Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions

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Abstract **We have previously shown that plasma lipoproteins can be separated by analytical capillary isotachophoresis (ITP) according to their electrophoretic mobility in a defined buffer system. As in lipoprotein electrophoresis, HDL show the highest mobility followed by VLDL, IDL, and LDL. Chylomicrons migrate according to their net-charge between HDL and VLDL, because ITP has negligible molecular sieve effects. Three HDL subfractions were obtained which were designated fast-, intermediate-, and slow-migrating HDL. To further characterize these HDL subfractions, a newly developed free-solution ITP (FS-ITP)-system was used, that allows micro-preparative separation of human lipoproteins directly from whole plasma (Böttcher, A. et al. 1998.** *Electrophoresis*. 19: **1110–1116). The fractions obtained by FS-ITP were analyzed for their lipid and apolipoprotein composition and by two-dimensional nondenaturing polyacrylamide gradient gel electrophoresis (2D-GGE) with subsequent immunoblotting. fHDL are characterized by the highest proportion of esterified cholesterol of all three subfractions and are relatively enriched in LpA-I. Together with iHDL they contain the majority of plasma apoA-I, while sHDL contain the majority of plasma apoA-IV, apoD, apoE, and apoJ. Pre-β-HDL were found in separate fractions together with triglyceride-rich fractions between sHDL and LDL. In summary, ITP can separate the bulk of HDL into lipoprotein subfractions, which differ in apolipoprotein composition and electrophoretic mobility. While analytical ITP permits rapid separation and quantitation for diagnostic purposes, FS-ITP can be used to obtain these lipoprotein subfractions on a preparative scale for functional analysis. As FS-ITP is much better suited for preparative purposes than gel electrophoresis, it represents an important novel tool for the functional analysis of lipoprotein subclasses.**—Böttcher, A., J. Schlosser, F. Kronenberg, H. Dieplinger, G. Knipping, K. J. Lackner, and G. Schmitz. **Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions.** *J. Lipid Res.* **2000.** 41: **905–915.**

Supplementary key words isotachophoresis • apolipoproteins • pre-β HDL • two-dimensional gel electrophoresis

Lipoproteins may be classified according to their density, particle size, electrophoretic mobility, or apolipoprotein composition (1–5). It has become obvious that among the major lipoproteins, and particularly among HDL, there are functionally distinct particle subclasses. Various methods have been developed to characterize HDL subclasses, such as ultracentrifugation, agarose gel electrophoresis, gradient gel electrophoresis, chromatographic methods, and immunoaffinity chromatography (6–13). One of the most interesting approaches is two-dimensional gradient gel electrophoresis (2D-GGE) followed by immunoblotting for apoA-I. Besides the bulk of HDL with α-mobility, usually three pre-β-migrating subclasses can be identified: pre-β_1-, pre-β_2-, and pre-β_3-LpA-I (14, 15). Pre-β_1 HDL are considered as initial acceptors of cellular cholesterol and therefore critical in reverse cholesterol transport (16). The same technique was also used to characterize apoE- and apoA-IV-containing HDL (17–20). A major drawback of 2D-GGE is its poor suitability for preparative purposes.

Another method for separation of lipoproteins by their electrophoretic mobility is capillary isotachophoresis, which is a support-free technique with negligible molecular sieve effects. It is performed in a discontinuous buffer system with the advantages of concentration and self-sharpening effects. Lipoproteins may be stained with lipophilic dyes

Abbreviations: ACES, N-2-acetamido-2-aminoethanesulfonic acid; ammediol, 2-amino-2-methyl-1,3-propanediol; apo, apolipoprotein; GlucAc, glucuronic acid; HDL, high density lipoprotein; fHDL, fast migrating HDL; iHDL, intermediate migrating HDL; sHDL, slow migrating HDL; HPMC, hydroxypropylmethylcellulose; LDL, low density lipoprotein; LpA-I, apoA-I-containing lipoproteins without apoA-II; LpA-I:A-II, apoA-I and apoA-II-containing lipoproteins; NBD, 7-nitro-benz-2-oxa-1,3-diazole; VLDL, very low density lipoprotein; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TAPSO, 3-N-tris(hydroxymethyl) methylamino-2-hydroxy-propanesulfonic acid; 2D-GGE, two-dimensional nondenaturing polyacrylamide gradient gel electrophoresis.

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such as Sudan Black B (21) or Fat Red 7B (22). With the introduction of NBD-ceramide by our group, the first quantitative label of lipoproteins became available (23). ITP can be automated for the analysis of large sample series.

So far, analytical ITP protocols could not be transferred to a preparative scale. Therefore, it was impossible to fully characterize the subfractions observed by analytical ITP. Continuous free-flow ITP was reported for preparative characterization of HDL and LDL subfractions (24, 25). The latter method has the drawback that steady state is not reached when the number of sample components increases and only small differences in electrophoretic mobility exist. Therefore, ultracentrifugally prepared lipoproteins and not total plasma had to be used in these protocols. In a recent paper based on our methodology Schlenck et al. (26) correlate peak areas obtained by analytical ITP with lipoprotein data. They show that the subfractions change specifically with changes in lipoprotein concentrations or composition. This was taken as an indication that analytical ITP may be useful for diagnostic purposes.

We recently developed an automated, preparative free solution isotachophoresis (FS-ITP) system suitable for separation of total plasma proteins in the milligram range (27). It is based on a support-free "thin film" electrophoresis device. FS-ITP separates lipoproteins from whole plasma in the batch mode without the problems inherent to continuous flow systems. We report here the use of FS-ITP to further characterize the composition of HDL fractions separated by analytical ITP. These data will provide the basis for a broader use of analytical ITP as a method for lipoprotein analysis.

MATERIALS AND METHODS

Blood sampling

Blood was drawn into EDTA-containing tubes (final concentration: 1.5 mg/ml) from healthy normolipidemic volunteers after an overnight fast and immediately cooled on ice. Plasma was obtained by centrifugation for 15 min at 2000 *g* and 4°C. The protease inhibitors benzamidine, phenylmethylsulfonylfluoride (final concentration 1 mm each), and aprotinin (10,000 U/l) were added to the plasma samples which were either maintained at 4°C until ITP within 5 h of venipuncture or divided into aliquots and stored frozen at −70°C.

