decay; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; CHD, coronary heart disease.

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MATERIALS AND METHODS

Subjects and study protocol

Fifty-eight subjects volunteered for the study (38 males and 20 females, age ranging from 20 to 86 years). The study group consisted of subjects with a broad range of lipoprotein lipid values and with various lipoprotein abnormalities such as hypercholesterolemia (Type IIa), hypertriglyceridemia (Type IV), and combined hyperlipidemia (Type IIb). The categorization of the hyperlipidemias was based on the measurements of plasma total cholesterol and triglycerides: Type IIa plasma total cholesterol >6.0 mmol/l and triglycerides are normal; Type IIb plasma total cholesterol >6.0 mmol/l and triglycerides >2.0 mmol/l; and Type IV plasma total cholesterol is normal and triglycerides >2.0 mmol/l. Also, one patient with heterozygous familial hypercholesterolemia and five patients with chronic renal failure were recruited. The study group was divided into two different sets. A calibration set of 15 subjects (the plasma samples from no. 1 to 15 in **Table 1**) with broadly varying lipoprotein lipid values was set up to ensure a good preliminary calibration basis for the ^{1}H NMR-based quantitation method. VLDL, IDL, LDL, and HDL fractions were isolated from all these subjects. The second set, a double-blind test set, consisted of 51 plasma samples from 43 persons (the plasma samples from no. 16 to 66 in Table 1). The quantitation of the plasma samples in the test set were done by both the ^{1}H NMR and the biochemical analysis methods in a double-blinded fashion. The final results of both methods were calculated before any comparisons were made and both analyses were completed without information on the clinical status of the subjects. VLDL, IDL, LDL, and HDL fractions were also isolated from five subjects of the test set. Thus, 20 samples of each lipoprotein fraction were isolated. In Table 1, both the NMR and biochemical analysis based estimates of the VLDL triglycerides, LDL cholesterol, and HDL cholesterol are given together with the clinical category of the subjects.

Lipoprotein isolation

The blood samples were drawn after an overnight fast of 12 h into EDTA-containing tubes and plasma was separated by centrifugation at 1200 *g* for 15 min (+4°C). Lipoproteins were isolated from 3.6 ml of plasma by sequential ultracentrifugation (8). VLDL (d ≤ 1.006 g/ml) was first separated from the plasma on a fixed-angle rotor at 114,000 *g* for 18 h (+15°C). The VLDL fraction was removed from the ultracentrifuged preparation by tube slicing. The density of the infranatant was adjusted to 1.019 g/ml with NaCl–NaBr solution and IDL (d 1.006–1.019 g/ml) was spun to the surface by ultracentrifuging the preparation as described above. The density of the infranatant was raised to 1.063 g/ml and LDL (d 1.019–1.063 g/ml) was floated off. Finally, HDL (d 1.063–1.210 g/ml) was separated from the remaining infranatant by adjusting the density to 1.210 g/ml and ultracentrifuging the preparation for 48 h as described above. After isolation, the lipoprotein fractions were dialyzed against 0.15 M NaCl solution containing 0.01% EDTA, pH 7.4 (EDTA-saline). The volumes of the dialyzed fractions were adjusted to 3.6 ml, i.e., to the same concentrations as in the original plasma samples.

Biochemical lipid and protein assays

Total cholesterol, free cholesterol, triglyceride, and phospholipid concentrations were determined with Specific Chemistry Analyzer (Kone, Finland) using enzymatic colorimetric methods (kits by Boehringer Diagnostica, Mannheim GmbH, FRG, cat. nos. 236691, 310328, and 701912, and a kit by Wako Chemicals GmbH, Neuss, FRG, cat. no. 990-54009, respectively). Cholesteryl ester concentrations were calculated as the difference between the total and the free cholesterol concentrations. The protein contents were measured by the method of Lowry et al. (9). The total masses of the various lipoprotein fractions were calculated by adding the measurements of concentrations of triglycerides, phospholipids, free cholesterol, esterified cholesterol, and total proteins. To correct the lipoprotein losses in LDL and HDL fractions during the repeated ultracentrifugations, HDL cholesterol was measured from plasma after precipitation of apolipoprotein B-containing lipoproteins with heparin-manganese (10) and LDL cholesterol was calculated as follows: LDL cholesterol = plasma cholesterol − VLDL cholesterol − IDL cholesterol − HDL cholesterol (heparin-manganese precipitation method). Lipid and protein concentrations in LDL and HDL fractions were then corrected by the ratio of the calculated LDL or the HDL cholesterol concentration measured with heparin-manganese precipitation to the LDL or HDL cholesterol concentration measured after repeated ultracentrifugations.

Experimental NMR spectroscopy

The ^{1}H NMR spectra of all the samples were recorded at +37°C on a Jeol JNM-GX400 FT NMR spectrometer. A double tube system was used. The sealed external reference tube (o.d. 5 mm) containing the reference and locking substances (sodium 3-trimethylsilyl[2,2,3,3-D_4]propionate (TSP) 4 mmol/l, $MnSO_4$ 0.3 mmol/l in 99.8% D_2O) was placed coaxially into the NMR sample tube (o.d. 10 mm) containing 2.5 ml of a sample. The same reference tube was used with every sample. The homogeneity of the magnetic field was adjusted before the measurements with an identical tube system using 2.5 ml D_2O as a sample. The depth of each sample tube in the spinner was carefully measured before inserting to the magnet. Therefore, only slight shimming was necessary before the measurements to obtain the cor-

TABLE 1. ^{1}H NMR (NMR) and biochemical (BIO) analyses based estimates and their differences (NMR-BIO) for the VLDL triglycerides, LDL cholesterol, and HDL cholesterol (in mmol/l)

