

## An efficient chromatographic system for lipoprotein fractionation using whole plasma

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**Abstract** We have validated a semi-automatic procedure for the efficient isolation of plasma lipoproteins from 300  $\mu$ l of whole plasma (actual injection volume 200  $\mu$ l) by Fast Phase Liquid Chromatography (FPLC). Modified enzymatic assays were established to allow the determination of low concentrations (1–20 mg/dl) of triglycerides and cholesterol using the Beckman CX-5 Autoanalyzer. The sum of the cholesterol contents in the fractions corresponding to low density (LDL) and high density lipoprotein (HDL) can be demonstrated to be highly correlated to values obtained with dextran sulfate/MgCl<sub>2</sub> precipitation for HDLc (slope = 0.98,  $r^2$  = 0.997) and ultracentrifugation (beta-quant) for LDLc (slope = 1.03,  $r^2$  = 0.988). Using pure lipoprotein fractions isolated by ultracentrifugation, linear ranges of detection for HDLc and HDL apoA-I were performed at 18–95 mg/dl and 59–262 mg/dl, respectively. The ranges for LDLc were 41–435 mg/dl and 21–280 mg/dl for LDL apoB. The mean (range) fractional standard deviations for quadruplicate runs for 15 individual plasma samples ranging widely in lipoprotein concentrations were 0.97 (0.29–2.86%) for LDLc (range: 101.5–258.5 mg/dl), 3.67 (0.62–14.11%) for HDLc (range: 27.1–85.1 mg/dl) and 2.19 (0.16–6.56%) for VLDL-TG (range: 6.1–515.0 mg/dl).—**Innis-Whitehouse, W., X. Li, W. V. Brown, and N-A. Le.** An efficient chromatographic system for lipoprotein fractionation using whole plasma. *J. Lipid Res.* 1998. **39**: 679–690.

**Supplementary key words** fast phase liquid chromatography (FPLC) • lipid distribution • apolipoprotein distribution

Plasma lipoproteins represent a heterogeneous mixture of lipid-carrying particles varying in size and composition. Procedures for the fractionation of the lipoprotein classes in whole plasma depend either on their flotation characteristics or on their size, ranging from 8–15 nm for high density lipoprotein (HDL) to 50–100 nm for very low density lipoprotein (VLDL) and chylomicrons. Based on data using the analytical ultracentrifuge (1), three major density ranges have been accepted as representing the major classes of lipoproteins (1–3). Several ultracentrifugal procedures have been reported for the isolation of these major classes of lipoproteins,

including isopycnic ultracentrifugation (3), density gradient ultracentrifugation (2, 4), zonal ultracentrifugation (5), and, more recently, ultracentrifugation using the vertical rotor (6). All of these ultracentrifugal approaches require plasma samples that have never been frozen. With the exception of a modified protocol using the vertical rotor (7), most of these centrifugal procedures require approximately 1 to 3 ml of plasma for analysis.

Another disadvantage of the ultracentrifugal methods for the sequential isolation of the lipoprotein density classes is the possibility that certain apolipoproteins (apo), in particular apoCs and apoE, may be displaced from the surface of the lipoproteins with repeated centrifugation steps in the presence of a high salt concentration (8). In addition, apolipoprotein distribution may not correspond exactly to lipoprotein subpopulations defined by conventional density ranges. For instance, studies that examine the distribution of apoE among plasma lipoproteins separated by column chromatography have suggested that this apolipoprotein overlaps several of these density-defined lipoprotein classes (9, 10).

It has become increasingly important to be able to better describe the heterogeneity of plasma lipoproteins. While column chromatography using either 4 or 6% agarose has been demonstrated to be an effective approach to fractionating plasma lipoproteins (10), this procedure is time-consuming (36–48 h per sample) and very labor-intensive (30–40 fractions to be analyzed). As with ultracentrifugal procedures, the conventional column chromatographic protocol requires 2–3 ml of plasma per column run (10). Fast phase liquid

Abbreviations: FPLC, fast phase liquid chromatography; TG, triglycerides; CHOL, cholesterol; VLDL, very low density lipoproteins ( $d < 1.006$  g/ml); LDL, low density lipoproteins ( $d 1.006$ – $1.063$  g/ml); LDLc, LDL-cholesterol; HDL, high density lipoproteins ( $d 1.063$ – $1.21$  g/ml); HDLc, HDL-cholesterol.

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chromatography (FPLC), on the other hand, is based on the filtration property of pressure-packed Superose 6B and can be operated at pressures of up to 215 psi (11) thus allowing for more efficient (1–2 h) separation of macromolecules in the molecular weight range of 5,000 to  $5 \times 10^6$ .

In order to use the enzymatic methods available on the automatic chemistry analyzers, early reports on the FPLC used 1–2 ml of whole plasma (12, 13). With this volume of sample applied to the column, however, two Superose 6B columns had to be arranged in tandem to achieve complete resolution of the major lipoprotein classes. Consequently, a total of 30–40 fractions had to be collected and analyzed to obtain the complete profile (12, 13). When smaller plasma volumes (300–500  $\mu$ l) are applied to the column, lipid concentrations in the eluted fractions range from 1 to 10 mg/dl, a range which is typically too low for accurate determination using conventional automated enzymatic assays. By directly combining enzymatic reagents with the eluate in special heated coils, on-line enzymatic assay can be carried out providing a continuous tracing of the absorbance as a qualitative assessment of cholesterol distribution (14–17). With an electronically controlled splitter valve, the eluate can be divided into two lines for the simultaneous determination of triglyceride (TG) and cholesterol (CHOL) profiles (18). In such a system, the eluted fractions are not collected and multiple aliquots of the same plasma sample would have to be analyzed by FPLC to obtain the fractions for other measurements, e.g., apolipoprotein determinations. Anywhere from 1 to 1.5 ml of plasma could be required for multiple injections onto the FPLC system for the determination of various analytes.

In the present report we describe a modification of the standard enzymatic assays which allow the determinations of TG and CHOL in the ranges of 0.5–20 mg/dl by the CX-5 chemistry autoanalyzer (Beckman Diagnostics Instruments) using standard reagents (Beckman Diagnostics Instruments). These sensitive lipid assays allow the efficient analysis of lipid profiles from the elution of 200  $\mu$ l of whole plasma (a total volume of 300  $\mu$ l is required to allow for dead space). These fractions are also available for other determinations such as apolipoproteins by ELISA or free cholesterol and phospholipids.

## METHODS

### Equipment and materials

The FPLC system (Pharmacia LKB) consisted of a single Superose 6HR 10/30 column, one P-500 pump, a P-

100 peristaltic pump to load samples from an MV-8 eight-place multi-injection valve into a 200- $\mu$ l loop, a Super-Frac fraction collector, and a P-500 Plus controller.