Capillary isotachophoresis of lipoproteins

Analytical capillary ITP of plasma samples was performed as described earlier (23) with minor modifications. Lipoproteins were stained with the fluorescent lipophilic dye 7-nitro-benz-2-oxa-1,3-diazole-(NBD) ceramide (Molecular Probes, Eugene, OR). Plasma was diluted 1:3.5 (v/v) with leading buffer which consists of 10 mm HCL, 0.35% w/v hydroxypropylmethylcellulose (HPMC), adjusted with 2-amino-2-methyl-1,3-propanediol (ammediol) to pH 8,8. The diluted plasma (20 μl) was incubated for 1 min with 10 μl NBD-ceramide solution (0.1 mg/ml in ethylene glycol–methanol, 9:1 (v/v). Twenty μl of this solution was mixed with 50 μl of spacer mixture (1:6 v/v with leading buffer). The spacer mixture was prepared from stock solutions of 10 mg/ml of the following compounds in order of decreasing electrophoretic mobilities: 100 μl N-2-acetamido-2-aminoethanesulfonic acid (ACES), 50 μl glucuronic acid, 50 μl octanesulfonic acid, 80 μl 3-N-tris(hydroxymethyl)methylamino-2-hydroxy-propane sulfonic acid (TAPSO), 120 μl N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), 60 μl serine, 80 μl glutamine; 80 μl methionine, and 50 μl glycine. As internal standard 10 μl of 5-carboxy-fluorescein (10 μg/ml) was added. The terminating electrolyte contained 20 mm alanine and was adjusted to pH 9.4 with saturated barium hydroxide solution. Separations were performed on a P/ACE 5510 system (Beckman Instruments, Inc., Fullerton, CA) equipped with a 27 cm (20 cm length to detector) dimethyl polysiloxane modified fused silica capillary, inner diameter 180 μm (Restek Rtx-1, purchased from Alltech, Unterhaching, Germany). The capillary, samples, and buffers were thermostated at 20°C during separation. Samples were injected into the capillary using pressurized injection for 18 s at 3.44 kPa. Separation was performed at constant 25 μA. The separated zones were monitored with laser-induced fluorescence detection (excitation 488 nm; emission 520 nm).

Preparative isotachophoresis of lipoproteins

Preparative separation of lipoproteins was performed as recently described (27), with minor modifications for the separation of lipoproteins. Briefly, the separation chamber was cooled to 4°C and filled with the chamber buffers. The leading chamber buffer contained 10 mm HCl and was adjusted with ammediol to pH 8.8; the terminating chamber buffer was 100 mm alanine adjusted with ammediol to pH 9.4. Both buffers were prepared in 0.35% w/v HPMC solution which had been filtered through a paper filter, and subsequently through a 0.8 μm pore size membrane filter. The fractionation buffer and leading electrode buffer consisted of 10 mm HCl adjusted with ammediol to pH 8.8 without HPMC. The terminating electrode buffer was 40 mm alanine adjusted with $Ba(OH)_2$ to pH 9.4. Samples were prepared by dilution of 500 μl plasma with 500 μl spacer-mixture. As indicator for the start of fractionation, 2 μl of a solution of 5-carboxy-fluorescein (concentration 1 mg/ml) was added to the sample. The spacer mixture was prepared from stock solutions of 10 mg/ml in leading buffer with 0.5% HPMC of the following compounds in order of decreasing electrophoretic mobilities: ACES (200 μl); glucuronic acid (150 μl); octane sulfonic acid (150 μl); TAPSO (200 μl); TAPS (300 μl); serine (150 μl); glutamine (200 μl); methionine (200 μl); glycine (100 μl). The sample was injected with a 1-ml syringe and the separation was started with constant current of 2 mA. The voltage remained constant for the first 10 min at 190 V and increased within 80 min to 950 V. The current was then reduced to 1 mA and the voltage decreased to 500 V. After a further 5 min the line of the added marker dye was 1 mm in front of the fractionation channel and the voltage had reached a value of 530 V. This value was adjusted at the interface, as the signal to start collecting fractions. The dye line moved with an effective velocity of 1.5 mm/min. Thirty fractions of 270 μl were collected. After 110 min the separation was stopped. The voltage had reached a constant final value of 580 V.

Two-dimensional nondenaturing gradient gel electrophoresis (2D-GGE)

For the first dimension, 5 μl plasma or 20 μl ITP-fraction was separated in 0.7% (w/v) agarose (SeaKem LE) gels containing 0.25% fatty acid-free BSA in 50 mm barbital buffer (pH 8.6). Samples were electrophoresed at 100 V constant voltage and 10°C for about 1 h when the albumin stained with bromophenol blue had moved 4 cm. For the second dimension, the agarose gel strips from the first dimension were transferred to a 4–15% polyacrylamide gradient gel (Ready Gels, Bio-Rad Laboratories, Munich, Germany). Separation in the second dimension was performed at 20 mA per gel for 4 h at 4°C. Molecular mass standards (Pharmacia, Freiburg, Germany) containing thyroglobulin, 669

kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; and albumin, 67 kDa were run simultaneously with the sample.

Immunoblotting and chemiluminescent detection

After electrophoresis, proteins were electrophoretically transferred to fluorotrans transfer membranes of 0.2 μm pore size (Pall, Dreieich, Germany). Transfer was carried out for 18 h in 20 mm Tris and 150 mm glycine buffer, in a trans-blot cell (Bio-Rad Laboratories) at constant 30 V and 10°C. The lane with molecular mass standards was cut off and stained with Coomassie Blue. Membranes were then blocked by incubation for 1 h in phosphate-buffered saline (PBS) containing 5% non-fat milk powder and 0.1% Tween 20. For the detection of the individual apolipoproteins, membranes were incubated for 1 h with the corresponding antibodies in a dilution of 1:5.000 in PBS with 1% non-fat milk powder and 0.1% Tween 20. The following antibodies were used: rabbit anti-human apoA-I antiserum, rabbit anti-human apoA-II antiserum (both from Behring, Marburg, Germany), rabbit anti-human apoA-IV antiserum (28), goat anti-human apoC-III antiserum (WAK-Chemie, Bad Homburg, Germany), rabbit anti-human apoD antiserum (29), affinity-purified goat polyclonal anti-human apoE antibody, goat anti-human apoJ antiserum (both from Biodesign International, Kennebunk, ME), rabbit anti-human factor H antiserum (Serotec, Oxford, England).

After antibody incubation, the membrane was washed three times (10 min each) in 50 ml PBS containing 0.1% Tween 20 and then incubated for 1 h with anti-rabbit or anti-goat immunoglobulin–horseradish peroxidase conjugate (dilution 1:10,000). Before chemiluminescent detection the washing step was repeated. The membrane was developed with the ECL-Plus Western blotting detection system (Amersham/Pharmacia, Freiburg, Germany) according to the manufacturers instructions and analyzed by using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany). For reprobing with another antibody, membranes were stripped of bound antibodies by incubation in 100 mm 2-mercaptoethanol, 2% SDS, 62.5 mm Tris-HCl, pH 6.7, at 50°C for 30 min with gentle agitation. After washing for 2 × 10 min with PBS containing 0.1% Tween 20, membranes were again blocked in 5% non-fat dry milk in PBS for 1 h at room temperature. The second immunodetection was performed as outlined above.

