methods

The differential apoA-I enrichment of preβ$_1$ and αHDL is detectable by gel filtration separation

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Abstract The aim of the study was to assess the isolation of HDL by fast protein liquid chromatography (FPLC) to perform kinetics studies of apolipoprotein (apo)A-I-HDL labelled with a stable isotope. Comparison between FPLC and ultracentrifugation has been made. ApoA-I-HDL kinetics were studied by infusion of [5.5.5-$^2H_3$]leucine for 14 h in five subjects. Using FPLC, preβ$_1$ HDL and αHDL (HDL$_2$ and HDL$_3$) were separated from 200 μl of plasma samples. Total HDL was isolated by sequential ultracentrifugation (HDL-UC). The tracer-to-tracee ratio was higher in preβ$_1$ HDL than in total HDL-UC. The higher leucine enrichment found in total HDL-UC compared to αHDL suggested the existence of a mixture of apoA-I-HDL sub-classes. From this difference in enrichments, the turnover rate of total HDL-UC, usually assumed to be αHDL, was probably overestimated in previous studies. To our knowledge, this study is the first report which provides a convenient tool to distinguish enrichments of apoA-I in preβ$_1$ HDL and αHDL from total HDL previously used for kinetic measurements. This original and new method should help to understand the kinetics of HDL in humans and the reverse cholesterol transport dynamics.—Chétiveaux, M., H. Nazih, V. Ferchaud-Roucher, G. Lambert, Y. Zaïr, M. Masson, K. Ouguerram, D. Bouhours, and M. Krempf. **The differential apoA-I enrichment of preβ$_1$ and αHDL is detectable by gel filtration separation. *J. Lipid Res.* 2002.** 43: **1986–1993.**

Supplementary key words fast protein liquid chromatography • apolipoprotein A-I • kinetic analysis

Plasma HDL and apolipoprotein A-I (apoA-I) concentrations are inversely correlated with the risk of coronary heart disease (1, 2). Kinetics studies are a good tool for a better understanding of the role that HDL play in the reverse cholesterol transport. Preβ$_1$ HDL are considered as the initial acceptor of cellular cholesterol and are transformed into spherical HDL$_3$ as a result of the LCAT activity. Nascent discoidal preβ$_1$ HDL are either directly synthesized by the liver and the intestine or generated when other classes of lipoproteins including αHDL are remodeled by hepatic lipase (3). In plasma, preβ$_1$ HDL represented 5.5 ± 3.3% and 7.2 ± 4% of total apoA-I in women and men, respectively (4).

ApoA-I, a major protein component of HDL, could be endogenously labeled using stable isotopes to study HDL metabolism in humans (5). Plasma lipoprotein and HDL are usually separated by ultracentrifugation based on differential flotation using either sequential (6) or one-step gradient methods (7). It appears that the combination of high salt concentrations and high centrifugal forces may cause structural damages to the lipoproteins (8) that could change results of kinetics studies (9). Alternatively, plasma lipoproteins may also be separated by size-exclusion chromatography on agarose. Fast Protein Liquid Chromatography (FPLC), based on the gel filtration property, is a rapid, quantitative, and non-destructive method used to separate lipoproteins. Nascent preβ$_1$ HDL can also be separated from αHDL by FPLC (10). The aim of the present study is to validate the separation of HDL subclasses by FPLC in metabolic study after an endogenous labeling with stable isotopes. In this study, we have compared the leucine enrichment of apoA-I-HDL separated by FPLC to that of HDL separated by the traditional ultracentrifugation of plasma samples.

EXPERIMENTAL PROCEDURES

Experimental protocol

The experimental protocol was approved by the ethics committee of the Nantes University Hospital and written consents were obtained from each volunteer before inclusion in the study.

The kinetic protocol was already described in a previous study (11). Endogenous labelling of apoA-I was performed by administration of L-[5.5.5-$^2H_3$]leucine (99.8 atom %; Mass Trace,

Manuscript received 28 June 2002.
Published, JLR Papers in Press, September 1, 2002.
DOI 10.1194/jlr.D200024-JLR200

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Woburn, MA), dissolved in a 0.9% NaCl solution, and tested for sterility and pyrogenicity before the study. All subjects fasted overnight for 12 h prior to the study, and remained fasting for the entire procedure. They received intravenously 10 μmol.kg^{-1} of tracer, immediately followed by a constant tracer infusion (10 μmol.kg^{-1}.h^{-1}) for 14 h. Venous blood was drawn into EDTA tubes (Venoject, Paris, France) at baseline every 15 min during the first hour, every 30 min during the next 2 h, and then hourly until the end of the study. Plasma was immediately separated by centrifugation for 30 min at 4°C; sodium azide, an inhibitor of bacterial growth, and Pefabloc SC (Interchim, Montluçon, France), a protease inhibitor, were added to blood samples at final concentrations of 1.5 and 0.5 mmol/l, respectively.