Sample	VLDL TG			LDL CHO			HDL CHO			Clinical Category[a]
	BIO	NMR	Difference (NMR-BIO)	BIO	NMR	Difference (NMR-BIO)	BIO	NMR	Difference (NMR-BIO)	
Calibration set										
1	2.41	2.25	−0.16	6.48	6.80	0.32	0.92	0.77	−0.15	FH, Type IIb
2	0.17	0.34	0.17	1.83	1.86	0.03	1.57	1.58	0.01	
3	1.29	1.43	0.14	2.02	2.41	0.39	1.18	1.30	0.12	
4	0.82	0.86	0.04	4.85	4.38	−0.47	1.32	1.50	0.18	Type IIa
5	0.74	0.76	0.02	4.79	3.96	−0.83	1.34	1.54	0.20	Type IIa
6	0.75	0.78	0.03	1.21	1.98	0.77	1.51	1.40	−0.11	
7	0.33	0.36	0.03	2.28	2.43	0.15	1.69	1.50	−0.19	
8	0.83	0.74	−0.09	4.51	3.83	−0.68	1.75	1.70	−0.05	Type IIa
9	1.94	1.88	−0.06	3.08	3.40	0.32	1.06	1.17	0.11	Type IV
10	2.07	2.21	0.14	3.90	3.96	0.06	1.26	1.33	0.07	Type IIb
11	0.91	0.83	−0.08	4.01	4.13	0.12	0.94	1.10	0.16	
12	0.60	0.34	−0.26	3.96	4.83	0.87	1.29	1.39	0.10	
13	0.43	0.45	0.02	3.23	3.01	−0.22	1.77	1.47	−0.30	
14	0.52	0.53	0.01	3.81	3.03	−0.78	1.52	1.44	−0.08	
15	0.60	0.66	0.06	2.81	2.75	−0.06	1.27	1.24	−0.03	
Double-blinded set										
16	0.53	0.50	−0.03	1.88	1.68	−0.20	1.50	1.44	−0.06	
17	0.43	0.56	0.13	1.97	1.94	−0.03	0.84	1.05	0.21	
18	0.66	0.71	0.05	3.69	3.48	−0.21	0.91	1.07	0.16	
19	0.40	0.32	−0.08	2.37	1.92	−0.45	1.65	1.64	−0.01	
20	0.37	0.36	−0.01	2.36	2.51	0.15	1.87	2.11	0.24	
21	0.57	0.56	−0.01	3.86	3.30	−0.56	1.22	1.48	0.26	
22	0.47	0.35	−0.12	3.02	3.47	0.45	1.87	2.02	0.15	
23	0.71	0.76	0.05	2.92	3.07	0.15	0.93	1.26	0.33	
24	0.38	0.26	−0.12	2.97	2.75	−0.22	1.63	1.83	0.20	
25	1.86	1.94	0.08	4.70	4.42	−0.28	0.73	0.88	0.15	Type IIb
26	0.58	0.47	−0.11	2.54	2.56	0.02	1.37	1.52	0.15	
27	0.66	0.27	−0.39	5.14	3.73	−1.41	2.18	2.43	0.25	Type IIa
28	0.80	0.67	−0.13	4.28	3.77	−0.51	1.20	1.25	0.05	
29	2.57	2.69	0.12	3.88	4.42	0.54	1.06	1.08	0.02	Type IIb
30	0.94	0.93	−0.01	5.01	4.15	−0.86	1.12	1.35	0.23	Type IIa
31	0.38	0.37	−0.01	3.32	3.31	−0.01	1.27	1.39	0.12	
32	0.41	0.41	0.00	3.41	2.74	−0.67	1.29	1.41	0.12	
33	0.84	0.91	0.07	3.38	3.87	0.49	1.07	0.95	−0.12	
34	2.08	2.00	−0.08	3.12	3.44	0.32	0.90	0.91	0.01	Type IV
35	0.27	0.40	0.13	2.31	2.41	0.10	2.08	2.10	0.02	
36	0.67	0.69	0.02	3.49	3.36	−0.13	1.12	1.26	0.14	
37	0.88	0.99	0.11	1.94	2.51	0.57	1.05	1.25	0.20	CRF
38	4.32	4.34	0.02	3.32	2.99	−0.33	0.90	0.87	−0.03	CRF, Type IIb
39	0.99	1.27	0.28	5.51	5.24	−0.27	1.19	1.50	0.31	Type IIa
40	1.37	1.32	−0.05	1.96	3.06	1.10	0.75	1.03	0.28	CRF, Type IV
41	0.14	0.34	0.20	2.80	3.05	0.25	1.14	1.16	0.02	
42	1.97	1.85	−0.12	3.59	6.01	2.42	1.22	1.19	−0.03	CRF, Type IIb
43	0.53	0.49	−0.04	3.84	3.43	−0.41	1.10	1.45	0.35	
44	1.35	1.18	−0.17	3.06	3.90	0.84	0.99	1.21	0.22	CRF, Type IV
45	0.68	0.90	0.22	3.02	2.95	−0.07	1.35	1.42	0.07	
46	0.68	0.87	0.19	3.28	2.95	−0.33	1.38	1.39	0.01	
47	0.74	0.87	0.13	3.13	2.89	−0.24	1.39	1.40	0.01	
48	0.75	0.89	0.14	2.96	2.96	0.00	1.39	1.42	0.03	
49	0.72	0.81	0.09	3.34	3.15	−0.19	1.36	1.36	0.00	
50	0.94	1.02	0.08	4.25	3.77	−0.48	1.64	1.62	−0.02	Type IIa
51	0.61	0.57	−0.04	4.34	3.64	−0.70	1.35	1.59	0.24	Type IIa
52	1.43	1.73	0.30	3.01	3.24	0.23	0.99	1.12	0.13	Type IV
53	0.36	0.44	0.08	3.84	3.40	−0.44	1.24	1.37	0.13	
54	0.39	0.37	−0.02	2.44	2.54	0.10	1.62	1.70	0.08	
55	0.51	0.63	0.12	2.63	2.54	−0.09	1.21	1.28	0.07	
56	0.53	0.36	−0.17	4.11	4.03	−0.08	2.59	2.41	−0.18	Type IIa
57	0.37	0.43	0.06	2.35	2.58	0.23	1.19	1.32	0.13	
58	0.65	0.53	−0.12	3.36	3.41	0.05	1.49	1.61	0.12	
59	0.55	0.51	−0.04	3.20	3.02	−0.18	1.51	1.59	0.08	
60	1.68	1.63	−0.05	3.82	4.48	0.66	1.36	1.46	0.10	Type IIb
61	0.15	0.34	0.19	1.43	1.93	0.50	1.04	1.06	0.02	
62	0.22	0.38	0.16	2.90	3.08	0.18	1.58	1.60	0.02	
63	0.22	0.38	0.16	2.71	3.19	0.48	1.59	1.67	0.08	
64	0.24	0.39	0.15	2.74	3.09	0.35	1.60	1.60	0.00	
65	0.22	0.40	0.18	2.78	3.28	0.50	1.56	1.70	0.14	
66	0.22	0.40	0.18	2.65	3.17	0.52	1.56	1.63	0.07	

[a]Only abnormal cases are specified; FH, familial hypercholesterolemia; CRF, chronic renal failure.

rect Lorentzian shape and width (1.1 ± 0.1 Hz) for the reference peak. The water signal in the samples was suppressed by the standard binomial $1 - \bar{1}$ pulse sequence. In each experiment, 256 FID signals (28 min) were accumulated using a spectral width of 5 kHz, 64 k datapoints, a 45 degree pulse of 28–33 μs and a pulse repetition time of 6.6 s. For some plasma samples also 128 (14 min) and 32 FIDs (3.5 min) were accumulated. The measured FIDs were Fourier-transformed (no apodization) to the frequency domain spectra, which were then moved to a 486 IBM-compatible PC for lineshape fitting analyses.

The temperature of a sample was stabilized before placing it to the magnet by prewarming the sample in a water bath at +37°C for about 30 min. The different heating environment of the probe caused temperature gradients to the sample and therefore an additional equilibration time of about 15 min in the magnet was also necessary. However, the effective heating time needed was only about 15 min, because the sample(s) can be placed to the water bath at the same time as another sample is in the magnet.

NMR lineshape fitting analyses

The resolvable individual ^{1}H NMR resonances of the lipoprotein lipids have been shown to be well described by a Lorentzian lineshape (1–3, 11). Although both the real and imaginary parts of the generally complex Lorentzian signal are used in the parameter estimation, the real part is adequate for illustration purposes:

$$L_i(v) = A_i(v)\cos(\phi_i) + D_i(v)\sin(\phi_i) = \frac{a_i^2 I_i}{a_i^2 + 4(v - v_i)^2}\cos(\phi_i) + \frac{2a_i I_i(v - v_i)}{a_i^2 + 4(v - v_i)^2}\sin(\phi_i) \quad Eq.\ 1)$$

in which a_i is the half linewidth, I_i the intensity, v_i the resonance frequency, and ϕ_i the phase angle of the individual Lorentzian i (see **Fig. 1** and **Fig. 2** for the shape of a Lorentzian line). $A_i(v)$ stands for the absorption and $D_i(v)$ for the dispersion part of the signal. If the phase correction of the spectrum is exact, the real spectrum is purely absorptive and the imaginary spectrum purely dispersive. The phase correction of the ^{1}H NMR spectra of plasma and lipoproteins is so difficult in practice that a few degree's inaccuracies easily result. Thus both absorption and dispersion parts of the Lorentzian are used in the lineshape fitting analyses with the phase as one of the variable parameters. All analyses were performed using the sophisticated lineshape fitting analysis algorithm FITPLA^C developed in our laboratory (1–3, 11).