Lipid and apolipoprotein analyses

Phospholipids were determined by an enzymatic method (30) using a reagent kit from BioMèrieux (Marcy-l'Etoile, France). Total and free cholesterol were determined by a cholesterol oxidase assay with fluorimetric detection. For the determination of free cholesterol, no cholesterol esterase was added to the incubation. Concentrations of enzymes and detergents relative to cholesterol were chosen as described (31, 32). Fluorescence intensities of the calibrators and the samples were measured by monitoring emission at 400 nm (λ_{ex} = 320 nm).

ApoA-I was determined by a sandwich type enzyme-linked immunosorbent assay according to Koren et al. (33). ApoA-IV concentrations were determined by a previously described sandwich type enzyme-linked immunosorbent assay (28). Quantification of apoD was performed by an immunoassay with time-resolved fluorescence spectroscopy (29). ApoB and apoE were measured by a sandwich type in-house ELISA. A polyclonal rabbit anti-apoB antiserum (Behring) or a polyclonal goat anti- apoE affinity-purified IgG fraction (Biodesign International), respectively, were used for coating. The amounts of bound apolipoproteins were determined with a monoclonal anti-human apoB antibody (Calbiochem, Bad Soden, Germany) or a monoclonal anti-human apoE antibody (clone IX8FR39D) and horseradish peroxidase-labeled anti-mouse IgG (Dianova, Hamburg, Germany). A reference standard for human apolipoproteins (Immuno, Vienna, Austria) was used for calibration.

The relative concentrations of apoC-III, apoJ, and factor H in the ITP fractions were determined by scanning the intensities of protein bands from Western blots by use of a LumiImager. Aliquots from each fraction (10 μl) were diluted with SDS sample buffer (1:2), boiled, and electrophoresed on 4–14% denaturing gradient gels. Immunoblotting and chemiluminescent detection were performed as outlined above. For the relative distribution of apoC-III, 16.5% Tris-Tricine gels (34) were used. The relative concentrations were calculated from calibrated intensity values (Boehringer Light Units, BLUs) as described by the manufacturer.

Lipoproteins containing apoA-I (LpA-I) as the only apolipoprotein were determined by an electroimmundiffusion technique in agarose gels using kits from Sebia (Issy-les-Moulineaux, France).

RESULTS

Analytical ITP

When normal human plasma stained with NBD-ceramide is separated by analytical capillary ITP with the spacers described in Materials and Methods, eight lipoprotein fractions are obtained (**Fig. 1A**). By addition of purified lipoproteins, these fractions can be further identified: peaks 1–3 represent HDL subfractions, peak 4 represents chylomicrons (elevated in postprandial serum and type I hyperlipoproteinemia), peak 5 represents VLDL/IDL, and peaks 6–8 represent LDL (Fig. 1B–D). According to their mobility on analytical ITP, the HDL subfractions have been defined as fast-migrating HDL (fHDL) (peak 1), intermediate-migrating HDL (iHDL) (peaks 2a and b), and slow-migrating HDL (sHDL) (peak 3).

When the same sample is separated repeatedly and the peak areas in the HDL range (peaks 1–3) are determined, there is a between-run coefficient of variation (CV) of 4.0% for the total area of peaks 1–3 and 4.8%, 4.1%, and 1.8% for the individual peaks 1, 2, and 3, respectively. The CV for the LDL range (peaks 6–8) is similar (3.1%). In **Fig. 2** several representative examples of analytical ITP separations are shown. It is apparent that there are specific changes in the HDL region associated with certain diseases or conditions.

Preparative FS-ITP

During FS-ITP a total of 30 fractions of equal volume were collected. The fractionation begins with appearance of the added marker dye. **Figure 3A** shows the concentration of total cholesterol in the FS-ITP fractions and the reproducibility of the method. The latter was assessed by performing 6 separations on 2 different days from the same sample. The CV was 5.0% for combined fractions 4–14 and 5.3% for combined fractions 19–29. When only single fractions were analyzed, the CV was 3.7% for fHDL (fractions 4–6). 5.0% for iHDL (fractions 7–11), and 15% for sHDL (fractions 12–14).

The distribution of apoA-I-containing lipoproteins was determined after electrophoresis through 0.7% agarose by immunoblotting with subsequent chemiluminescent detection. Figure 3B shows that the majority of apoA-I is present in fractions 5–13 and has α-mobility as expected

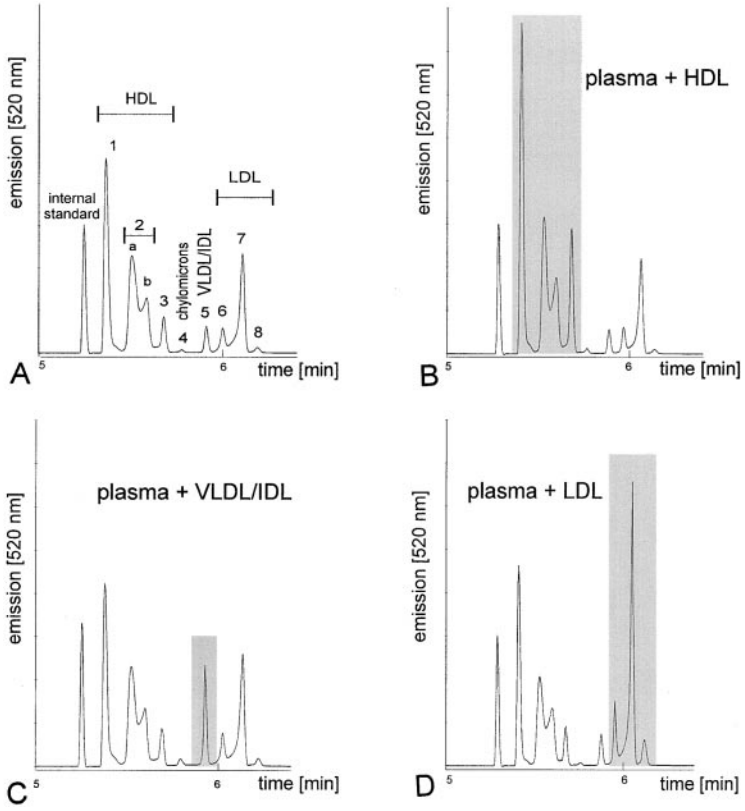

Fig. 1. Representative lipoprotein pattern obtained by analytical ITP (panel A). Peak 1 represents fHDL, peak 2 a and b iHDL, and peak 3 sHDL. The identification of lipoproteins was possible by addition of purified lipoproteins to serum samples. A representative experiment is shown in panels B–D (HDL, panel B; VLDL, IDL, panel C; LDL, panel D).

for HDL particles. A minor amount of apoA-I is found in fractions 17–21 with pre-β mobility. The migration distance from origin shows, furthermore, that the mobility of the α-HDL decreases from fraction 5 to fraction 13 with fraction 5 containing only fHDL and fraction 13 only sHDL. As the exposure time of the gel had to be quite long to visualize the pre-β particles, the overlap between fractions appears exaggerated.