### Buffers

The column was equilibrated in 50 mm phosphate-buffered saline (PBS, containing 0.1 m NaCl, 0.02%  $\text{NaN}_3$ , and 0.001 m EDTA, pH 7.4). The column was washed on a daily basis or after the injection of 8–10 plasma samples by programming the injection of two samples each of 0.01% Tween-20 followed by 20% ethanol, and discarding the 25 ml of the eluate with each injection. By incorporating the extensive washing procedure recommended by the manufacturer after every 30 injections, identical elution profiles can be obtained for up to 1 year or approximately 1,000 injections.

### FPLC conditions

A minimum sample volume of 300  $\mu$ l was required in the sampling vial for the automatic sampler to completely fill the 200- $\mu$ l sample loop. Excess sample volume would be shunted to the waste collection tray during the wash procedure and discarded prior to the injection of the next sample. Elution was performed in a phosphate-buffered saline solution containing 0.01% EDTA and 0.02%  $\text{NaN}_3$ , at a flow rate of 0.3 ml/min. Approximately 0.6 ml was collected for each fraction and 20 fractions were available for lipid and protein analysis. An entire lipoprotein profile (20 fractions) was completed within 40 min and the system was programmed for a 40-min wash cycle with the mobile phase prior to the injection of the next sample to minimize potential carry-over effects.

### Determination of low levels of cholesterol and triglycerides

For these determinations, user-defined channels were implemented on the Beckman CX-5 chemistry analyzer and programmed to pick up 25  $\mu$ l of sample for each lipid analysis instead of the standard 3  $\mu$ l volume commonly used for measurements in whole plasma. These dedicated low-range channels were calibrated with standards containing 30–35 mg/dl of CHOL and TG.

### Identification of the FPLC peaks

The cholesterol contents of the peaks corresponding to low density lipoprotein (LDL) and HDL were compared to CDC reference methods. The reference method for LDL consisted of the isolation of VLDL at density  $d < 1.006$  g/ml using the Optima tabletop ultracentrifuge (100,000 rpm for 2 h at 15°C). LDLc is defined as the difference between the cholesterol in the infranate ( $d > 1.006$  g/ml) and HDLc determined

using dextran sulfate/MgCl<sub>2</sub>. All analyses were performed in the Emory Lipid Research Laboratory, a participant in the CDC/NHLBI Lipid Standardization Program. Lipid determinations in the individual fractions can be imported from the autoanalyzer as an EXCEL spreadsheet and the lipid contents for VLDL can be calculated automatically by summing the contents of fractions 4 through 8 (elution volume 7.2–9.6 ml), fractions 9 through 16 for LDL (elution volume 10.2–14.4) and fractions 17–23 for HDL (elution volume 15–18.6).

However, for the analysis of severely lipemic samples with high concentrations of remnant lipoproteins and samples with high concentrations of Lp[a], resolution between the VLDL and LDL peaks could not be obtained by inspection of the lipid profiles. For the analysis of these more complex elution profiles, we have used the computer program PeakFit (Jandel Scientific, San Rafael, CA) to fit the elution profile to a series of chromatographic distribution curves. The lipid contents associated with each peak were calculated as the product of the percent area under the curve and the appropriate plasma concentration. This program requires considerable user guidance to identify the position and width of the peaks before the iterative process can be initiated. For the majority of the plasma samples from free-living individuals, however, this step was not found to be necessary.

#### **Reproducibility of the elution profile and lipoprotein lipids**

To examine the reproducibility of the lipoprotein fractionation procedure using the FPLC, four aliquots of plasma from the same donor were analyzed by FPLC on successive days. Plasma samples were obtained from a group of normal, hypertriglyceridemic, and hypercholesterolemic subjects ranging in TG from 44 to 446 mg/dl and in CHOL from 166 to 308 mg/dl. Lipid contents in each of the three major lipoprotein classes were defined as the sum of the lipid concentrations in the eluted fractions corresponding to VLDL (fractions 4–8), LDL (fractions 9–16), and HDL (fractions 17–23).

The reproducibility of the FPLC procedure was also examined by applying different concentrations of purified lipoproteins isolated by ultracentrifugation, LDL: 1.019–1.063 g/ml and HDL: 1.063–1.021 g/ml. For LDL, fractions 9–16 were pooled and assayed for CHOL and apoB. For HDL, fractions 17–23 were pooled and assayed for CHOL and apoA-I. Apolipoprotein levels were determined by sandwich ELISA using polyclonal antibodies isolated by immunoaffinity chromatography.

#### **Effect of lipid contents on recovery**

To examine the recoveries of TG and CHOL after the FPLC procedure, freshly isolated plasma samples

from human subjects with TG ranging from 55 to 840 mg/dl and CHOL ranging from 100 to 295 mg/dl were analyzed by FPLC. The sum of the lipid masses (concentration  $\times$  0.6 ml/fraction) in all 20 fractions collected (fractions 4–23) was compared to the total lipid mass in the 200  $\mu$ l of plasma injected.

#### **Effect of sample storage**

The effect of storage was examined in a series of samples that had been stored at  $-80^{\circ}\text{C}$  for 1 year. The samples were obtained from patients with primary hypertriglyceridemia who had participated in a multicenter trial. LDLc in these samples were determined by ultracentrifugation (beta-quant) within 24 h of blood collection and HDLc were determined by dextran sulfate/MgCl<sub>2</sub> at the Core Laboratory for Clinical Trials (Washington University, St. Louis, MO). Total plasma lipids were re-measured when the samples were thawed for FPLC analysis. LDLc and HDLc as determined by FPLC were obtained by multiplying the fraction of the cholesterol associated with LDLc ( $\Sigma[\text{Fr. 9–16}] / \Sigma[\text{Fr. 4–23}]$ ) by the total plasma CHOL. Similarly, HDLc were defined as the product of the total CHOL and the fraction of the cholesterol eluted in fractions 17–23, inclusively, corresponding to HDL ( $\Sigma[\text{Fr. 17–23}] / \Sigma[\text{Fr. 4–23}]$ ). In this experiment, two separate samples were available from each participant, one at baseline and one after treatment with either a high (80 mg/day) or moderate (20 mg/day) dose of atorvastatin, a new HMG-CoA reductase inhibitor (18). Plasma TG ranged from 240 to 2250 mg/dl in these samples.

#### **Effect of remnant lipoproteins and Lp[a] on the FPLC elution profile**

The ability of the FPLC procedure to estimate lipoprotein lipids from more complex profiles was examined using plasma samples from individuals with elevated Lp[a]. To examine the ability of the FPLC procedure to resolve remnant lipoproteins and/or  $\beta$ -VLDL, we used plasma samples from a group of New Zealand white rabbits that had been maintained on an atherogenic diet (0.5% coconut oil and 1 mg/kg cholesterol) for up to 35 days.