Isolation of lipoproteins by ultracentrifugation

Plasma lipoproteins were isolated by ultracentrifugation (6) using a LKB ultracentrifuge with a RP55T rotor at 40,000 rpm for 24 h at 4°C. The density of the plasma was adjusted to the density of each lipoprotein (1.006, 1.063, and 1.025 g/ml for VLDL, LDL, HDL respectively) with NaCl solutions. Then, the lipoprotein fractions were dialysed against NaCl 0.15M for 24 h at 4°C.

Isolation of lipoproteins by FPLC

FPLC analysis was performed at room temperature using two P-500 pumps (1 MPa), a MV-7 place multi-injection with a 200 μl loop, an ultraviolet (UV) monitor UV-1, a FRAC-100 fraction collector, and a LCC-501 Plus controller. All operations were monitored by absorbance at 280 nm. The system was controlled by FPLC DIRECTOR software (Amersham Pharmacia Biotech Inc., Orsay, France) (12–16).

Elution was performed in a 1 mM EDTA, 154 mM NaCl, and 0.02% NaN_3 (pH 8.2) buffer. The buffer was filtered through a 0.22 μm filter (Membrane filters type GV, Duropore®, Millipore). The column was washed with an aqueous ethanol solution (20%).

Isolation of whole HDL. Total HDL separations (total-HDL-FPLC) by FPLC were performed on two Superose 6 HR 10/30 columns in series (Amersham Pharmacia Biotech Inc., Orsay, France). Two hundred microliters of plasma was injected and elution was performed at a flow of 0.35 ml/min. Approximately 0.35 ml fraction were collected. An entire profile was completed within 105 min. The columns were washed for 50 min at the end of each run.

Isolation of apoA-I containing sub-classes. ApoA-I-containing HDL sub-classes separations by FPLC were performed on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech Inc., Orsay, France) (10). Samples of 200 μl of plasma were injected and elution was performed at a flow of 0.35 ml/min. Approximately 0.2 ml was collected for each fraction. An entire profile was completed within 50 min. The columns were washed for 50 min at the end of each run.

Determination of the FPLC peaks. First, human VLDL, LDL, HDL_2, and HDL_3 isolated by ultracentrifugation were used to determine the elution volumes of the various lipoproteins isolated by FPLC.

In another way, lipids and apolipoproteins levels were measured in each FPLC fractions. Total cholesterol and triglycerides contents of lipoproteins and choline containing phospholipids in HDL subpopulations were measured using enzymatic kits (Biomerieux, Marcy-L'Etoile, France). ApoA-I and apoB-100 concentrations were measured by immunoturbidimetry (Biomerieux, Marcy-L'Etoile, France).

Isolation and preparation of apoA-I

ApoA-I of HDL isolated by FPLC and ultracentrifugation was then separated from other proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% to 20% discontinuous gradient and 4% stacking gel. Electrophoresis was run overnight at 70 V and 4°C in Tris-Glycine buffer. ApoA-I was identified by comparing with molecular weight standards (Biorad, Ivry-Sur-Seine, France). ApoA-I bands were excised from the polyacrylamide gels and hydrolysed for 24 h in 4 N HCl at 110°C.

Determination of tracer-to-tracee ratios

Hydrolysates were evaporated to dryness and amino acids were purified by cation exchange chromatography using a Dowex 50WX8-200 resin (Aldrich, Saint-Quentin Fallavier, France). Amino acids were esterified with propanol/acetyl chloride and derivatized using heptafluorobutyric anhydride (HFBA, Fluka, Saint-Quentin Fallavier, France).

Electron-impact ionization gas chromatography-mass spectrometry (GC-MS) was performed on a 5890 A gas chromatograph connected with a 5971 A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). The isotopic ratio was determined by selected ion monitoring at *m/z* of 282 and 285.

Determination of apoA-I containing subpopulations

Non-denaturing two-dimensional PAGE. Non-denaturing two-dimensional PAGE was realized on HDL isolated either by FPLC or by ultracentrifugation, as well as on αHDL and preβ_1 HDL to validate their separations. Elution volume of albumin (67 KDa) was used as exclusion limit between these two populations.