In a next step the content of apolipoproteins A and B and of lipid constituents was determined in the FS-ITP fractions (**Fig. 4**). It should be noted that the distributions are derived from individual plasma samples from normolipidemic subjects. Even though there are interindividual differences, the results are representative for normolipemic persons. The distribution of the two major apolipoproteins apoA-I and apoB in FS-ITP fractions was determined by ELISA (Fig. 4A). As expected, apoA-I was mainly found in fractions 5–14 which represent α-HDL with a maximal concentration in fractions 5 and 6. ApoB was mainly found in the slower migrating fractions 20–26. Fractions 17–21 shown to contain pre-β-HDL by immunoblotting contained only traces of apoA-I and apoB. The recovery rate of apoA-I was 98% and of apoB 93%. The distribution of total and free cholesterol in the FS-ITP fractions is presented in Fig. 4B. The ratio of unesterified cholesterol to cholesteryl esters varies across the apoA-I-containing HDL fractions with a minimal value of about 0.09 in fractions 5 and 6, about 0.48 in fractions 7–11, and 0.24 in fractions 12–14. In the apoB-containing fractions 20–27, the ratio of cholesteryl esters to cholesterol was fairly constant at approximately 0.43. In fractions 15–20, where both apoA-I and apoB are low, only minor amounts of cholesterol are found. The recovery rate of total cholesterol was 92%. The relative amount of phospholipids to cholesterol is greater in apoA-I-containing particles than in apoB particles (Fig. 4C). The actual distribution shown is derived from one plasma sample. However, it is representative for healthy probands.

Comparison of analytical ITP and FS-ITP

To further characterize the HDL subfractions detected by analytical ITP, the spacers used for this procedure were added in higher concentrations to preparative FS-ITP. This results in a broader spacer zone between the lipoprotein fractions. The position of the spacers relative to the peaks on analytical ITP is shown in **Fig. 5A**. The FS-ITP fractions were then subjected to 2D-GGE and immunoblotting for apoA-I (Fig. 5B). The separation conditions for 2D-GGE were the same for all samples. The fractions with faster mobility than Gluc-Ac representing peak 1 or fHDL on analytical ITP (Fig. 5A) contain only particles with α-mobility when analyzed by 2D-GGE. The fractions between TAPSO and Gln, which are equivalent to peak 3 or sHDL, and peaks 4 and 5 contain slow-migrating α-HDL and pre-β-HDL. When only TAPS and Ser are used, which are the spacers flanking peak 4, almost pure pre-β_2-HDL is obtained. Between Ser and Gln, pre-β_1-HDL is found. The positions of the spacers are shown in an original blot (Fig. 5C). Molecular mass standards are

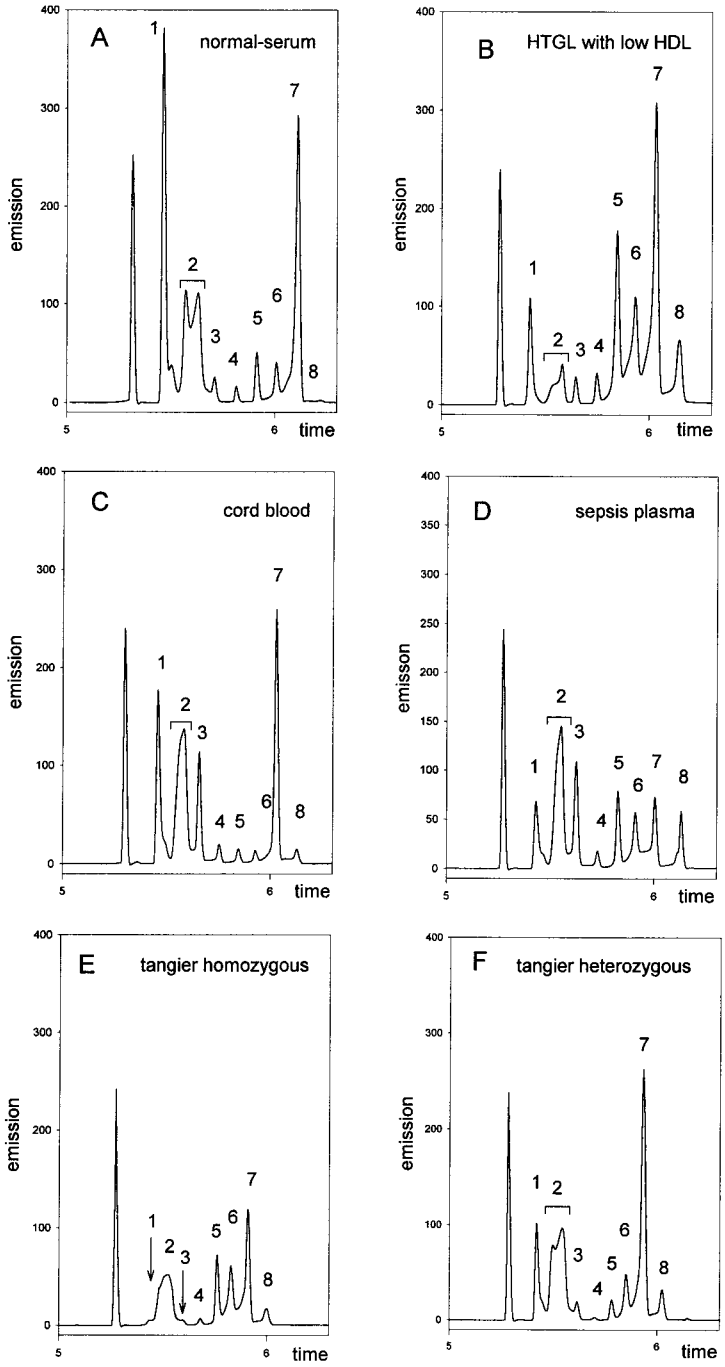

Fig. 2. Examples of analytical ITP patterns from different individuals. The patterns represent typical examples for normal individuals (panel A) as well as for the underlying lipoprotein abnormalities or systemic disorders indicated in the panel. While some patients show a generalized reduction of HDL (e.g., panel B), others show changes in the proportion of fHDL to sHDL. The remaining peak 2 in plasma from a patient with homozygous Tangier disease is caused by staining of albumin.

indicated, but it should be noted that migration of the proteins does not exactly reflect true molecular mass. These data clearly show that it is possible to obtain lipoprotein subfractions from FS-ITP representing the composition of the corresponding peaks of analytical ITP. Due to the addition of surplus spacers, there is no relevant overlap between the fractions.