## **RESULTS**

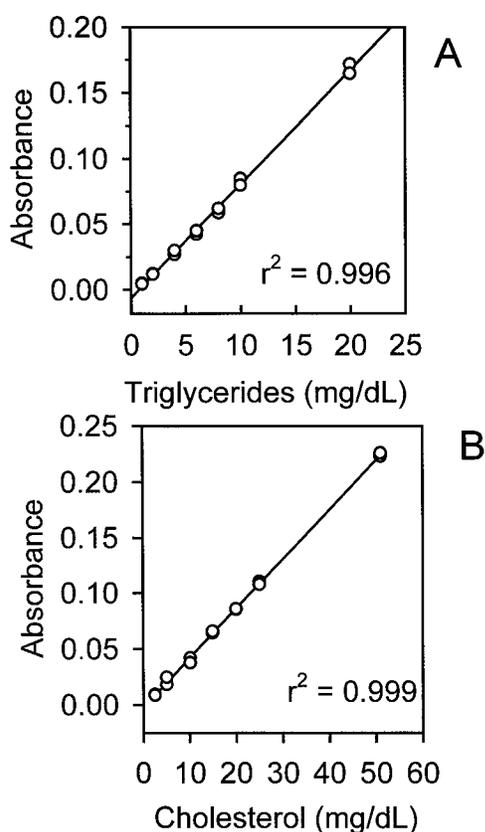
#### **Determination of low concentrations of TG and CHOL by Autoanalyzer**

Using the sensitive channels defined on the CX-5 chemistry analyzer with standard Beckman reagents for TG and CHOL, the coefficient of correlation between

the absorbance and TG concentrations was 0.998 for TG ranging from 0.4 to 25 mg/dl (Fig. 1A). Similarly, the coefficient for the linear regression between the absorbance and CHOL concentrations was 0.999 for CHOL concentrations ranging from 0.2 to 50 mg/dl. Table 1 presents the inter- and intra-assay CV for two plasma pools with low lipid concentrations as determined by the CX-5 chemistry analyzer using the sensitive channels for TG and CHOL.

### Identification of the elution profile

The cholesterol peaks corresponding to LDL and HDL can be confirmed by several methods. First, the eluted fractions were assayed for apoB and apoA-I in order to identify the fractions corresponding to LDL and HDL, respectively. Figure 2 and Figure 3 illustrate the elution profiles for CHOL (Figs. 2A and 3A), TG (Figs. 2B and 3B), apoB (Figs. 2C and 3C), and apoA-I (Figs. 2D and 3D) for two hyperlipidemic individuals. Figure 2 represents the FPLC profile for a hypercholesterolemic individual with CHOL of 266, TG of 71, and HDLc of 67 mg/dl. The plasma apoA-I and apoB con-



**Fig. 1.** Determination of low concentrations of TRIG and CHOL. Linearity between absorbance readings (520 nm) and TRIG and CHOL concentrations using serial dilutions of whole plasma.

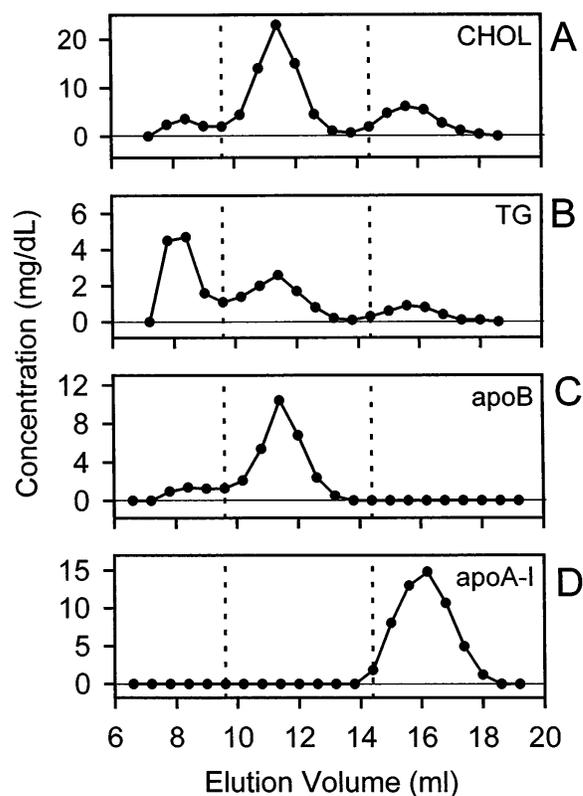
**TABLE 1.** Reproducibility of lipid determinations using the sensitive channels

	Inter-Assay	Intra-Assay
	mg/dl	
TG		
Low	4.7 ± 0.07	5.3 ± 0.06
High	14.8 ± 0.1	14.8 ± 0.1
CHOL		
Low	3.6 ± 0.13	3.6 ± 0.12
High	26.8 ± 0.3	26.8 ± 0.2

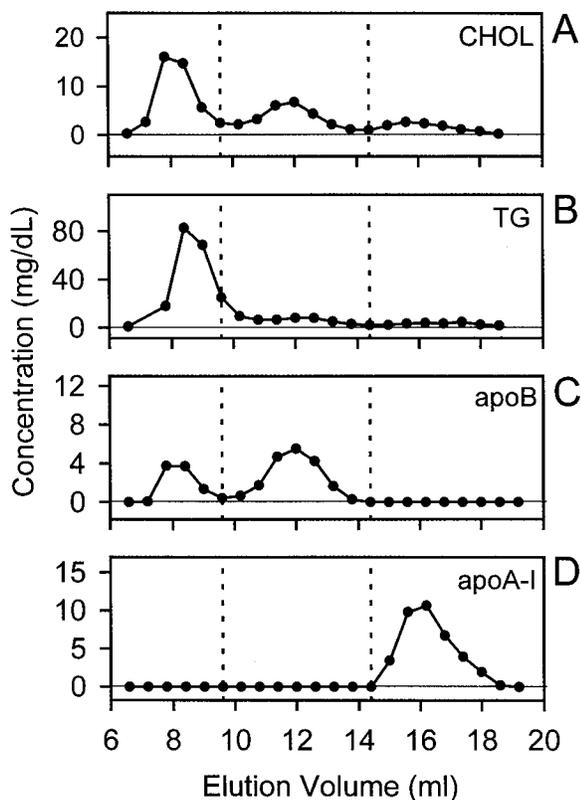
Results are mean ± SD from 20 determinations each day for two control samples over a period of 30 days.

centrations in this sample were 159.7 and 104.5 mg/dl, respectively. Figure 3 represents the FPLC profile for a hypertriglyceridemic subject (CHOL = 293, TG = 1030, HDLc = 42, apoA-I = 113.0, and apoB = 98 mg/dl).