Three individual Lorentzians were needed in the case of the VLDL and LDL fractions and one in the case of the HDL fractions to give an accurate description of the methyl resonances of the lipoprotein lipids (see Figs. 1 and 2). Some parameters of these individual Lorentzians (half linewidths, relative resonance frequencies and intensity ratios of the individual components) were uniform within each lipoprotein fraction. Thus, mathematical lineshape models

$$M_{VLDL}(v) = L_1(v)^{VLDL} + L_2(v)^{VLDL} + L_3(v)^{VLDL},$$
$$M_{LDL}(v) = L_1(v)^{LDL} + L_2(v)^{LDL} + L_3(v)^{LDL} \text{ and,}$$
$$M_{HDL}(v) = L_1(v)^{HDL} \quad Eq.\ 2)$$

could be constructed for the methyl resonances of the VLDL, LDL, and HDL fractions. The uniform parameters of the individual Lorentzians in equation 2 were fixed to the values obtained as an average of 20 analyses of ^{1}H NMR spectra of each fraction from different volunteers. They are as follows (see equation 1 and Figs. 1 and 2 for the numbering): for VLDL the half linewidths a_1^{VLDL} = 6.45 Hz, a_2^{VLDL} = 8.84 Hz, and a_3^{VLDL} = 7.72 Hz, the intensity ratios I_1^{VLDL}:I_2^{VLDL} = 0.2748 and I_3^{VLDL}:I_2^{VLDL} = 0.4782, and the relative resonance frequencies $v_1^{VLDL} - v_2^{VLDL}$ = 6.29 Hz and $v_2^{VLDL} - v_3^{VLDL}$ = 7.19 Hz; for LDL the half linewidths a_1^{LDL} = 4.64 Hz, a_2^{LDL} = 10.98 Hz, and a_3^{LDL} = 11.38 Hz, the intensity ratios I_1^{LDL}:I_2^{LDL} = 0.1254 and I_3^{LDL}:I_2^{LDL} = 1.0019, and the relative resonance frequencies $v_1^{LDL} - v_2^{LDL}$ = 7.20 Hz and $v_2^{LDL} - v_3^{LDL}$ = 6.65 Hz; and for HDL the half linewidth a_1^{HDL} = 18.44 Hz.

These lineshape models of the lipoprotein methyl resonances were then used in the analyses of the methyl lineshapes of the plasma spectra. The theoretical description of the methyl resonance region in a plasma spectrum could thus be expressed as

$$M_{PLASMA}(v) = M_{VLDL}(v) + M_{LDL}(v) + M_{HDL}(v) + c_0 + c_1(v) \quad Eq.\ 3)$$

in which $c_0 + c_1(v)$ takes into account the varying underlying background resonances relating to residual water, albumin-bound immobile fatty acids, and proteins. For every plasma spectrum, equation 3 was fitted to the experimental methyl envelope in a χ^2 sense using the Levenberg-Marquardt method for solving the nonlinear matrix equations (1–3, 11). A correct position (resonance frequency) and total intensity for every model signal was mathematically derived. The areas of the model signals were scaled in every spectrum by using the area of the external reference signal (obtained as a result of a fit of one Lorentzian). The area under a Lorentzian signal i is $A_i = \frac{\pi}{2} a_i I_i$.

The prior knowledge of the lipoprotein methyl lineshapes M_{VLDL}, M_{LDL}, and M_{HDL} (Fig. 2) is necessary to ensure that the extremely overlapping information in the ^{1}H NMR spectra of the plasma samples can be separated in an accurate and biochemically consistent way. In nonlinear problems, the final solution might also depend on the initial values of the estimated parameters. Thus we have incorporated an automatic guess-system to the

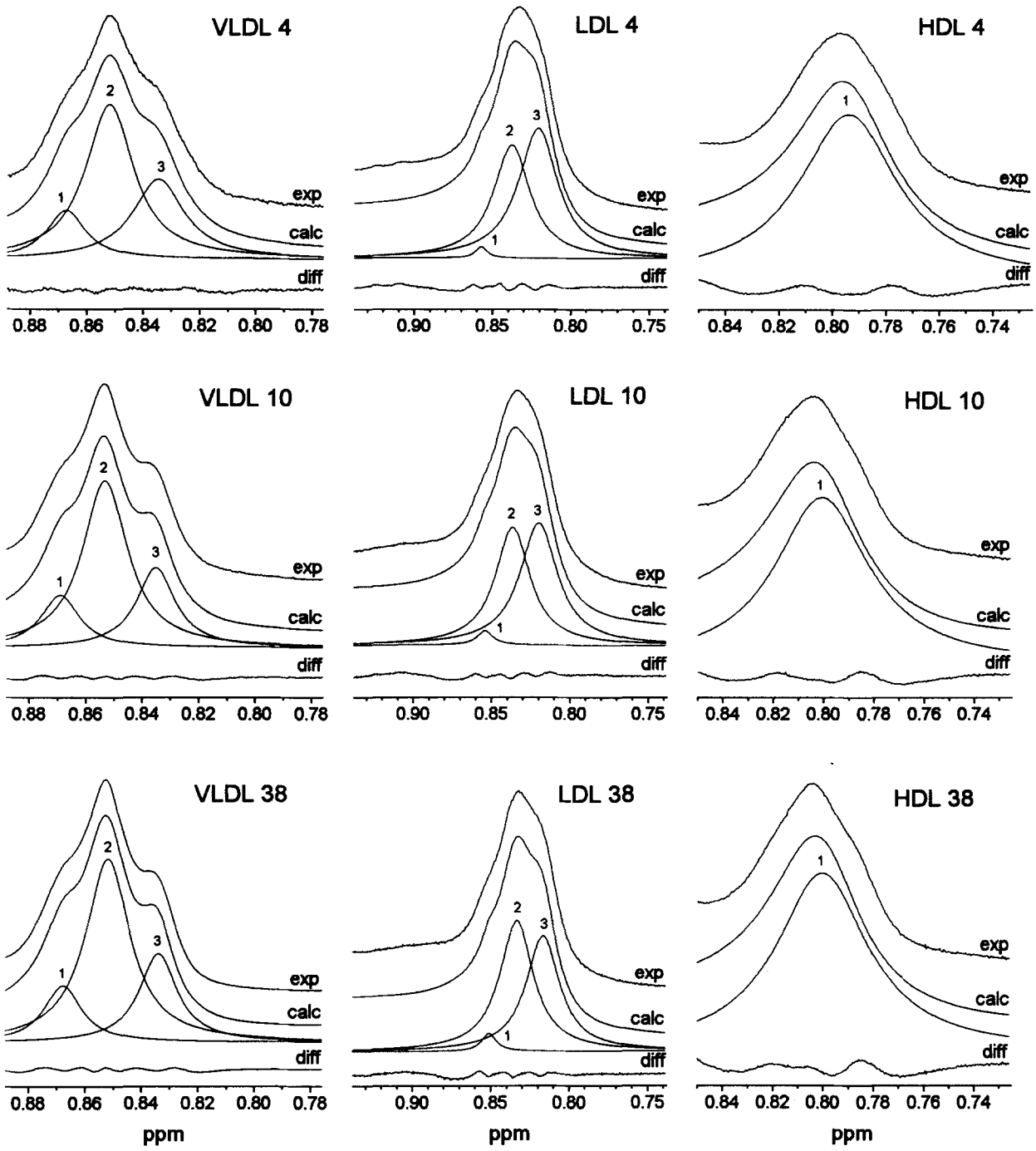

Fig. 1. The lipoprotein methyl lineshapes in the ^{1}H NMR spectra of VLDL, LDL, and HDL fractions of the subjects no. 4, 10, and 38. The experimental spectra (exp) are shown at the top and the calculated spectra (calc) below them. The individual Lorentzian components used in the modeling are also shown and the difference spectra (diff = exp − calc) are drawn at the bottom. The individual Lorentzians are numbered in each lipoprotein fraction (see equation 2).