In **Fig. 6**, 2D-GGE-immunoblots for apoA-I of some fractions of a representative preparative FS-ITP are shown. These results confirm the data obtained by supplementing specific spacers to FS-ITP separations. Furthermore, they show that the overlap between fractions is small.

Distribution of apolipoproteins and other lipoprotein-associated proteins

The distribution of other apolipoproteins in the FS-ITP fractions of normal plasma was analyzed by ELISA and quantitative Western blots. As a comparison, the pattern of 2D-GGE and subsequent immunoblotting of total plasma is shown for each protein analyzed. **Figure 7 A–G** shows the distribution of apoA-II, apoA-IV, apoE, apoD, apoC-III, apoJ, and factor H. The distribution for apoA-I and apoB as shown in Fig. 4A is included as a reference. ApoA-II, which represents LpA-I:A-II particles, is found in fHDL, iHDL, and sHDL fractions. The relation of LpA-I to LpA-I:A-II particles is found highest in fHDL. In iHDL,

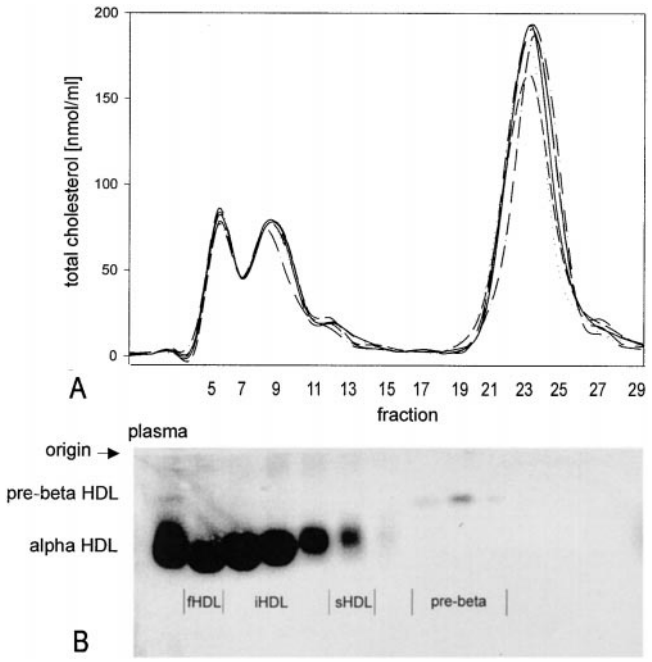

Fig. 3. Separation of plasma by FS-ITP into 30 fractions results in two major peaks of lipoproteins as determined by cholesterol concentration. In panel A the reproducibility of the separation is shown. The same plasma was separated 6 times on 2 different days and cholesterol was determined in all fractions and the distribution curves superimposed. In panel B fractions were analyzed by electrophoresis in an 0.7% agarose gel, followed by immunoblotting with an anti apoA-I antibody. The different HDL subfractions—fHDL, iHDL, and sHDL—are indicated in panel B. There is some apparent overlap between the fractions, which is, however, exaggerated by the strong signal of the major HDL fractions. Pre-β-HDL particles are fully separated from the other fractions.

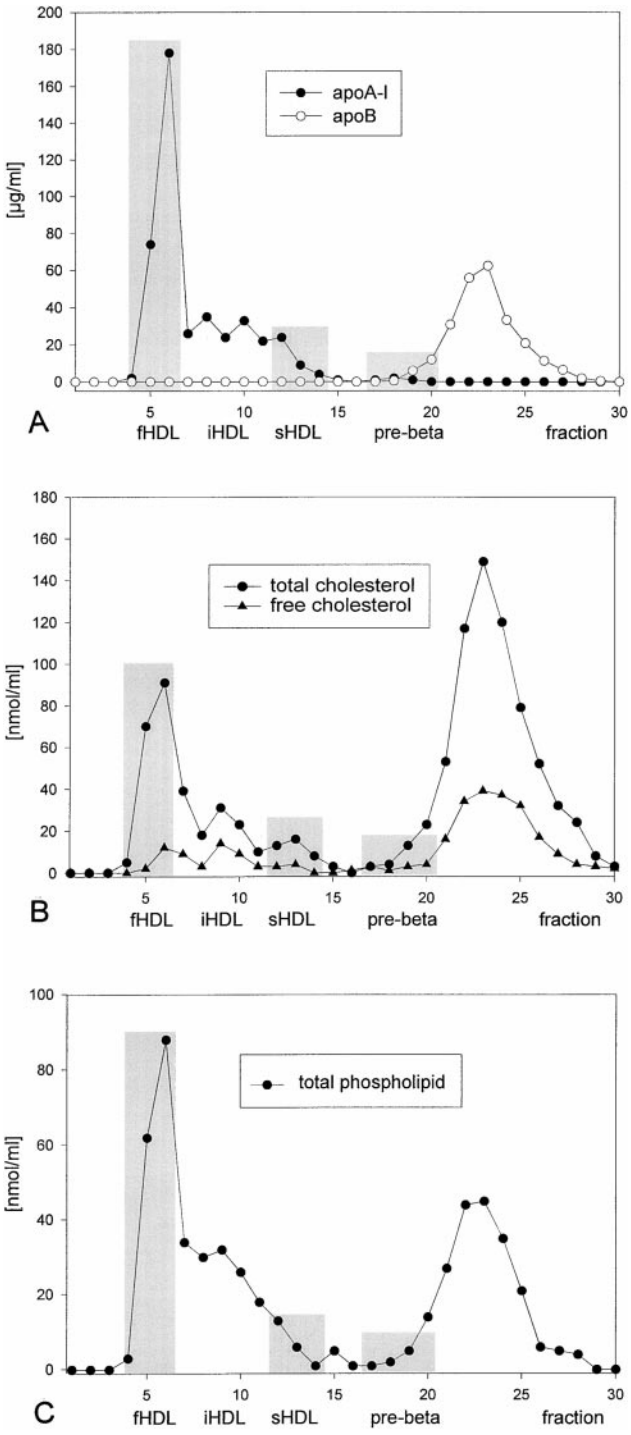

Fig. 4. Representative distribution of apoA-I and apoB (panel A), total and free cholesterol (panel B), and total phospholipids (panel C) in the fractions obtained by FS-ITP. The relative mobility associated with the individual fractions is indicated underneath each panel and by the shaded areas. It should be noted that even though there are minor differences in the pattern the general distribution of apoA-I, apoB, cholesterol, and phospholipids is very similar between different normolipemic individuals.