Second, estimates for LDLc and HDLc obtained by FPLC were compared with values obtained by ultracentrifugation for LDLc and by dextran sulfate/MgCl<sub>2</sub> pre-



**Fig. 2.** FPLC elution profiles for a representative plasma sample from a hypercholesterolemic individual as assessed by cholesterol (panel A), triglycerides (panel B), apoB (panel C), and apoA-I (panel D). The elution volume for VLDL ranges from 7.2 to 9.6 ml, from 9.6 to 14.4 ml for LDL, and from 14.4 to 19.2 ml for HDL. The lipid profile for this individual was TG = 71, CHOL = 266, HDLc = 67, LDLc = 172, apoB = 104.5, and apoA-I = 159.7 mg/dL.



**Fig. 3.** FPLC elution profiles for a representative plasma sample from a hypertriglyceridemic individual as assessed by cholesterol (panel A), triglycerides (panel B), apoB (panel C), and apoA-I (panel D). The lipid profile for this individual was TG = 1030, CHOL = 293, HDLc = 42, LDLc = 55, apoB = 98.0, and apoA-I = 113.0 mg/dL.

cipitation for HDLc. A high correlation was obtained between LDLc determined by the ultracentrifuge method (beta-quant) and the sum of the cholesterol masses (concentration  $\times$  0.6 ml/fraction) in fractions 9–16 defined as LDL (Fig. 4A). The slope of the linear regression was 1.026 with an intercept of 9.70 mg/dl ( $r^2 = 0.988$ ). The mean ( $\pm$ SD) difference between the two estimates was  $-13.25 (\pm 8.83)$  mg/dl. Of the 20 samples analyzed with these two methods, only 5 (25%) had differences outside 1 SD and only 1 sample (5%) had a difference greater than 2 SD of the mean difference (Fig. 4B). For the same set of samples, the slope of the linear regression between the estimates of HDLc by FPLC and the values by precipitation was 0.98 with a Y-intercept of 0.76 mg/dl ( $r^2 = 0.997$ ). The range of HDLc in these samples was 40–60 mg/dl (data not shown).

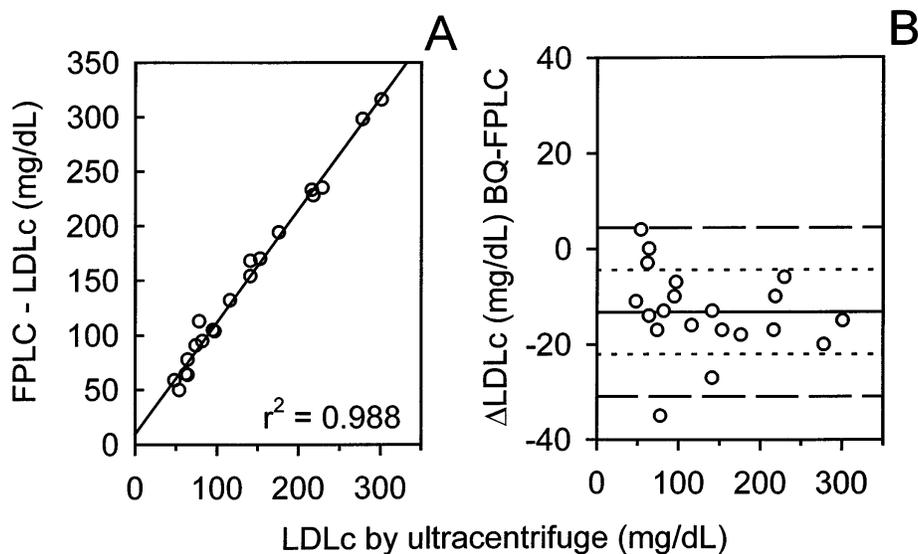
To demonstrate that the relationship between the two estimates of HDLc (FPLC vs. precipitation) remained significant for a wider range of total cholesterol and HDLc values, we used plasma samples from a large

number of New Zealand white rabbits ( $n = 75$ , Fig. 5). Total cholesterol in these samples obtained ranged from 40 mg/dl at baseline to 1100 mg/dl after 35 days on the atherogenic diet and HDLc ranged from 12 to 65 mg/dl. In this comparison between the two estimates, each animal only contributed one data point, either the baseline or the post-diet sample, and all samples were analyzed within 24 h of blood collection. In the analysis of these severely hypercholesterolemic samples, HDLc was calculated as the product of the total CHOL by the % area under the HDL peak as determined by PeakFit (Fig. 5A). The slope of the linear regression was 0.965 with an intercept of 1.16 mg/dl ( $r^2 = 0.87$ ). The mean ( $\pm$ SD) difference between the two estimates was  $-0.125 (\pm 3.88)$  with 12% of the samples (9 out of 75) falling outside 1 SD and 6.7% (5 out of 75) falling outside 2 SD (Fig. 5B).

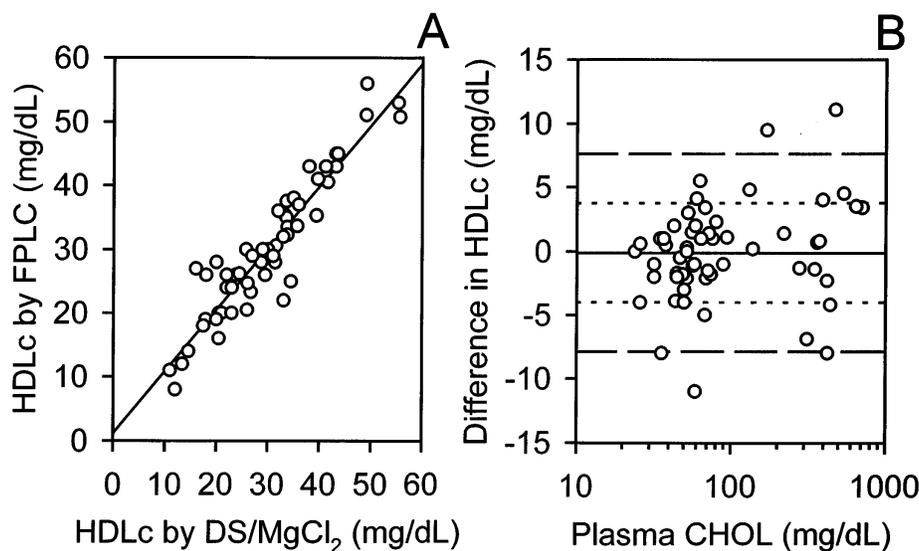
Last, by analyzing the pooled fractions defined as LDL (fractions 9–16, elution volume 9.6–14.4 ml) and HDL (fractions 17–23, elution volume 15.0–18.6 ml) for apoB and apoA-I, we demonstrated that all of the LDL and HDL apolipoproteins can be recovered. For this experiment LDL was isolated by sequential ultracentrifugation ( $d 1.019$ – $1.063$  g/ml) from pooled human plasma, dialyzed back to plasma density and diluted with saline (1.006 g/ml) to achieve a wide range of LDLc concentrations (50–450 mg/dl). These samples were applied to the FPLC which was programmed to collect a single pooled fraction corresponding to the elution range from 9.6 to 14.4 ml, i.e., fractions 9 through 16. This single fraction was subsequently assayed for cholesterol and apoB and Fig. 6A presents the linear regression between the injected LDLc and the recovered LDLc which had a slope of 0.960 and an intercept of  $-14.3$  ( $r^2 = 0.985$ ). The slope for the linear regression between the injected LDL apoB and the recovered LDL apoB was 1.039 with an intercept of  $-4.09$  ( $r^2 = 0.971$ , Fig. 6B). Similarly, HDL was isolated by sequential ultracentrifugation ( $d 1.063$ – $1.21$  g/ml), dialyzed back to 1.006 g/ml, and diluted with saline to achieve a range of HDLc from 20 to 95 mg/dl. The FPLC was programmed to collect a single pooled fraction eluting between 15.0 and 18.6 ml which was subsequently analyzed for cholesterol and apoA-I. The slopes for the linear regressions were 1.006 for cholesterol ( $r^2 = 0.990$ , Fig. 6C) and 0.980 for apoA-I ( $r^2 = 0.985$ , Fig. 6D). The values for the Y-intercept were  $-6.0$  and  $-4.6$  mg/dl for HDLc and HDL apoA-I, respectively.