FITPLA^C program; the initial values of the variable parameters are randomly chosen from given wide (but reasonable) limits, but the final parameters are not constrained to these limits. This technique is, therefore, used only to effectively probe the multidimensional parameter space and to ensure reliable decision about the unequivocality and physicality of the solutions. In practice, this means that about 5 to 25 different solutions are calculated for each spectrum; the solution with lowest RMS error is selected as the final one if it is physically correct. The different solutions are easily checked with the graphical utility of the FITPLA^C program. We have carefully

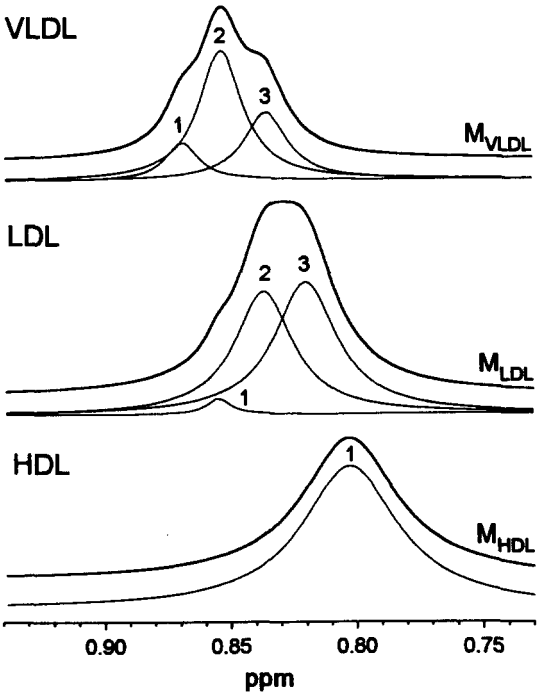

Fig. 2. The mathematical methyl lineshape models M_{VLDL}, M_{LDL}, and M_{HDL} and their individual components (see equations 2 and 3).

tested that both mathematical unequivocality and physicality of the solutions can be fulfilled for every spectrum using the model lineshapes and analysis procedure described above (equations 1, 2, and 3). This means that unique results for all the lipoprotein values are obtained in each case. At the moment, the calculation time of one solution is about 20 to 30 sec in a 486 33 MHz PC and normally 10 solutions are calculated for each spectrum. Hence, the mathematical analysis of one ^{1}H NMR spectrum of a plasma sample can be completed in less than 10 min.

A copy of the lineshape fitting analysis program FITPLA^C, ©University of Oulu, Department of Physics, NMR Research Group, can be ordered by writing a signed letter to M. A-K. in which use of the program for research purposes only in the stated laboratory is guaranteed. The program will be sent free of charge and with full instructions for its use.

RESULTS AND DISCUSSION

The methyl resonance in the ^{1}H NMR spectrum of a plasma sample is a sum of heavily overlapping individual components of all the lipoprotein fractions. In reconstructing an experimental ^{1}H NMR signal of plasma (or an isolated lipoprotein fraction), mathematical difficulties should be kept in mind all the time (1, 3, 11). In the case of plasma spectra, considerable amount of prior knowledge connecting the system biochemistry to the data analysis is needed to ensure mathematically unequivocal and physically consistent solutions. In this case, sufficient prior knowledge can be obtained from separate ^{1}H NMR experiments and lineshape fitting analyses of ultracentrifuged lipoprotein fractions VLDL, LDL, and HDL (3).

We measured the ^{1}H NMR spectra of VLDL, IDL, LDL, and HDL fractions from 20 subjects (from all the 15 volunteers in the calibration set and from 5 subjects from the blinded set). All the methyl resonance regions of the fraction spectra were analyzed by using the program FITPLA^C (1-3, 11). The methyl lineshapes in the experimental ^{1}H NMR spectra of VLDL, LDL, and HDL fractions together with the calculated regions, the individual Lorentzian components, and the difference spectra of three subjects (nos. 4, 10, and 38) are shown as an example in Fig. 1. As Fig. 1 implies, the parameters of the individual Lorentzians were uniform for all the subjects in every lipoprotein category (see equation 2), as previous qualitative experimental observations have also proposed (4). This indicates that the main variable in the ^{1}H NMR spectra of the lipoprotein fractions of different persons is the total amount, i.e., concentration, of the fraction. This permits construction of the lineshape models, based on equation 2 and shown in Fig. 2, for the methyl resonances of the lipoprotein fractions and their use in the analyses of ^{1}H NMR spectra of plasma.

In order to determine the relation between the biochemical lipid and protein measurements and the ^{1}H NMR-based (reference scaled) areas of the lipoprotein model signals, we carried out both measurements for plasma samples of 15 volunteers (from no. 1 to 15 in Table 1). All the ^{1}H NMR spectra of these plasma samples were analyzed using the developed lipoprotein model lineshapes. The total intensity and chemical shift, i.e., the correct position in the frequency axis, of every model signal (VLDL, LDL, and HDL) and the parameters c_0 and c_1 of the linear background were variable parameters in the calculations. Also, one common phase parameter ϕ for all the signals was varied (1, 11). The methyl regions of the experimental ^{1}H NMR spectra of plasma of four different subjects (nos. 1, 6, 7, and 10) from this calibration set are shown in **Fig. 3** together with the calculated reconstructions, the lipoprotein lineshape models, and the difference spectra. Linear correlations between the external reference scaled areas of the lipoprotein model signals and the biochemically estimated VLDL, LDL, and HDL triglycerides, phospholipids, total cholesterol, free cholesterol, esterified cholesterol, total proteins, and total masses were calculated. As is seen from **Table 2,** the correlations were excellent, ranging from 0.84 to 0.99 in VLDL, from 0.88 to 0.95 in LDL, and from 0.40 to 0.84 in HDL. The correlation lines for VLDL triglycerides, LDL cholesterol, and HDL cholesterol are shown in **Fig. 4.** The

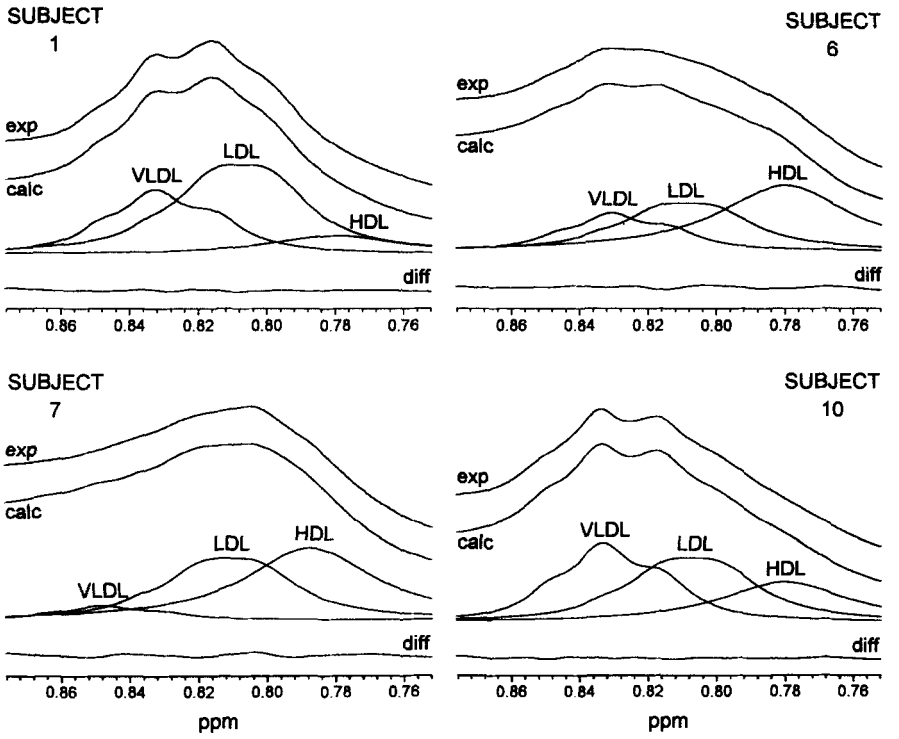

Fig. 3. The methyl regions of the ^{1}H NMR spectra of plasma for the subjects no. 1, 6, 7, and 10 from the calibration set (see Table 1). The experimental spectra (exp) are shown at the top and the calculated spectra (calc) below them. The lipoprotein lineshape models used in the modeling are also drawn and the difference spectra (diff = exp − calc) are given at the bottom.

reference scaled areas of the lipoprotein model signals, estimated mathematically from the ^{1}H NMR spectrum of plasma, thus reflect the absolute concentrations of all the lipids, proteins, and total masses of the lipoprotein fractions. Therefore, these existing correlation equations can be taken as conversion equations between the NMR and biochemical assays (Table 2) and used in further studies as a basis to estimate the lipoprotein lipid and protein values directly from the ^{1}H NMR spectrum of an unknown plasma sample. Especially for the clinically most essential lipid values, i.e., the VLDL triglycerides, LDL cholesterol, and HDL cholesterol, the correlations were striking; the correlation coefficients were 0.99, 0.93, and 0.84, respectively.