LpA-I and LpA-I:A-II particles are present in almost equal concentrations. On 2D-GGE with immunoblotting, especially in the fHDL region, there is a more prominent immunoreactivity for apoA-I than for apoA-II when the blot is probed successively with both antibodies.

ApoA-IV is mainly found in fractions with slow-α- and pre-β-mobility with maximal concentration in fractions 12 to 16 (Fig. 7B). 2D-GGE immunolocalization shows two regions with an apparent molecular mass of approx. 67 and 140 kDa with a widely varying electrophoretic mobility, and one spot with a more homogenous electrophoretic mobility, which overlaps the molecular mass range between the two bands. Interestingly, apoA-IV immunoreactivity is distinctly separated from the position of pre-β_1- and pre-β_2-HDL.

ApoE is detectable over a wide range in ITP-fractions 8 to 22 (Fig. 7C). Within HDL its maximal concentration is found in sHDL (fractions 12 to 14). A second maximum is in the apoB-containing fractions. ApoE particles were visualized in 2D-GGE immunoblots in three groups of different mobility. The majority of apoE is found in large HDL-sized particles with pre-β-mobility. Only a small proportion with slow-α-mobility overlaps with apoA-I-containing HDL. A significant amount of apoE was associated with particles that were too large to enter the gel and remained on top of the gel. These particles are apparently the source of apoE-containing particles with pre-β-mobility. The used anti-apoE antibody similar to all other commercially available apoE antibodies (data not shown) showed

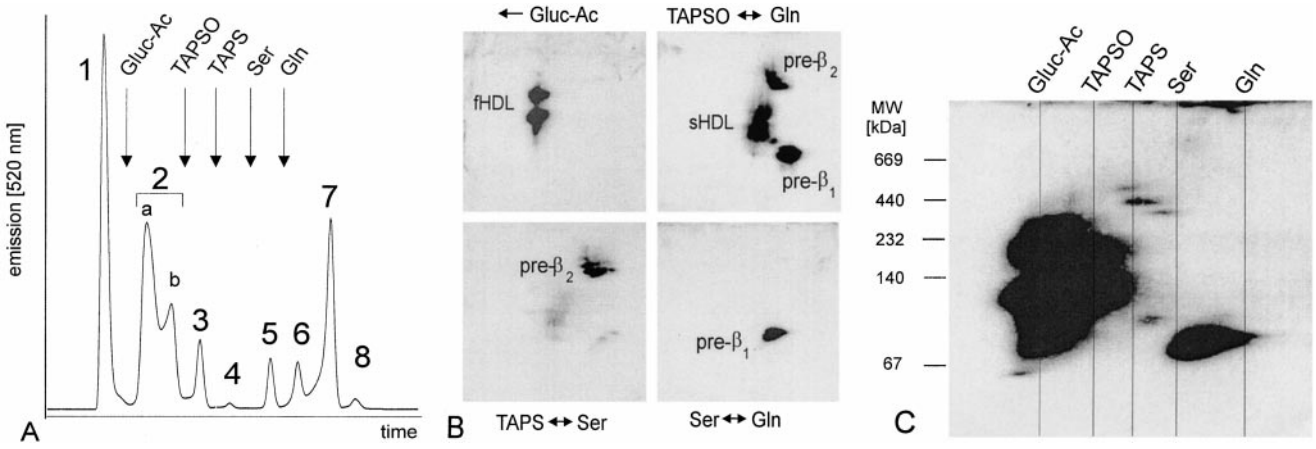

Fig. 5. In order to identify the position of the spacers used in analytical ITP and FS-ITP relative to the lipoprotein subfractions, plasma samples were spiked with surplus spacers. In panel A the position of the spacers relative to the ITP peaks is indicated. In panel B fractions isolated by the addition of surplus spacer as indicated were analyzed by 2D-GGE and immunoblotted for apoA-I. The fractions obtained in front of Gluc-Ac, between TAPSO and Gln, TAPS and Ser, and Ser and Gln are shown (panel B). The results of this experiment are summarized in panel C which represents a two dimensional gel of whole plasma immunoblotted for apoA-I. The relative position of the respective spacers is indicated.

unspecific crossreactivity with human immunoglobulins as indicated in the figure.

The distribution of apoD in ITP fractions is shown in Fig. 7D. ApoD is apparently associated with several distinct particle populations in iHDL and sHDL. A large proportion of apoD is also found at an apparent molecular mass of approximately 40–50 kDa with pre-β-mobility which could represent particles with apoD only or lipid-free apoD.

ApoC-III is found mainly in iHDL, sHDL, and the fractions containing triglyceride-rich lipoproteins. This in accordance with the distribution found by 2D-GGE (Fig. 7E). The majority of apoJ is found in the sHDL fractions. There is almost no apoJ detectable in the fractions containing triglyceride-rich lipoproteins and LDL (Fig. 7F). Factor H shows a distribution similar to that of apoJ with the majority in the sHDL-containing FS-ITP fractions. By 2D-GGE it appears that a significant proportion of factor H is associated with particles with even slower mobility than sHDL that do not contain apoA-I and are different from pre-β1 and pre-β2 HDL (Fig. 7G).

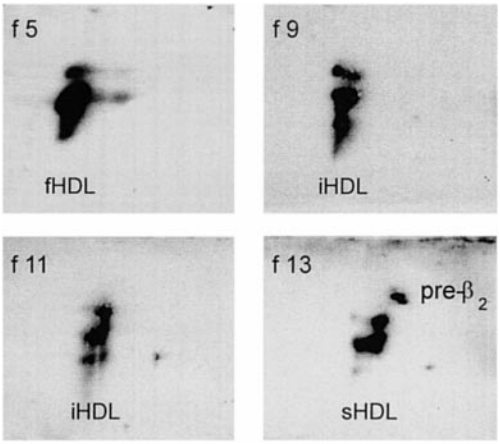

Fig. 6. Representative fractions obtained by preparative FS-ITP were subjected to 2D-GGE and immunoblotted for apoA-I. Compared to panel 5B in which fractions were separated by addition of specific spacers the fractions obtained by regular FS-ITP do not show much more overlap. This indicates that FS-ITP is a good means to characterize the peaks observed on analytical ITP.

DISCUSSION

Classically, lipoproteins are separated by sequential ultracentrifugation according to their buoyant density. However, during ultracentrifugation, components with an intermediate or low affinity to the lipoprotein particle will be lost. This problem is circumvented by chromatographic or electrophoretic techniques. Theoretically, ITP is the preferable electrophoretic technique because it has only negligible molecular sieve effects compared to agarose gel electrophoresis. Therefore, even large particles like chylomicrons can be analyzed in this system (35).