The one-dimensional electrophoresis was performed on a low endosmosity agarose gel, 0.7% (Agarose, Type I: Low EEO, Sigma, Saint-Quentin Fallavier, France) in tris-tricine buffer (25 mM, pH 8.6) containing 3 mM calcium lactate at constant voltage (250 V) at 10°C until bromophenol blue run 3.5 cm. After electrophoresis was completed in the one-dimensional, the agarose strip was cut and placed on polyacrylamide gel as well as molecular weight standards (High molecular weight electrophoresis, Amersham Pharmacia Biotech Inc., Orsay, France). These agarose strips were sealed with the same agarose. The two-dimensional electrophoresis was performed in a 2% to 36% non-denaturing, concave gradient polyacrylamide gel at 220 V for 24 h at 4°C in a buffer containing 90 mM tris, 80 mM boric acid, and 2.5 mM EDTA (pH 8.3).

After electrophoresis, lipoproteins were transferred to a 0.45 μm pore size nitrocellulose membrane (Membrane Protran BA85, Schleicher and Schuell, Dassel, Germany) at 30 V for 24 h at 10°C in a Tris-Glycine buffer containing 25% methanol.

Immunoblotting. After transfer, the bands with molecular weight standards were cut and stained with red Ponceau and the membrane was completely wetted and blocked in phosphate-buffer saline (PBS) containing 3% BSA for 2 h. Then, the membrane was incubated for 2 h with the rabbit anti-human apoA-I (apoA-I, Lpu 1 mg, Institut Pasteur Lille, France) at a dilution of 1:1,000 in PBS with 1% BSA. After primary antibody incubation, the membrane was washed three times (10 min each) in PBS and then incubated with a secondary goat-anti-rabbit IgG peroxidase conjugate at a dilution of 1:1,000 for 2 h in a PBS-BSA (1%) solution. The membrane was then washed in PBS and peroxidase reaction was performed with 4 Chloro-1 Naphtol method (Sigma, Saint-Quentin Fallavier, France).

Determination of the purity of apoA-I

The purity of apoA-I contained in HDL subpopulations was determined with a denaturing two-dimensional gel electrophoresis: isoelectric focusing (IEF)/SDS-PAGE; process which allowed the separation of proteins according to their Isoelectric point (p*I*)