After calibrating the ^{1}H NMR-based method as explained above, a double-blinded test of 51 unknown plasma samples from 43 volunteers (from no. 16 to 66 in Table 1) was performed using both the biochemical lipid and protein assays and the new ^{1}H NMR-based method utilizing the specified conversion equations. This serves to assess the capabilities of the new method in true analytic situations. The methyl regions of the experimental ^{1}H NMR spectra of plasma of six different subjects (nos. 16, 25, 34, 38, 39, and 61) from this blinded set are shown as an example in **Fig. 5** together with the calculated reconstructions, the lipoprotein lineshape models, and the difference spectra. The molarities of VLDL triglycerides, LDL cholesterol, and HDL cholesterol for every subject (including the 15 plasma samples used in the calibration) with the differences of the NMR values compared to the biochemical ones are given in Table 1. From Table 1 and Figs. 3 and 5 it is obvious that different lipoprotein lipid profiles give different plasma methyl lineshapes. These different ^{1}H NMR characteristics lead to lipoprotein lipid and protein values that are in excellent agreement with the biochemically estimated ones. In Table 2 the correlation coefficients between the NMR and biochemical assays are given together with the conversion equations necessary to convert the VLDL, LDL, and HDL model signal areas in the ^{1}H NMR spectrum of a plasma sample to the VLDL, LDL, and HDL lipid concentrations (mmol/l), total protein amounts (mg/dl), and total masses (mg/dl). No correlation was found for HDL triglycerides ($r = -0.04$). In **Fig. 6**, correlations between the final results of the NMR and biochemical assays of VLDL triglycerides, LDL cholesterol, and HDL cholesterol are drawn. Differences between the NMR and the biochemical estimates of the VLDL triglycerides, LDL cholesterol, and HDL cholesterol are presented for all the 66 plasma samples in **Fig. 7.** Moreover, the VLDL model signal area correlated with the

TABLE 2. Correlation coefficients (r) and conversion equations (BIO = A + B × NMR) between the reference scaled ^{1}H NMR areas of the VLDL, LDL and HDL methyl lineshape models and the biochemical lipoprotein lipid and protein assays

		BIO = A + B × NMR	
Fraction	r	A[× 10^3]	B[× 10^3]
VLDL			
TG (n = 15)	0.99	103.45	4.34
TG (n = 66)	0.98	83.92	4.30
PL (n = 15)	0.96	25.04	1.64
PL (n = 63)	0.96	37.40	1.54
CHO (n = 15)	0.89	59.28	1.99
CHO (n = 66)	0.88	− 7.10	2.50
FREE CHO (n = 15)	0.93	− 5.78	0.95
FREE CHO (n = 66)	0.91	− 7.47	1.03
EST CHO (n = 15)	0.84	65.07	1.03
EST CHO (n = 66)	0.85	0.37	1.47
PROT (n = 15)	0.93	4375.11	64.41
PROT (n = 66)	0.91	4883.34	71.76
MASS (n = 15)	0.97	19469.83	679.82
MASS (n = 63)	0.97	16666.98	702.69
LDL			
TG (n = 15)	0.94	− 138.18	0.90
TG (n = 65)	0.63	12.46	0.68
PL (n = 15)	0.94	− 59.84	2.92
PL (n = 62)	0.90	− 7.33	2.79
CHO (n = 15)	0.93	72.80	8.10
CHO (n = 65)	0.88	10.03	8.28
FREE CHO (n = 15)	0.88	58.75	2.02
FREE CHO (n = 64)	0.82	60.84	2.02
EST CHO (n = 15)	0.93	14.06	6.08
EST CHO (n = 64)	0.89	− 42.36	6.23
PROT (n = 15)	0.95	− 22890.73	244.31
PROT (n = 65)	0.89	− 12779.11	227.10
MASS (n = 15)	0.95	− 36571.66	1023.02
MASS (n = 61)	0.92	− 10045.92	981.50
HDL			
PL (n = 15)	0.68	166.08	2.63
PL (n = 64)	0.83	57.58	2.67
CHO (n = 15)	0.84	− 15.90	3.49
CHO (n = 66)	0.93	− 67.04	3.42
FREE CHO (n = 15)	0.67	− 64.10	0.68
FREE CHO (n = 66)	0.85	− 121.61	0.82
EST CHO (n = 15)	0.83	48.19	2.80
EST CHO (n = 66)	0.91	54.58	2.60
PROT (n = 15)	0.40	101973.49	187.68
PROT (n = 66)	0.75	57567.21	279.76
MASS (n = 15)	0.61	161220.59	518.58
MASS (n = 64)	0.84	80905.04	683.53

The NMR areas of the lipoproteins were obtained for each plasma sample from the lineshape fitting analysis of the methyl resonance region in the ^{1}H NMR spectrum. For each substance two equations are given; the first is calculated from the calibration set of 15 samples and the second from the whole study population. These conversion equations enable calculation of the lipoprotein lipid concentrations (mmol/l), protein amounts (mg/dl), and total masses (mg/dl) directly from the VLDL, LDL, and HDL lineshape model areas obtained from the lineshape fitting analysis of a ^{1}H NMR spectrum of an unknown plasma sample. Abbreviations: TG, triglycerides; PL, phospholipids; CHO, total cholesterol; FREE CHO, free cholesterol; EST CHO, cholesterol esters; PROT, total proteins; and MASS, total mass (calculated as MASS (mg/dl) = 88.5 × TG (mmol/l) + 77.5 × PL (mmol/l) + 38.7 × FREE CHO (mmol/l) + 64.9 × EST CHO (mmol/l) + PROT (mg/dl)).

plasma triglycerides (r = 0.97, n = 66, plasma triglycerides (mmol/l) = 0.55385 + 0.00537 × VLDL area %) and the total area of the methyl resonance, i.e., the summed integrated intensity of the VLDL, LDL, and HDL model signals, with the plasma cholesterol (r = 0.86, n = 66, plasma cholesterol (mmol/l) = 0.07988 + 0.00521 × (VLDL + LDL + HDL area %)).

The reproducibility and accuracy of the ^{1}H NMR and biochemical analyses were assessed by analyzing five identical plasma samples of two different volunteers. The results in the case of the VLDL triglyceride, LDL cholesterol, and HDL cholesterol estimates are shown in Table 1; the first set contained the samples from no. 45 to 49 and the second set the samples from no. 62 to 66. The results were analogous and the ^{1}H NMR-based estimates were as accurate as the biochemical ones. The variability of both methods seems to be less than 0.2 mmol/l. The means (± standard deviations) of the absolute differences between the methods, calculated using all the samples, were 0.10 ± 0.08 mmol/l for VLDL triglycerides, 0.40 ± 0.38 mmol/l for LDL cholesterol, and 0.12 ± 0.09 mmol/l for HDL cholesterol. Using these two sets of identical plasma samples, we also tested the effect of NMR data collection time, i.e., the number of the accumulated FIDs (see Experimental NMR spectroscopy), to the quantitation results. All the collection times (from 28 min corresponding to 256 FIDs to 3.5 min corresponding to 32 FIDs) gave identical results. This makes it possible to use only 32 FIDs (instead of 256) in the future applications.