#### Reproducibility of the lipoprotein composition as isolated by FPLC

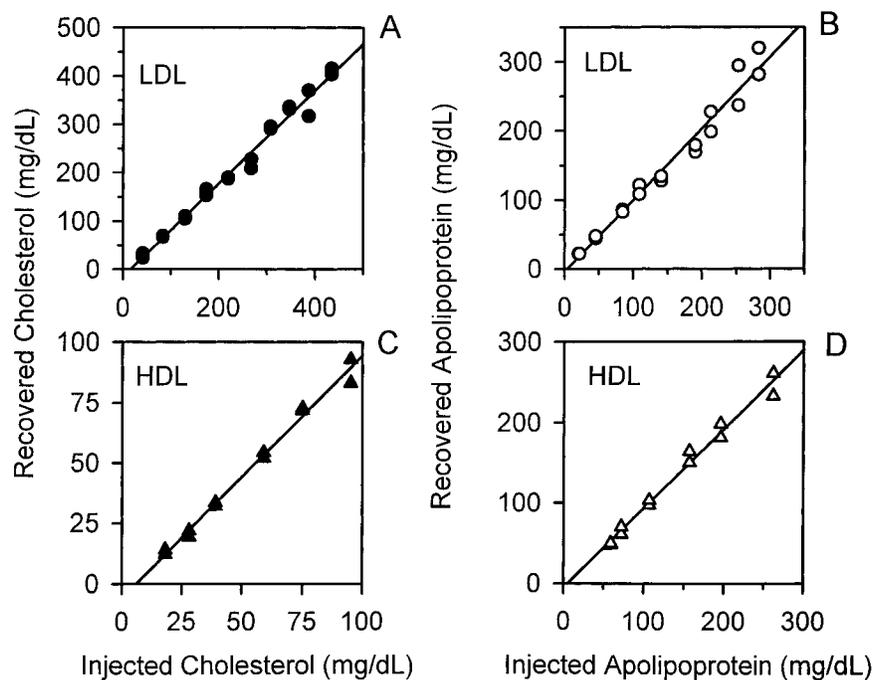
Table 2 presents the reproducibility for VLDL, LDL, and HDL lipids based on 4–6 separate injections of plasma samples from normolipidemic controls, patients



**Fig. 4.** Comparison of estimates for LDLc as determined by ultracentrifugation and by FPLC using freshly collected plasma samples from a group of volunteers with total cholesterol ranging from 130 to 495 mg/dL. Panel A: The linear regression between the two estimates had a slope of 1.026 with the estimates from the FPLC being slightly higher than that by ultracentrifugation ( $r^2 = 0.988$ , intercept = 9.7 mg/dL). Panel B: The net difference between the two estimates was plotted as a function of LDLc as determined by the ultracentrifuge method. The mean ( $\pm$ SD) difference in the two estimates was 13.25 ( $\pm$ 8.83) mg/dL for this small number of samples with LDLc ranging from 50 to 300 mg/dL. The difference was not statistically different by two-tail paired *t*-test.



**Fig. 5.** Comparison of the estimates of HDLc as determined by dextran sulfate/MgCl<sub>2</sub> precipitation and by FPLC using freshly isolated plasma samples from New Zealand White rabbits that have been maintained either on chow or on a high-fat/high-cholesterol regimen. Total cholesterol in these animals ranged from 40 to 1100 mg/dL. For all of these samples HDLc was calculated as the product of the % area under the HDL peak as defined by PeakFit and the total plasma cholesterol. Panel A: The slope of the linear regression was 0.965 ( $r^2 = 0.995$ , intercept = 1.16 mg/dL) with the FPLC estimates being lower than those from the precipitation method. Panel B: The mean ( $\pm$ SD) difference between the two estimates was  $-0.125 (\pm 3.88)$  and there was no statistical difference between the two estimates as assessed by two-tail paired *t*-test.



**Fig. 6.** FPLC elution of individual lipoprotein classes isolated by ultracentrifugation. LDL and HDL were isolated by sequential ultracentrifugation in the density range  $d$  1.019–1.063 g/ml and  $d$  1.063–1.21 g/ml, respectively. Different amounts of LDLc and HDLc in 200  $\mu$ l of sample were injected on the FPLC in duplicates. With each run, the FPLC was programmed to collect only one fraction corresponding to either an elution volume from 9.6 to 14.4 ml for LDLc or an elution volume from 14.4 to 18.0 ml for HDLc. Panel A: Linear regression between the injected LDLc and the recovered LDLc after FPLC ( $r^2 = 0.985$ ). Panel B: Linear regression between injected LDL apoB and recovered LDL apoB in the FPLC fraction collected ( $r^2 = 0.971$ ). Panel C: Linear regression between injected HDLc and recovered HDLc ( $r^2 = 0.990$ ). Panel D: Linear regression between injected HDL apoA-I and apoA-I in the FPLC fraction collected ( $r^2 = 0.985$ ).

with hypercholesterolemia, and patients with hypertriglyceridemia. For all of the samples used in this analysis, lipoprotein lipids were obtained by summing the lipid concentrations in the eluted fractions defined as VLDL (fractions 4–8), LDL (fractions 9–16), and HDL (fractions 17–23). The program PeakFit was not used for any of these analyses. The standard deviations for lipid measurements in all fractions averaged 3.5%, with a range of 0.7 to 5.6%.