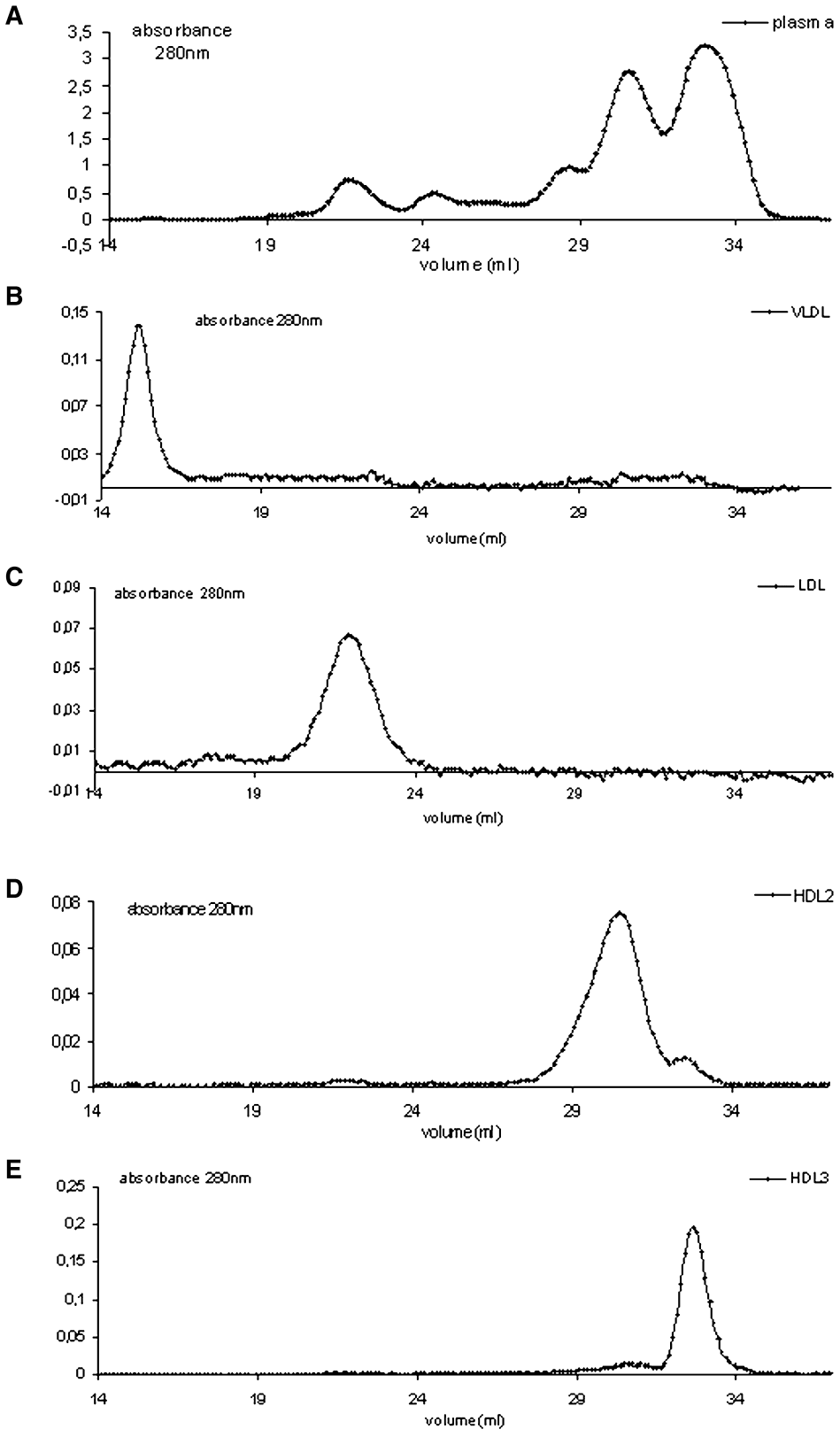

Fig. 1. FPLC elution profiles on two Superose 6 HR for a representative plasma (A) and VLDL (B), LDL (C), HDL_2 (D), and HDL_3 (E) obtained by ultracentrifugation.

and their molecular mass. All instrumentation, gels and protocols were from Amersham Biosciences. 50 ng of αHDL and 100 ng of preβ$_1$ HDL were diluted to a final volume of 200 μl in a solution containing 8 M urea, 2% CHAPS, 18 mM DTT, 0.2% Pharmalyte 4-7, and a few granules of bromophenol blue. IEF was carried out with Immobiline DryStrip gels (IPG, pH 4–7, 11 cm; and pH 5–10, 18 cm) in the IPFphor isoelectric focusing system, according to the manufacturer's protocol. Two-dimensional SDS-PAGE was performed using ExcelGel 2-D Homogeneous 12.5% in the Multiphor II electrophoresis unit, according to the manufacturer recommendations. After completion of the two-dimensional electrophoresis, the gels were silver stained as described by Heukeshoven and Dernick (17).

RESULTS

Determination of elution volume of lipoproteins isolated by FPLC

When human plasma samples were analysed on two Superose 6HR gel permeation columns, four distinct peaks of