Our NMR approach differs significantly from the currently used biochemical lipoprotein quantitation methods (7). All other methods require a physical separation of the lipoprotein categories followed by specific biochemical assays of the protein and lipid constituents. In practice, this often means prolonged and laborious measurements and may include considerable inaccuracies (7). Although our NMR results should be considered as quite preliminary ones, we emphasize that they clearly show the capability of this type of an NMR approach. The method can be integrated directly to many areas of NMR spectroscopic research. Many biomedical NMR studies (12–14), which are not necessarily aimed to study lipoproteins, will gain considerable benefit from the additional biochemically important quantitative lipoprotein information, the measurement of which by conventional biochemical methods is usually too laborious and high-priced to be adapted into the normal study protocols.

Figs. 3 and 5 give a clear picture of the effects of the different lipoprotein lipid levels on the shape of the plasma methyl envelope and also demonstrate that visually apparently very different situations can be correctly analyzed by our lipoprotein model lineshapes and

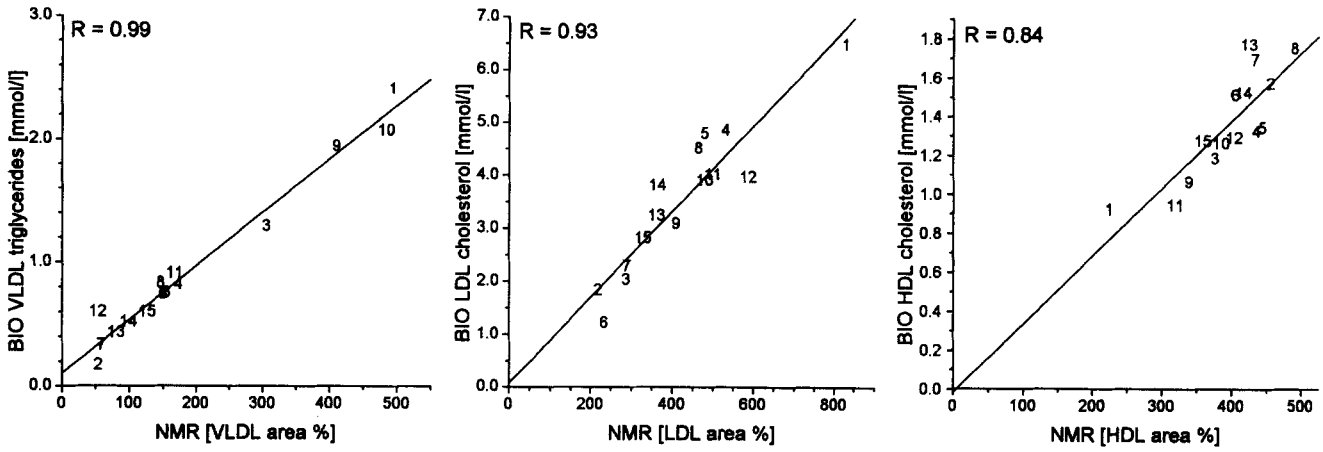

Fig. 4. The correlations between the areas of the lipoprotein methyl lineshape models estimated mathematically from the ^{1}H NMR spectra of plasma and the VLDL triglyceride, LDL cholesterol, and HDL cholesterol values from the biochemical assays. The numbers refer to the subjects in Table 1.

data analysis technique. We emphasize that proper attention to the data analysis mathematics and biochemical prior knowledge is necessary to achieve reliable and consistent results. In the FITPLA^C algorithm, all essential aspects have been taken into account and some special features facilitating the practical use of the program have also been incorporated (11). FITPLA^C estimates a linear background (the coefficients c_0 and c_1 in equation 3) from the experimental spectrum and thus allows for automatic correction of the broad underlying hump arising from the albumin-bound immobile fatty acids, some other protein, and residual water-related resonances (11, 15, 16). In this case the width of the analyzed methyl resonance regions was 50 Hz, which naturally allowed the above procedure to be correctly applied because all the underlying signals in the methyl resonance region are closely linear. The baseline is fitted to the experimental spectrum at the same time as the actual lipoprotein model signals. Thus the varying underlying background resonances are mathematically taken into account in every spectrum and are therefore separated from the actual lipoprotein lipid methyl resonances described by the lineshape models. We would also like to point out that the phase correction of the experimental spectrum in the case of complicated overlapping resonances is a difficult task and may induce inaccuracies because the phase-corrected spectrum may easily be misphased a few degrees. Thus, we have incorporated a phase correction to the analysis algorithm; every spectrum is at first phased at the spectrometer and, in the mathematical analysis of the methyl region, a refinement to the phase correction is automatically calculated. The FITPLA^C algorithm also allows inclusion of all the possible resonances present in the spectral region under study (e.g., the lactate doublet in the methylene region) (3, 11). Therefore, the problems encountered in the previously reported ^{1}H NMR-based lipoprotein quantitation approach (4–6) are avoided.

The FITPLA^C modeling algorithm is based on the Levenberg-Marquardt method which is the most sophisticated one for nonlinear optimization (1, 11). It will enable mathematically unequivocal and biochemically consistent solutions to be obtained for very complicated cases, as the methyl envelope in the ^{1}H NMR spectra of plasma, in an elegant and rapid fashion. A case-dependent amount of prior knowledge is generally required. This is now available in the form of the lipoprotein model lineshapes. Moreover, the program is easily convertible and can be adapted to studies of any spectral region (in any spectrum) (3, 11). Also, very recent comparative mathematical analyses have clearly verified the success of the FITPLA^C approach to analyze the heavily overlapping information in the ^{1}H NMR spectra of the lipoprotein fractions (11). Finally, an additional indication of the effectiveness and correctness of these mathematical procedures is the final result; the ^{1}H NMR-based lipid estimates agree extremely well with the biochemically measured ones as seen from the values in Table 1 and from the correlation coefficients in Table 2.

Our present study population serves as a good illustration of wide-ranging lipid values; e.g., VLDL triglycerides from 0.15 to 4.32 mmol/l, LDL cholesterol from 1.21 to 6.48 mmol/l, and HDL cholesterol from 0.73 to 2.59 mmol/l. Also, subjects with various lipoprotein abnormalities, familial hypercholesterolemia, and uremia were studied. This study population shows that the most common lipid anomalies such as hypercholesterolemia (Type IIa), hypertriglyceridemia (Type IV), and combined hyperlipidemia (Type IIb) are correctly identified by our ^{1}H NMR-based method (see Table 1). This is the most important property for a method to be used in an automatic fashion to establish persons at increased risk to CHD, i.e., to identify high LDL cholesterol and low HDL cholesterol levels. The analogous results in many very different subjects inspire confidence in the potential of the