#### Effect of lipemia on lipid recoveries

To rule out the possibility that large particles in hypertriglyceridemic samples might be selectively excluded from the FPLC thus resulting in poor recovery of lipids, we analyzed a series of samples ranging in TG from 55 to 840 mg/dl and from 110 to 295 mg/dl in CHOL. **Figure 7** illustrates the relationship between the lipid concentrations in whole plasma and the sums of the lipid concentrations in the 20 collected FPLC fractions. The slope of the linear regression for TG (Fig. 7A) was 0.98 with an intercept of 5.71 mg/dl ( $r^2 =$

0.995). The mean ( $\pm$ SD) fraction recovered (Fig. 7B) was 1.013 ( $\pm$ 0.072). The slope of the linear regression for CHOL (Fig. 7C) was 0.987 with an intercept of 4.38 mg/dl ( $r^2 = 0.970$ ). The mean ( $\pm$ SD) fraction recovered (Fig. 7D) was 0.990 ( $\pm$ 0.034).

#### Effect of sample storage

The samples used for this study were stored frozen at Washington University for approximately 13 months and shipped to Emory University for FPLC analysis. All samples were allowed to thaw in the refrigerator and total plasma CHOL were re-determined prior to their application on the FPLC. For these samples, the estimates of LDLc and HDLc by FPLC were calculated from the percent distribution (see Methods) and the newly determined values for total cholesterol. PeakFit was not required for the analysis of these elution profiles.

**Figure 8A** illustrates the linear regression of the estimates of LDLc determined by FPLC using frozen samples and values of LDLc measured by ultracentrifugation using fresh samples with TG ranging from 150 to

TABLE 2. Reproducibility of VLDL, LDL, and HDL lipids for normolipidemic, hypercholesterolemic, and hypertriglyceridemic plasma samples as assessed by FPLC fractionation

Subject	TG	CHOL	VLDLtg	VLDLc	LDLtg	LDLc	HDLtg	HDLc
	<i>mg/dl</i>		<i>mean (±SD) in mg/dl</i>					
1	44	197	6.1 (0.4)	nd	36.1 (1.0)	111.9 (1.2)	1.8 (1.4)	85.1 (1.2)
2	62	158	21.5 (0.8)	1.5 (1.3)	32.5 (1.1)	115.6 (0.9)	7.9 (1.3)	40.9 (0.5)
3	81	321	23.9 (0.6)	3.3 (1.2)	49.2 (0.4)	240.6 (0.7)	7.9 (0.4)	77.1 (1.5)
4	85	202	7.7 (0.6)	29.9 (1.0)	37.8 (0.5)	128.8 (0.4)	17.4 (0.7)	65.6 (0.4)
5	91	201	51.5 (0.8)	8.2 (0.6)	33.3 (1.0)	159.0 (1.5)	6.3 (0.3)	33.5 (1.7)
6	105	331	27.9 (1.7)	8.2 (0.5)	63.4 (0.7)	258.5 (1.8)	13.7 (1.5)	64.3 (2.1)
7	110	210	44.1 (0.7)	2.2 (0.4)	44.1 (0.4)	127.0 (1.0)	21.8 (0.4)	80.8 (1.1)
8	120	308	57.7 (0.8)	11.7 (0.5)	55.1 (0.7)	246.0 (1.2)	13.7 (1.5)	64.3 (2.1)
9	215	258	152.5 (0.9)	23.8 (0.3)	42.7 (0.5)	189.3 (1.1)	19.8 (0.9)	45.0 (1.1)
10	271	256	205.5 (1.4)	41.6 (0.5)	56.7 (1.2)	182.3 (3.0)	8.8 (0.2)	32.2 (3.4)
11	322	251	238.7 (3.9)	33.2 (2.2)	69.5 (3.8)	165.7 (2.9)	13.8 (1.3)	52.1 (1.1)
12	353	166	258.9 (3.4)	39.7 (0.7)	71.8 (2.2)	101.5 (2.9)	22.3 (1.7)	24.8 (3.5)
13	387	277	293.6 (2.2)	73.5 (0.9)	75.0 (1.6)	168.0 (0.7)	18.5 (1.1)	35.5 (0.6)
14	394	217	311.0 (2.2)	50.3 (0.7)	64.8 (1.2)	131.5 (1.2)	18.2 (1.2)	35.5 (1.1)
15	446	211	345.7 (6.1)	64.3 (1.4)	80.5 (3.4)	119.6 (1.3)	19.9 (3.0)	27.1 (0.8)

Four to six separate FPLC runs were available for each plasma sample over a period of 2–3 days. The lipid contents were determined as the sum of the contents in the designated eluted fractions and PeakFit was not used for any of these runs.

2500 mg/dl. The mean ( $\pm$ SD) ratio of the two estimates (Fig. 8B) for LDLc was 1.022 ( $\pm$ 0.116). When baseline samples with higher TG were excluded from the analysis, the two estimates for LDLc after therapy with atorvastatin (21) were more comparable and the fractional standard deviation ( $=$ SD/mean) for the ratio was reduced from 11.35% to 5.7%.

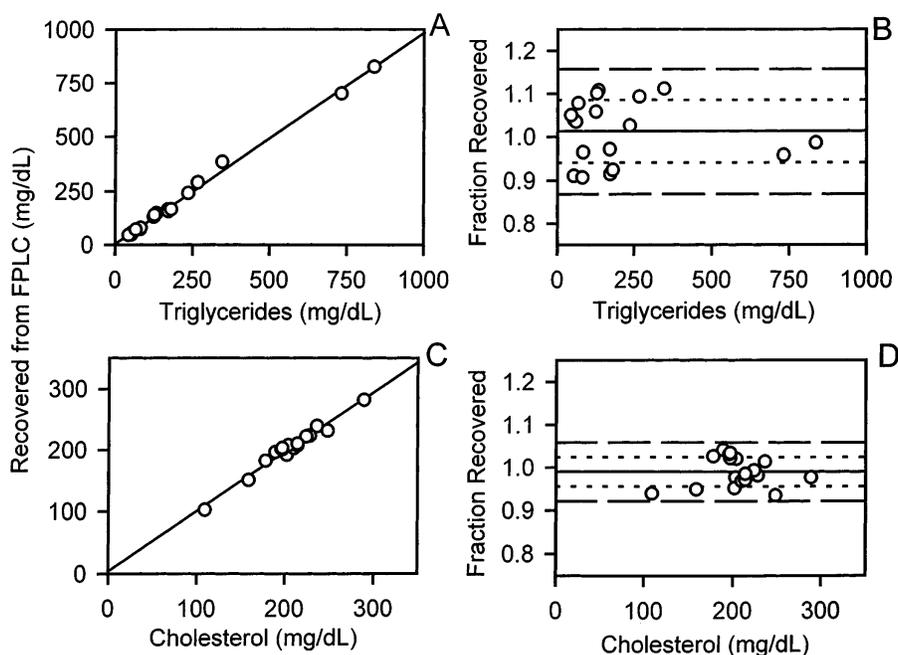
The linear regression between the two estimates of HDLc, FPLC in frozen plasma versus precipitation in fresh plasma, had a slope of 0.79 ( $r^2 = 0.48$ ). The mean ( $\pm$ SD) ratio of the two estimates was 1.099 ( $\pm$ 0.218) when all data points were used. By using only the post-treatment plasma samples with significantly lower TG levels, the difference between the two estimates was much less with a mean ( $\pm$ SD) ratio of only 1.028 ( $\pm$ 0.148). The slope of the regression line based on the post-treatment samples was 0.84 ( $r^2 = 0.68$ ).