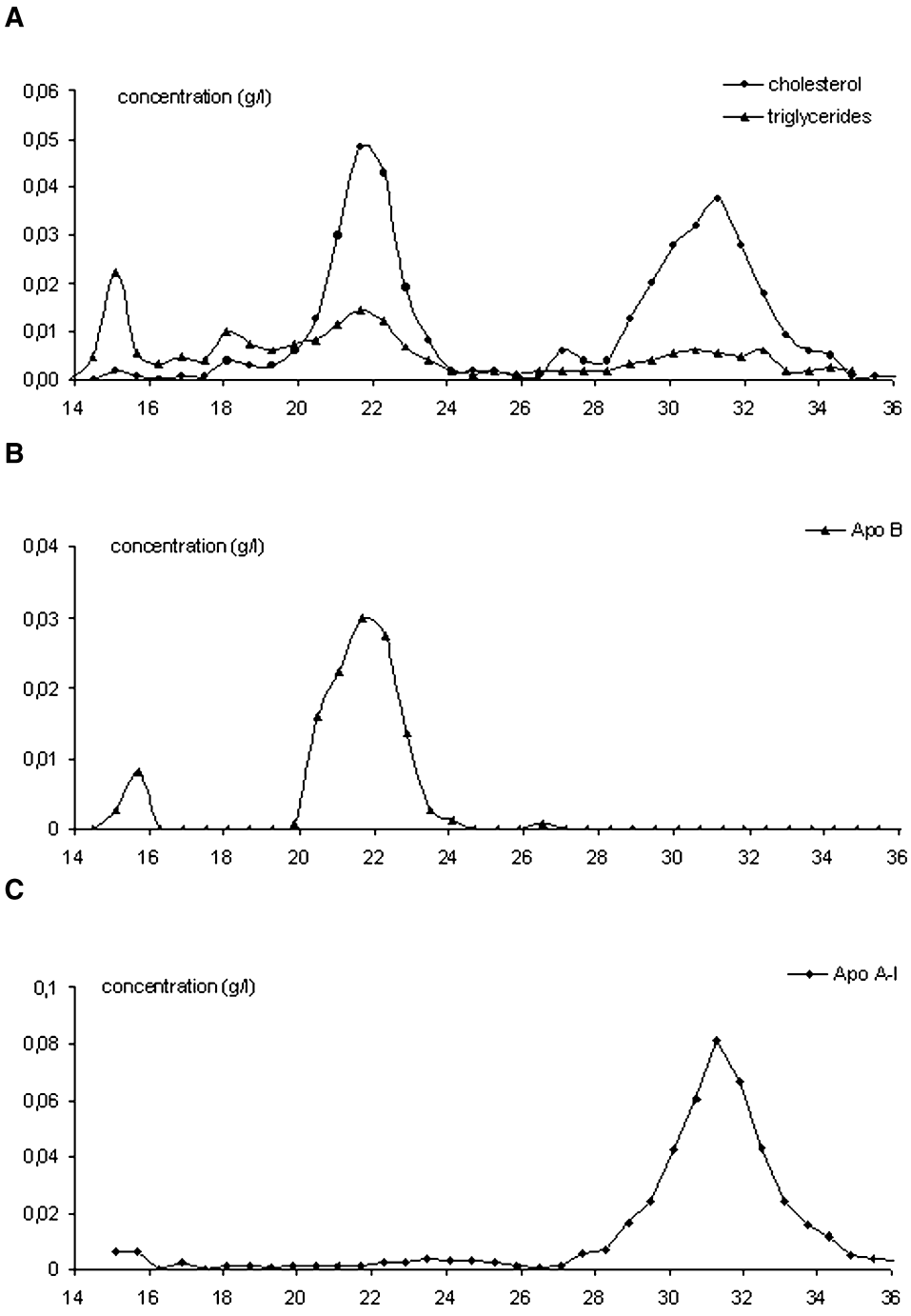

Fig. 2. FPLC elution profiles on two Superose 6 HR for a representative plasma sample as assessed by cholesterol and triglycerides (A) or apolipoprotein B (apoB) (B) or apoA-I (C).

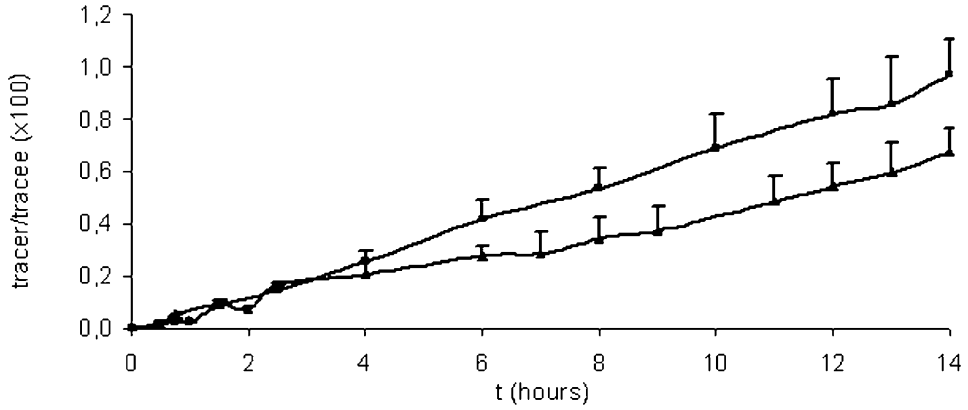

Fig. 3. Experimental values of the tracer-to-tracee ratio for total HDL-apoA-I isolated by ultracentrifugation (circles) and HDL-apoA-I isolated by FPLC (triangles). Data are shown as mean ± SEM.

lipoproteins were identified: VLDL, LDL, HDL_2, and HDL_3 (**Fig. 1A**). The elution profiles of VLDL (Fig. 1B), LDL (Fig. 1C), HDL_2 (Fig. 1D), and HDL_3 (Fig. 1E) isolated by ultracentrifugation and applied to FPLC in four different runs are shown in Fig. 1. In **Fig. 2**, the elution profiles of cholesterol, triglycerides (Fig. 2A), apoB (Fig. 2B), and ApoA-I (Fig. 2C) were analysed in order to identify and validate the fractions corresponding to VLDL, LDL, and HDL.