Analytical ITP separates lipoproteins reproducibly and quantitatively into several subclasses. Until now the biochemical analysis of ITP fractions was hampered by the lack of a preparative methodology with the same separation characteristics. FS-ITP, developed in our laboratory for the first time, permits reproducible preparative separation of proteins including lipoproteins under the same conditions as in analytical capillary ITP (27). It operates in batch mode with theoretically unlimited separation times and, unlike continuous flow systems, it ensures that the isotachophoretic steady state conditions will be achieved. FS-ITP can be applied to whole plasma like analytical ITP. The unavoidable mixing of the separated lipoproteins in the fractionation port can be overcome by adding surplus spacer, which improves the separation of specific fractions. With this approach the lipoprotein subclasses observed on analytical ITP can be purified for further biochemical analysis, which has not been feasible before. The

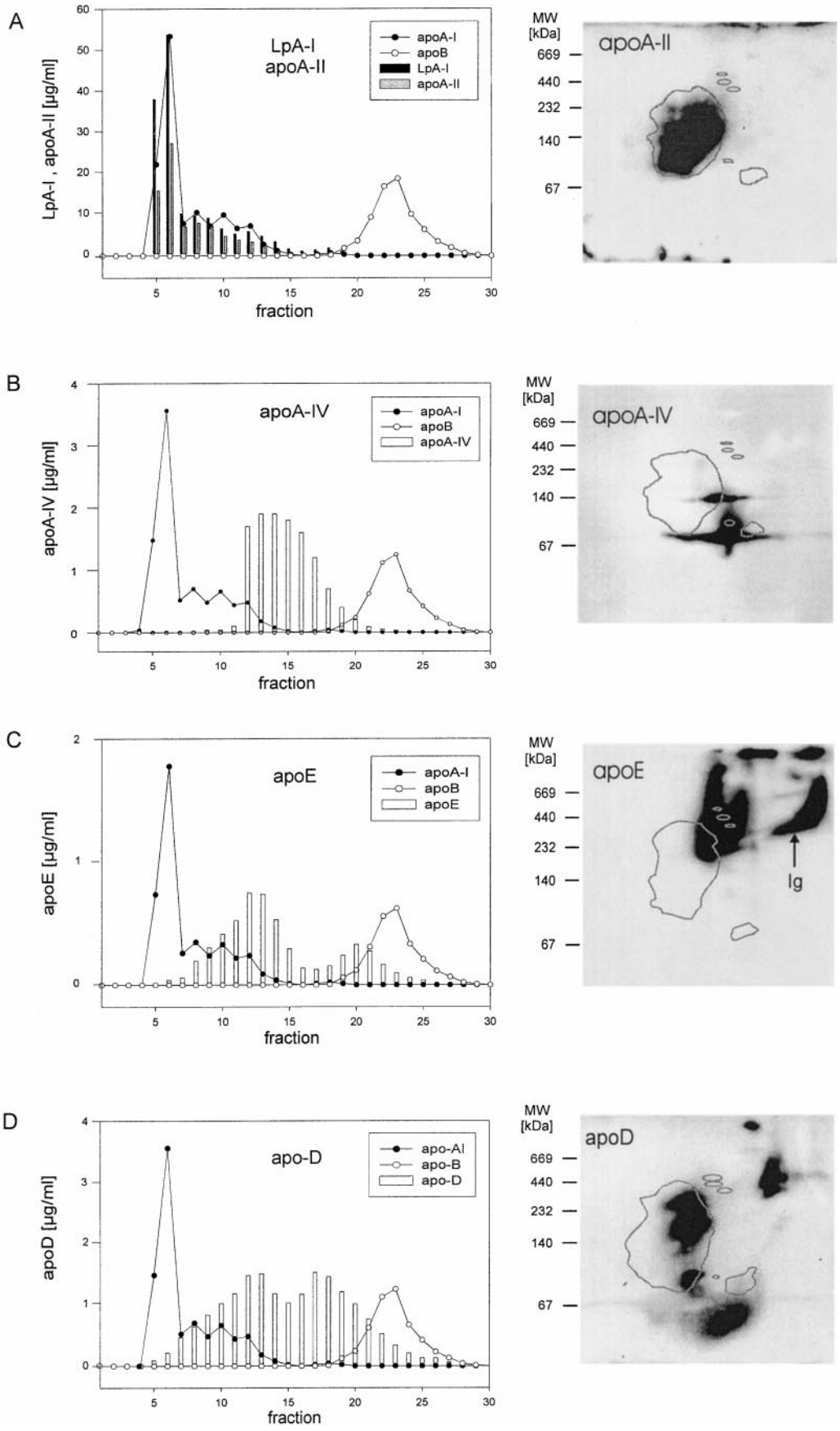

Fig. 7. (*Continued*)