#### FPLC analyses of special samples with complex lipoprotein profiles

Figure 9A illustrates representative FPLC elution profile for a plasma sample obtained from a patient with

elevated Lp[a]. The lipid concentrations in this sample were TG = 242, CHOL = 200, HDLc = 33, and Lp[a] = 158.2 mg/dl. Using PeakFit, we can resolve two peaks between fractions 8 and 16 of the elution profile. This corresponded to an Lp[a]-cholesterol of 43 mg/dl and LDLc of 102 mg/dl as compared to an LDLc estimate of 134 mg/dl by ultracentrifugation. The fractions designated as Lp[a] contained Lp[a] as confirmed by ELISA and three of the five fractions had a constant Lp[a]-to-apoB ratio (data not presented). Additional studies would be required to fully characterize the nature of this peak and the ability to determine Lp[a]-cholesterol with this method.

Figures 9B and 9C present the FPLC elution profile for plasma samples obtained from a New Zealand White rabbit at baseline (Fig. 9B) and after 5 weeks (Fig. 6D) on an atherogenic diet. The deconvoluted curves were obtained using the PeakFit program. By multiplying the percent area under each of these peaks by the total plasma cholesterol, cholesterol concentrations in VLDL, remnants, LDL, and HDL can be calculated.



**Fig. 7.** The effect of different degrees of lipemia in whole plasma was examined by comparing the total lipid recovered and the amount of lipid applied to the columns for a set of plasma samples with TG ranging from 50 to 850 mg/dL and CHOL ranging from 110 to 295 mg/dL. Panel A: The slope of the linear regression between the expected and observed TG concentrations was 0.98 ( $r^2 = 0.995$ ). Panel B: The mean ( $\pm$ SD) ratio of the two concentrations was 1.03 ( $\pm 0.072$ ). Panel C: The slope of the linear regression between the expected and the observed CHOL concentrations was 0.97 ( $r^2 = 0.98$ ). Panel D: The mean ( $\pm$ SD) ratio of the two concentrations was 0.990 ( $\pm 0.034$ ).

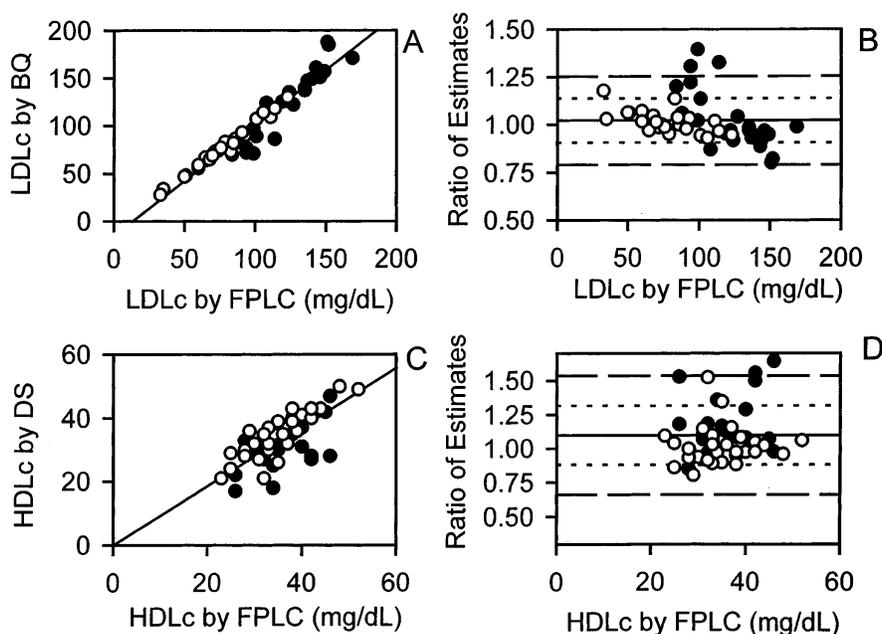
## DISCUSSION

The present report describes a semi-automated procedure for the isolation of plasma lipoproteins by column chromatography using the FPLC. Two major improvements of existing FPLC methodology are incorporated in the present report. First, we have validated sensitive automated enzymatic methods for the determination of TG and CHOL levels in the eluted fractions from only 200  $\mu$ l of plasma (the minimum volume required for each injection is 300  $\mu$ l to allow for dead space). By eliminating the necessity of using on-line enzymatic assays (16, 17), our procedure allows the potential analysis of other components in the lipoprotein fractions, e.g., apolipoproteins, phospholipids, free and esterified cholesterol. Second, we have demonstrated that the cholesterol contents of LDL and HDL as determined by FPLC are highly correlated to values obtained by the ultracentrifuge method for LDLc (beta-quant) and by precipitation with dextran sulfate/MgCl<sub>2</sub> for HDLc. This is consistent with the results of Marz et al. (17) using on-line enzymatic determination. Furthermore, by demonstrating that the lipid and apolipoprotein distribution among the FPLC fractions is stable in plasma samples that have been previously frozen

at  $-80^{\circ}\text{C}$  for extended periods of time, we have validated an efficient method for the study of lipoprotein composition in small volumes of frozen plasma samples.

Approximately 75–105% of the injected TG was recovered in the eluted FPLC fractions when freshly isolated plasma samples with TG ranging from 50 to 1800 mg/dl were analyzed. When samples containing CHOL in the range of 50 to 500 mg/dl were fractionated by FPLC, the recoveries of CHOL in the eluted fractions ranged from 85 to 102%. For most of these freshly collected plasma samples, lipoprotein lipids can be estimated simply by adding the lipid contents in the corresponding fractions (Table 2 and Figs. 4 and 5).