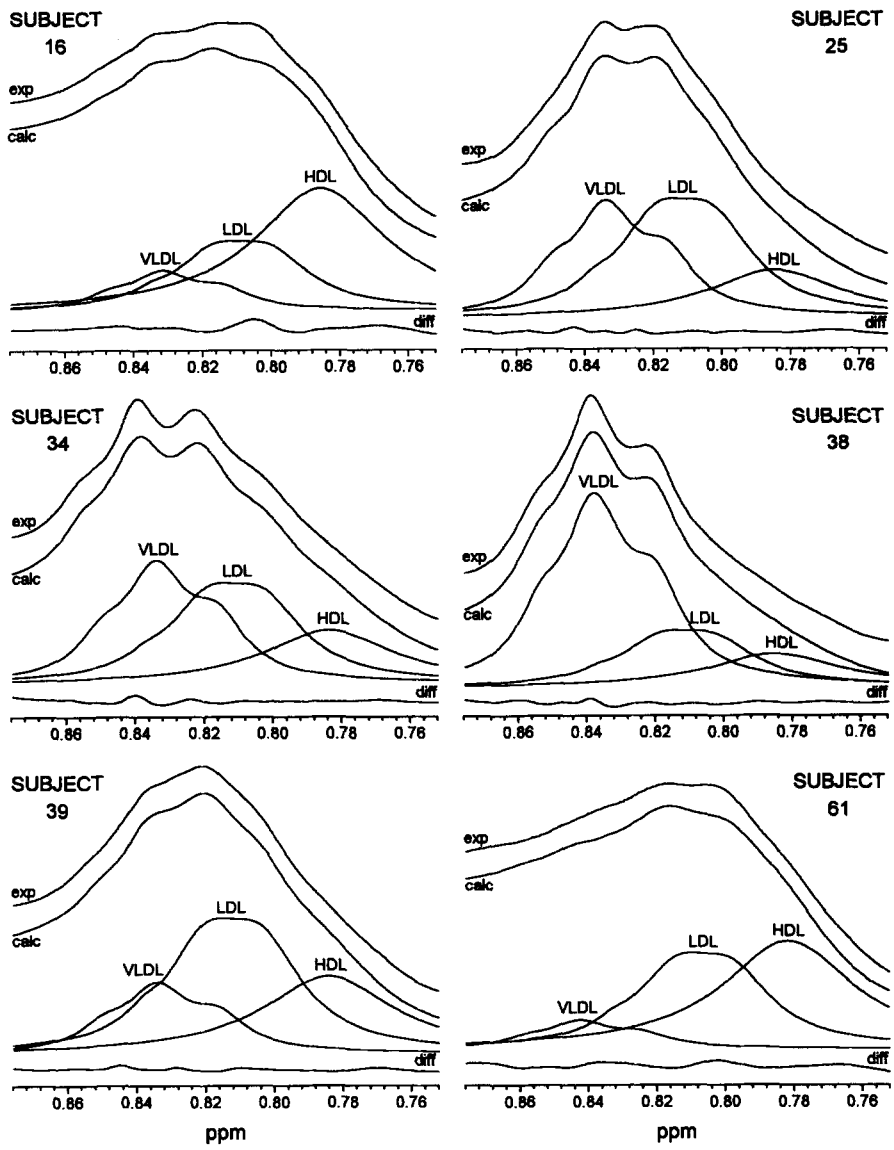

Fig. 5. The methyl regions of the ^{1}H NMR spectra of plasma of the subjects no. 16, 25, 34, 38, 39, and 61 from the blinded set (see Table 1). The experimental spectra (exp) are shown at the top and the calculated spectra (calc) below them. The lipoprotein lineshape models used in the modeling are also drawn and the difference spectra (diff = exp − calc) are given at the bottom.

method to be developed as a routine clinical assay to quantitate lipoprotein lipids, total proteins, and total masses. Naturally, more comparative experiments and analyses of plasma samples from subjects with distinct pathological conditions, e.g., Type I, Type V, and Type III hyperlipoproteinemias, are needed to assess the validity of the method and to examine its possibilities in rare clinical conditions.

To assess the limitations of the method and as a preliminary test towards a more specific classification of lipoproteins and estimation of their lipid concentrations, several volunteers with anomalously high IDL levels were studied (for example the patients with chronic renal failure, samples no. 37, 38, 40, 42, and 44). Normally, the IDL fractions exist in very low concentrations giving rise to insignificant contributions to the plasma methyl lineshapes. Thus the IDL fractions have been excluded from the present plasma analysis model to ensure reliable quantitation of the other fractions in the most abundant cases. In our present study group, we had 10 subjects with the IDL cholesterol $\geq$0.30 mmol/l (samples no. 1, 6, 10, 12, 34, 39, 40, 42, 44, and 53). For sample no. 42, an LDL cholesterol concentration of 3.59 mmol/l and an IDL cholesterol concentration of 1.08 mmol/l were estimated by the biochemical methods. In the NMR analysis, based on the VLDL, LDL, and HDL model lineshapes, an LDL con-

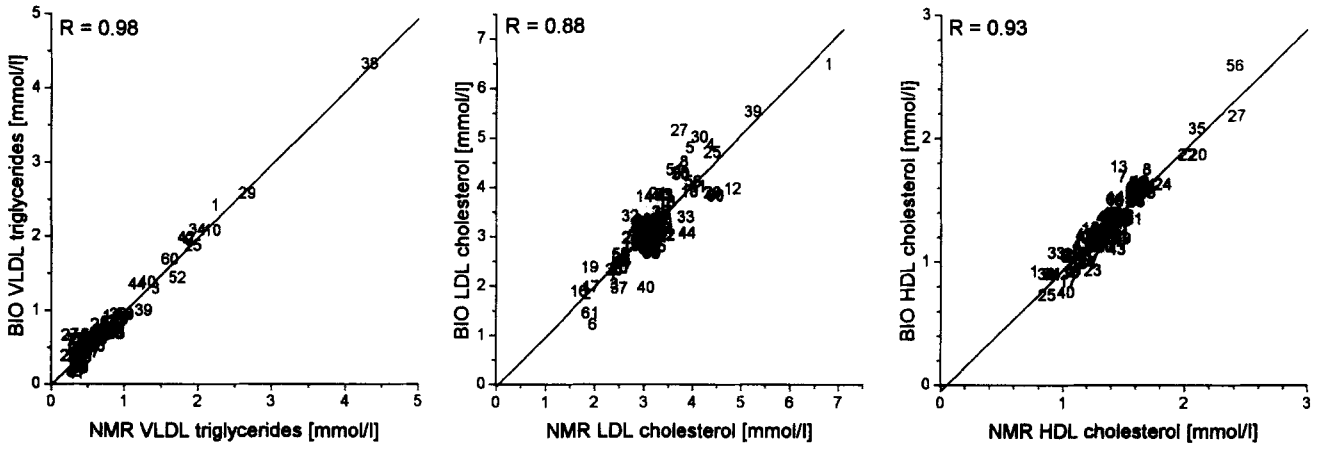

Fig. 6. The correlations between the ^{1}H NMR and biochemical assay estimates of the VLDL triglycerides, LDL cholesterol, and HDL cholesterol. The numbers refer to the subjects in Table 1.

centration of 6.01 mmol/l was estimated (the greatest deviation in Fig. 7); note that the VLDL triglyceride and HDL cholesterol estimates were still correct. In other samples with slightly elevated IDL levels, the NMR estimates of the LDL cholesterol tended to be slightly higher than the biochemical ones. Also, in these subjects, the VLDL triglyceride and HDL cholesterol estimates agreed well with the biochemical estimates. At first, this finding indicated that high IDL levels would exaggerate the LDL levels in the NMR analysis when only three lipoprotein model lineshapes are used. Because elevated IDL levels are rare, this tendency is not regarded as a serious problem, however. Second, it proposes an additional possibility for the ^{1}H NMR-based approach: if an enlargement of the plasma analysis model could be done, i.e., including IDL to the model by describing it by its own model lineshape, more specific quantitation of the lipoproteins could result. Indeed, based on our ^{1}H NMR measurements and lineshape fitting analyses of 20 different IDL fractions, we were able to develop the IDL model