Enrichment data

The tracer-to-tracee ratio curves in total HDL isolated by FPLC and ultracentrifugation are shown in **Fig. 3**. The tracer-to-tracee ratio for ApoA-I-HDL after FPLC were less than 35% compared to HDL isolated by ultracentrifugation for all subjects.

To rule out a possible retention of the tracer in the FPLC columns, we measured the tracer-to-tracee ratio of HDL-UC before and after FPLC as a control experiment. The tracer-to-tracee ratio curves were similar in these two different conditions (data not shown). These data indicated that the difference in the tracer-to-tracee ratio observed between ultracentrifugation and FPLC separation methods was related to the composition of HDL. To test this hypothesis, we have performed a two-dimensional electrophoresis of our HDL samples.

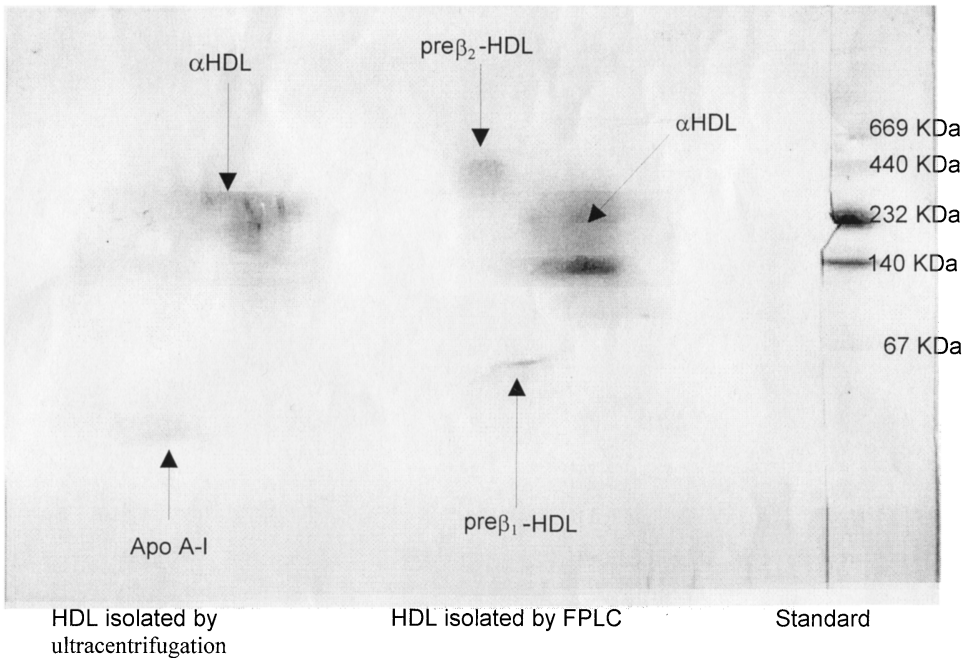

Fig. 4. Western blotting with anti-apoA-I of a two-dimensional electrophorese of total HDL isolated by ultracentrifugation and FPLC followed by apoA-I immunoblotting.