This was not the case with frozen samples, however. In our hands, the lipid recoveries in samples that have been kept at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  for more than 3 months are always lower. The distribution of the lipids among the different lipoprotein fractions was not affected, however. This was demonstrated by the high correlation between the FPLC estimates of LDLc and HDLc in frozen samples and the values obtained by ultracentrifuge and precipitation in fresh plasma (Fig. 8). For this comparison, the percent distributions of cholesterol were multiplied by the total plasma cholesterol to obtain the cholesterol contents in each lipoprotein fraction. We

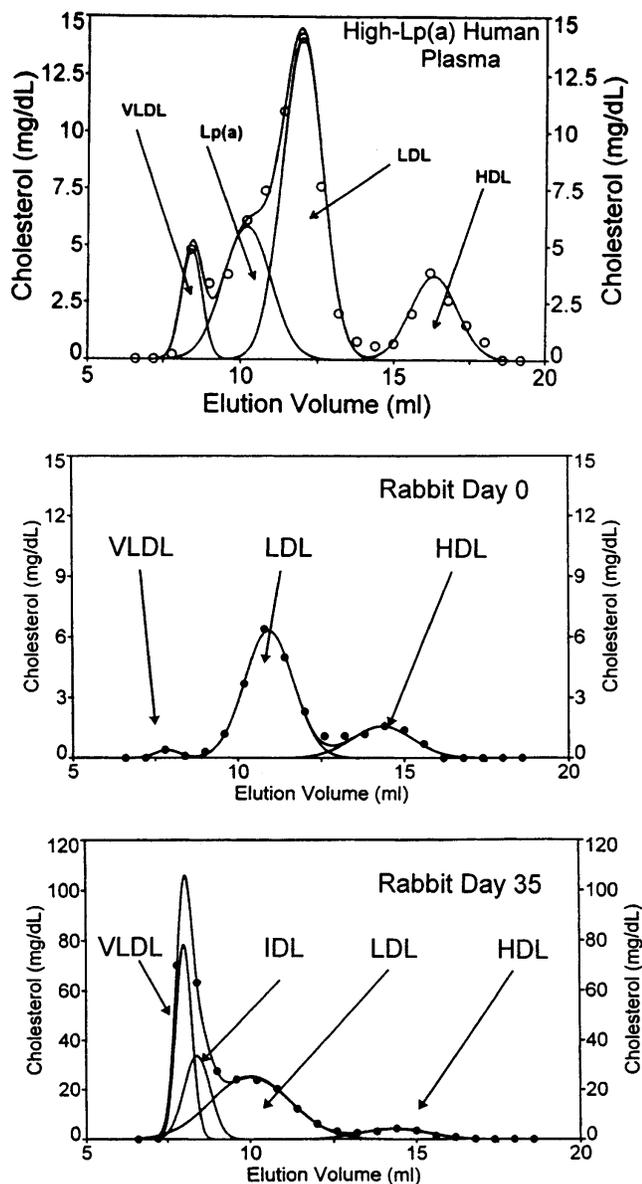


**Fig. 8.** Effect of storage at  $-80^{\circ}\text{C}$  on the estimates of LDLc and HDLc by FPLC. Plasma samples from 27 individuals with primary hypertriglyceridemia were analyzed at baseline (closed circles, TG: 450–2200 mg/dL) and after 2 weeks of therapy with atorvastatin (open circles, TG: 135–400 mg/dL). LDLc and HDLc were initially determined by ultracentrifugation and precipitation, respectively, using fresh plasma. Aliquots of these samples were thawed after 13 months of storage at  $-80^{\circ}\text{C}$  and analyzed by FPLC. Panel A: The slope of the linear regression between the two estimates was 1.16 ( $r^2 = 0.93$ ) when both the baseline and the post-therapy samples were used. When only the post-therapy samples which had lower TG were compared, the slope of the regression was 1.09 ( $r^2 = 0.98$ ). The values for the Y-intercept were  $-15.1$  and  $-7.3$  mg/dL, respectively. Panel B: The mean ( $\pm$ SD) ratio of the two estimates was 1.022 ( $\pm 0.116$ ) when samples from both visits were included in the analysis and 1.016 ( $\pm 0.058$ ) when only the post-therapy samples were used. Panel C: The slope of the linear regression between the two estimates for HDLc was 0.79 ( $r^2 = 0.48$ ) when samples from both visits were included in the analysis and 0.84 ( $r^2 = 0.62$ ) when only the estimates from the post-treatment samples were compared. Panel D: The mean ( $\pm$ SD) ratio of the two estimates was 1.099 ( $\pm 0.218$ ) when all samples were included in the analysis and 1.025 ( $\pm 0.148$ ) when only the post-treatment samples were compared.

believe that given the low-pressure system available for sample pick-up and injection with the present FPLC model, differences in either the viscosity of the samples or the protein concentration may affect the actual sample volume delivered by the pump to the column. Extended storage at freezing temperature could potentially affect either or both of these properties. Unless an internal standard is available to calibrate the exact volume of sample applied to the column, the use of the percent distribution and a determination of concentration in whole plasma would be the best approach to estimate the composition of the lipoprotein fractions isolated by FPLC. A similar use of the percent distribution and the total concentration in plasma to estimate lipoprotein contents was also recently described by other investigators to assess the contents of apoE and apoC in plasma lipoproteins after fractionation by FPLC (18).

In the estimation of the lipid concentration in the in-

dividual lipoprotein peaks, we have demonstrated that for the majority of the plasma samples, a sum of the lipid contents in the respective fractions was adequate. This is true even for samples ranging widely in TG and CHOL levels. When the concentrations of Lp[a] (Fig. 9A) or of  $\beta$ -VLDL (cholesterol-fed rabbits, Fig. 9B and 9C) are excessively elevated, distinct resolution of the major peaks is not possible (i.e., lipid contents did not return to zero between peaks), curve peeling programs such as PeakFit would be recommended to resolve the lipoprotein peaks. While we could propose to routinely use PeakFit for the analysis of all FPLC runs, this procedure would require considerable interaction with the technician to identify the peaks before the program could start the iteration process. We believe that to require the use of such a curve-fitting program may discourage the use of the FPLC to determine VLDL and LDL composition in frozen plasma.



**Fig. 9.** Identification of the lipoprotein classes by deconvolution of the FPLC elution profile using PeakFit. Panel A: The elution profile of the plasma from an individual with elevated Lp[a] (169 mg/dL) demonstrated a characteristic shoulder on the left of the LDL peak. Panel B: Elution profile for a New Zealand White rabbit maintained on normal rabbit chow demonstrated that the majority of the cholesterol was in the LDL fraction. Panel C: After 35 days on an atherogenic diet consisting of 1% cholesterol and 3% coconut oil, the majority of plasma cholesterol was associated with VLDL and IDL. In contrast to Lp[a], the IDL or  $\beta$ -VLDL peak was slightly smaller than VLDL and appeared as a shoulder to the right of the VLDL peak.

The sensitivity and resolution of this method can be useful in several applications. With the increasing use of the transgenic and knockout mouse models to study atherosclerosis, the ability to fractionate lipoprotein

classes from small volumes of whole plasma is critical (16). With sensitive enzymatic methods for lipid determination using the autoanalyzer, on-line assays will not be necessary and several other measurements can be performed in each eluted fraction. By collecting the actual eluted fractions it would also be possible to examine the apolipoprotein composition of the eluted fractions (18). ■

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