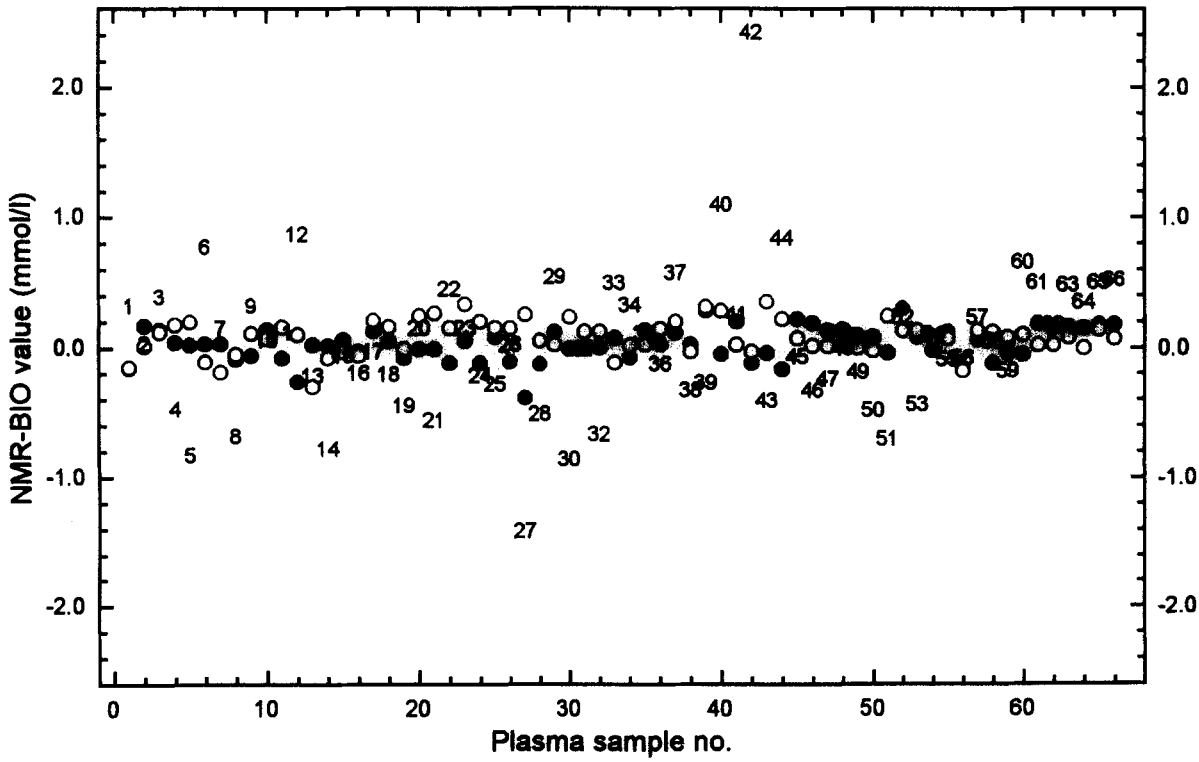

Fig. 7. Differences (NMR-BIO) between the ^{1}H NMR (NMR) and the biochemical (BIO) estimates of the VLDL triglycerides (●), LDL cholesterol (numbers refer to the samples in Table 1), and HDL cholesterol (○).

lineshape as a combination of three individual Lorentzian components ($M_{IDL}(v) = L_1(v)^{IDL} + L_2(v)^{IDL} + L_3(v)^{IDL}$, in which the half linewidths a_1^{IDL} = 9.20 Hz, a_2^{IDL} = 9.09 Hz and a_3^{IDL} = 6.70 Hz, and the intensity ratios I_1^{IDL}:I_2^{IDL} = 0.2632 and I_3^{IDL}:I_2^{IDL} = 0.5805, and the relative resonance frequencies $v_1^{IDL} - v_2^{IDL}$ = 6.36 Hz and $v_2^{IDL} - v_3^{IDL}$ = 6.70 Hz). Using this IDL model lineshape as an additional component in the normal model (equation 3), we proceeded to analyze the methyl region of the ^{1}H NMR spectrum of the plasma sample no. 42 again. As a promising indication of further capabilities of our NMR method, we found the mathematical solution to be unequivocal and the 'new' LDL cholesterol concentration (approximated by using the conversion equation in Table 2) to be 4.71 mmol/l (instead of 6.01 mmol/l), i.e., much closer to the biochemically estimated value of 3.59 mmol/l. It should also be noticed that other methods, e.g., the commonly used approximation based on the Friedewald formula, include the IDL cholesterol into the LDL cholesterol estimate as well (17). In the case of our sample no. 42, the Friedewald approximation-based LDL cholesterol value was 5.57 mmol/l. Because the sample no. 42 was highly anomalous, deviated clearly from the other samples, and was outside the capabilities of the present analysis model, it was not used in the LDL correlation equation calculations given in Table 2.

As we have already emphasized, mathematical analysis of heavily overlapping information, as in the case of the methyl envelope of the ^{1}H NMR spectra of plasma, is a difficult task requiring sufficient prior knowledge and proper analysis protocol to achieve biochemically meaningful and reliable results. If more lipoprotein model lineshapes are to be added to the plasma analysis model (equation 3), extreme care must be taken in the interpretation of the results. To ensure unique results for every spectrum, the necessary amount of prior knowledge in each model should be carefully tested to avoid overextraction of information from the experimental spectrum. For example, if one tries to include the IDL model lineshape in the same way as the other fractions for normal, i.e., very low (about 0.1 mmol/l) IDL cholesterol concentration, biochemically consistent and reliable solutions are not automatically obtained. This is because the model contains too many free parameters to be estimated from the experimental spectrum, in which information of the IDL particles is insignificant, and thus mathematical unequivocality is not reached. Nevertheless, as our preliminary IDL example shows, it might be possible to include IDL in the plasma analysis model and, in fact, addition of even more lipoprotein model lineshapes, i.e., chylomicrons and subfractions of VLDL, LDL, and HDL, could be realizable. This would considerably increase the information available from this kind of ^{1}H NMR-based technique because similar calibration of different lipid concentrations that has been applied here for VLDL, LDL, and HDL would be possible for all the included lipoprotein model fractions. The main practical difficulty is to find the most representative set of lipoproteins, which can be included in the final model in such a way that an unequivocal solution for every spectrum is automatically obtained. In order to resolve this problem, different lipoprotein model sets should be tested. Also, a special mathematical algorithm will be necessary for this kind of an application to ensure consistent results for all possible concentrations of the lipoproteins. Especially when one (or more) (sub)fractions have very low concentrations, there should be particular means to cope with this nonexisting experimental information in a biochemically sensible way. A decisive factor for the development of the final model and its usefulness will be the amount of sound prior knowledge of the lipoprotein model lineshapes (half linewidths and relative chemical shifts and relative intensities of the individual components) and their properties (chemical shifts). Studies to extend the proposed ^{1}H NMR-based lipoprotein quantitation method in the above sense are in progress in our laboratory.

CONCLUSIONS

A new method, based on ^{1}H NMR spectroscopy and sophisticated lineshape fitting analyses, was introduced for absolute quantitation of all the lipoprotein lipids, total proteins, and total masses. At the present stage the method is applicable to VLDL, LDL, and HDL and all the estimates can be obtained directly from a plasma sample in a total time of less than 1 h. Comparison of the ^{1}H NMR and the biochemical assay results of 66 plasma samples, of which 51 were analyzed in a double-blinded manner, showed excellent agreement between these two different techniques. For example, for the clinically most essential lipid values, i.e., the VLDL triglycerides, LDL cholesterol, and HDL cholesterol, the correlation coefficients between the NMR-based and the biochemical estimates were 0.98, 0.88, and 0.93, respectively. The presented ^{1}H NMR method can be directly integrated to many different kinds of biomedical NMR studies to offer additional biochemically important information from lipoprotein lipids. The method was shown to give correct estimates in subjects with the most common lipid anomalies such as hypercholesterolemia, hypertriglyceridemia, and combined hyperlipidemia, which are the most important to establish in screening of persons at increased risk to CHD. Thus, the proposed new ^{1}H NMR-based method has good potential to be developed for a clinical assay. There are also good possibilities to develop the method further by including more lipoprotein (sub)fractions to the analysis model.

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