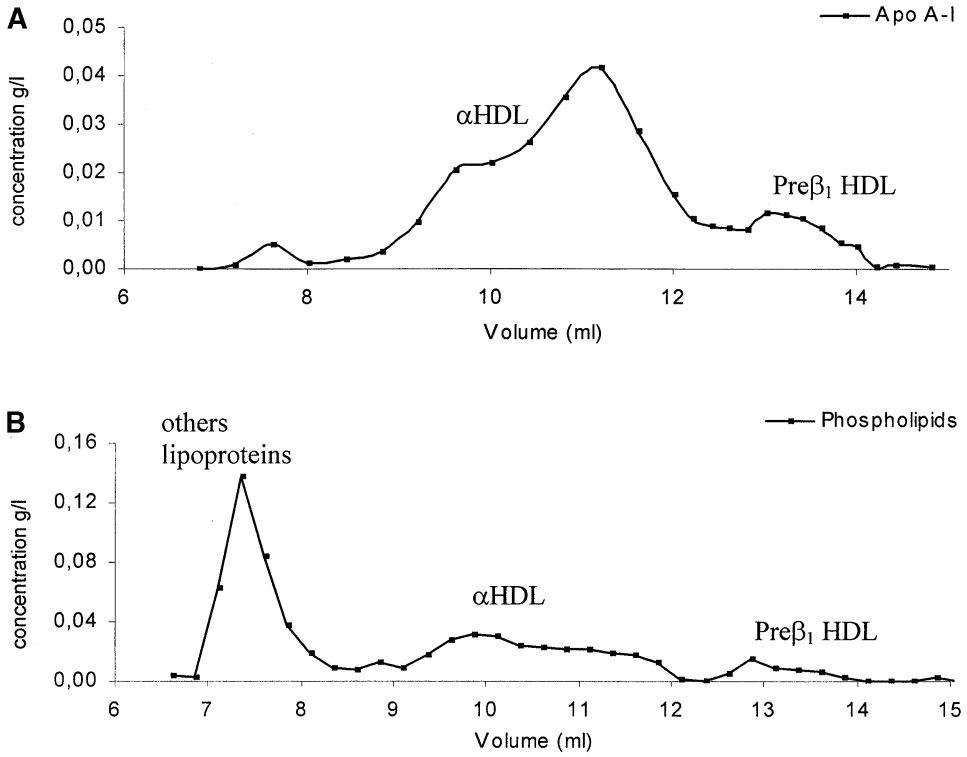

Fig. 5. ApoA-I (A) and phospholipids (B) composition of Superdex 200 HR column fractions of whole plasma.

Determination of apoA-I containing subpopulations in whole HDL

Figure 4 shows the two-dimensional electrophoresis of total HDL isolated by ultracentrifugation and FPLC followed by apoA-I immunoblotting. After ultracentrifugation preβ HDL was not found and only HDL with α electrophoretic mobility and free apoA-I was observed. Conversely, preβ as well as αHDL were recovered after FPLC separation of human plasma.

Separation and identification of HDL subclasses obtained by FPLC

When human plasma samples were separated by FPLC on a Superdex 200 HR gel permeation, three apoA-I-HDL subclasses, as well as three distinct phospholipids containing lipoproteins, were separated (**Fig. 5**).

The first peak represented a minor population of the largest particles >500 KDa (fraction 1 to 13). The second peak included a spherical αHDL of 100–500 KDa:

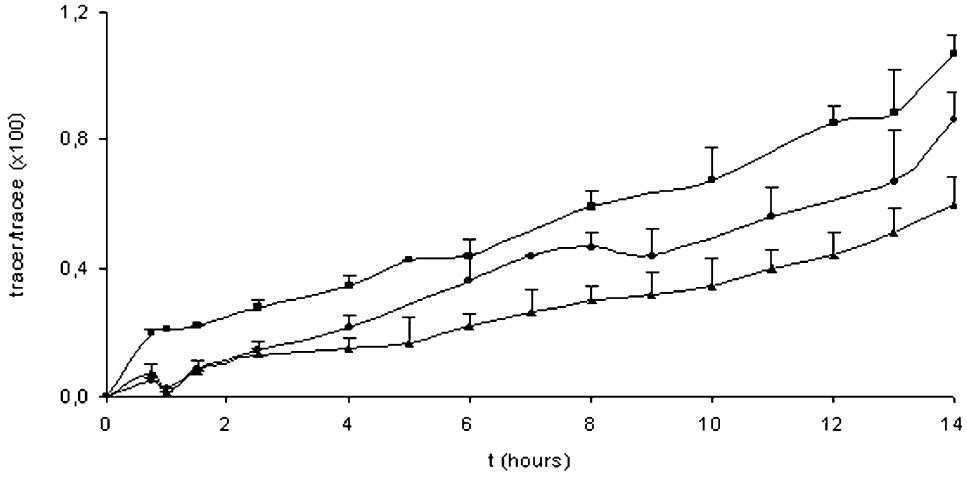

Fig. 6. Experimental values of the tracer-to-tracee ratio for total HDL-ApoA-I isolated by ultracentrifugation (circles); and αHDL (triangles) and preβ$_1$ HDL-ApoA-I (square) isolated by FPLC. Data from five individuals ± SEM.

HDL_2 and HDL_3. The third peak included a minor population of smaller particles of 40-60 KDa: $preβ_1$ HDL. The purity of this fractions was confirmed by a two-dimensional non denaturing electrophoresis and a immunoblotting methods.

Determination of the purity of apoA-I

With the denaturing two-dimensional gel electrophoresis, in both $preβ_1$ HDL and αHDL fractions, we did not observe another protein having the same molecular mass as apoA-I and a different p*I*. We could conclude that the apoA-I band is not contaminated by another protein. The only doubt was in αHDL fraction; Ig light chains were localised above apoA-I spot and could contaminate the isotopic ratio. To extract Ig from αHDL, 2.5 μg of αHDL were incubated for 7h at 4°C with rotation with protein A-Agarose, protein that bind to immunoglobulin classes. The Ig-protein A-Agarose complex was precipitated and the supernatant was separated on SDS-PAGE. We have obtained the same isotopic ratio for αHDL incubated or not with protein A-Agarose for several time of kinetic. Thus, we could conclude that apoA-I-αHDL was not contaminated by Ig light chains (data not shown).

Enrichment of HDL subclasses obtained by FPLC

The tracer-to-tracee ratio of αHDL, $preβ_1$ HDL isolated by FPLC, and total HDL isolated by ultracentrifugation were established on five subjects (**Fig. 6**). Experimental values of tracer-to-tracee ratio of $preβ_1$ HDL and αHDL were higher and lower, respectively, than those of total HDL.

DISCUSSION

The aim of this study was to compare two methods to separate HDL for the measurements of isotopic enrichment in apoA-I-HDL. HDL was isolated by standard sequential ultracentrifugation and by FPLC from plasma samples of five subjects who received a constant infusion of deuterated leucine during 14 h. The FPLC technique allowed the isolation of $preβ_1$ HDL from αHDL, and detectable isotopic enrichments in both fractions were found with GC-MS analysis. To our knowledge, this study is the first to distinguish enrichments data of $preβ_1$ HDL and αHDL kinetic compared to total HDL usually isolated in previous studies for kinetic measurements.

The linearity, reproducibility, and sensitivity of FPLC technique has been demonstrated previously to separate lipoproteins (16, 18, 19). With FPLC, the samples were not exposed during the separation process to the high g-forces and salt concentrations related to ultracentrifugation (12) that may shed some of the HDL components from the particle surface (e.g., apoA-I) (9). There was a progressive loss of apoA-I of about 35% with repeated ultracentrifugation of HDL at a density of 1.21 g/ml (8). Moreover, this latter procedure required approximately 1 ml to 3 ml of plasma for analysis, whereas only 200 μl were necessary with FPLC to isolate preβ and αHDL and to determine lipids and apolipoproteins concentrations in the eluted fractions. With the increasing use of the transgenic and knockout mouse models of study atherosclerosis, the ability to separate lipoproteins classes from small volumes of whole plasma is interesting. However, separation using gel filtration method resulted in sample dilutions.

The experimental values of tracer-to-tracee ratio for apoA-I-HDL after FPLC were less than those of HDL isolated by ultracentrifugation. This difference could be explained by the difference in the separation process with ultracentrifugation and FPLC and thus we did not analyse the same biological materials. Indeed, effects of ultracentrifugation separation on apoA-I subpopulations were dramatic. As demonstrated by Asztalos et al. (20), after ultracentrifugation only free apoA-I and HDL with α mobility were found, indicating the loss of $preβ_1$ and $preβ_2$ from the bulk of flotating HDL. Free apoA-I could come from denaturation of different HDL subpopulations as small particles ($preβ_1$ HDL). After FPLC, HDL with $preβ_1$, $preβ_2$, and α mobilities were detected. Therefore, FPLC reflects the biological diversity of lipoproteins better than ultracentrifugation.

$Preβ_1$ and αHDL were isolated by FPLC and the purity of each subpopulation was validated by two-dimensional PAGE. The purity of apoA-I contained in these lipoproteins was determined by a denaturing two-dimensional gel electrophoresis. The tracer-to-tracee ratios in $preβ_1$ HDL were higher than those of total HDL and αHDL. The fast and higher enrichment of $preβ_1$ HDL could be explained by the early role of these lipoproteins in the reverse transport. Total HDL obtained by ultracentrifugation was probably composed of principally αHDL and of small quantity of $preβ_1$ HDL. Thus, in previous studies, based on ultracentrifugation, tracer-to-tracee ratios in apoA-I-HDL could have been overestimated by the presence of apoA-I-$preβ_1$ HDL in αHDL population.

The authors thank Mrs Pascale Maugère for her excellent technical assistance and Dr Jean-François Bouhours for critical reading of the manuscript.

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