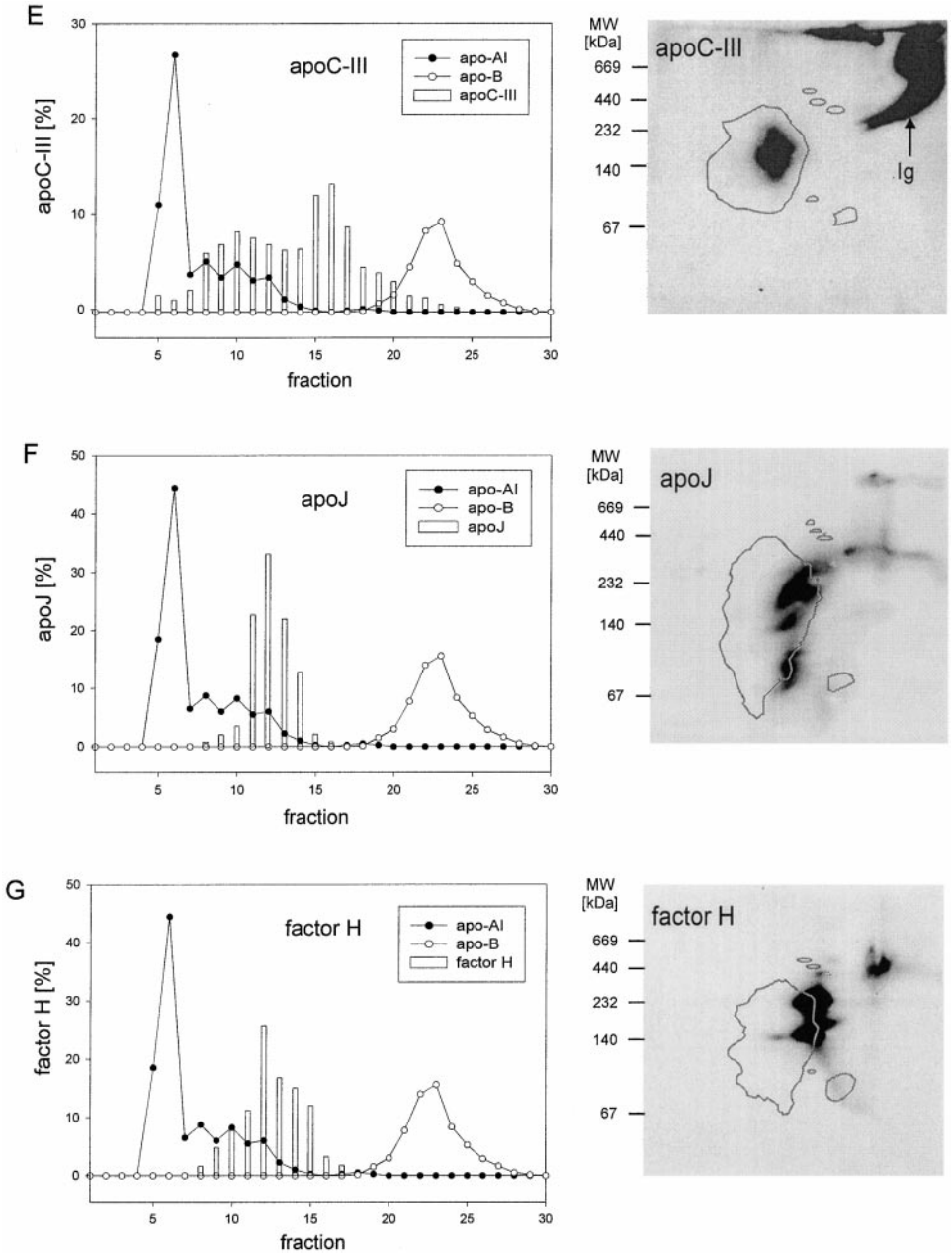

Fig. 7. Distribution of LpA-I and apoA-II in the fractions obtained by FS-ITP and immunoblot of 2D-GGE for apoA-II of normal plasma (panel A). The circled areas on the blot represent the immunoreactivity of apoA-I on the same blot. In panels B–G the equivalent data are shown for apoA-IV, apoE, apoD, apoC-III, apoJ, and factor H, respectively. The distribution for apoA-I and apoB as shown in Fig. 4A is included as a reference.

major aim of this study was the characterization of HDL subclasses separated by analytical ITP.

The first three peaks observed in analytical ITP represent HDL particles, because addition of purified HDL affects only these three peaks, and no apoB is found in the corresponding FS-ITP fractions. The three peaks were designated fHDL, iHDL, and sHDL. They contain mainly α-HDL. However, due to the lack of molecular sieve effects in ITP, they are not absolutely identical to α-HDL known from agarose gel electrophoresis. The three fractions defined in ITP differ in several relevant aspects. fHDL and iHDL contain the bulk of HDL and apoA-I. Compared to iHDL, fHDL have an increased ratio of LpA-I/LpA-I:A-II and an increased proportion of cholesteryl esters compared to unesterified cholesterol. The particle size of fHDL analyzed by 2D-GGE corresponds to HDL_{2a} and HDL_{2b}, which is on average larger than iHDL. However, there is considerable overlap and fHDL contains significant amounts of HDL_3. Apolipoproteins other than apoA-I and apoA-II (e.g., apoA-IV) were not detectable in fHDL or present only in trace amounts, e.g., apoE. sHDL contain several minor apolipoproteins such as apoA-IV, apoD, apoE, apoJ, and factor H. They also have an increased ratio of LpA-I/LpA-I:A-II. The presence of many different apo-

lipoproteins suggests that this fraction contains particles of specific functional relevance. The particular function of sHDL is also indicated by the presence of LCAT in this fraction (data not shown).

Besides α-migrating HDL particles, pre-β-HDL particles can be separated by ITP. Comparison with the mobility of the spacers indicates that peak 4 contains pre-β_2-HDL and peak 5 contains pre-β_1-HDL. However, peak 4 also contains chylomicrons and peak 5 VLDL and IDL, so that quantification of pre-β_2-HDL and pre-β_1-HDL is not feasible by analytical ITP as was indicated by Schlenck et al. (26). On the other hand FS-ITP permits fast prepurification of pre-β-HDL with substantially improved subsequent analysis by 2D-GGE. Therefore, FS-ITP provides a tool for preparative isolation of pre-β-HDL, perhaps combined with gel permeation chromatography.

The analysis of the association of specific proteins to the different HDL subclasses provided further information that confirmed and extended current knowledge. ApoA-II, the second major apolipoprotein of HDL was found exclusively in all α-migrating fractions that contained apoA-I immunoreactivity. In the pre-β-migrating HDL populations, no apoA-II was detectable. ApoA-IV was mainly found in particles with pre-β-mobility, the majority apparently not associated with apoA-I. This confirmed the existence of lipoprotein particles containing only apoA-IV and no apoA-I (20, 36, 37) consistent with an independent role of apoA-IV in reverse cholesterol transport (38).

In human plasma, apoE is almost entirely associated with lipoproteins containing apoB or apoA-I (39). However, several studies indicated that there are lipoprotein particles containing apoE as their sole apolipoprotein (17–19). These particles are either intermediate between LDL and HDL, as demonstrated by 2D-GGE (18, 19), or very small and having γ-mobility (17). The latter γ-LpE-particles have been claimed to be involved in reverse cholesterol transport (17). We observed apoE immunoreactivity in the γ-region, but its apparent molecular mass was >150 kDa, incompatible with γ-LpE. However, to firmly rule out the existence of a small γ-LpE particle would necessitate a much more detailed analysis, which was beyond the scope of the present study.

ApoD is found in all major lipoprotein fractions, with its majority in HDL particles that contain apoA-I and apoA-II (9). A major fraction of apoD appears to exist as a disulfide-linked heterodimer with other apolipoproteins, in particular apoA-II (40). Our data confirm these observations. In addition, it appears that there is a significant amount of apoD with pre-β-mobility and an apparent molecular mass of approximately 40–50 kDa not associated to any other apolipoprotein. This probably represents free apoD.

ApoC-III is found mainly in iHDL and sHDL with only little apoC-III in fHDL. This is consistent with the current view (41, 42). We did not detect any apoC-III in the pre-β_2-HDL particle population as has been reported (18).

By agarose gel electrophoresis, apoJ is found mainly with α_2-HDL particles and to a small extent with pre-β-HDL (43). ApoJ-containing particles are very heterogeneous in size and composition. There are particles with an apparent molecular mass >200 kDa as well as particles with an apparent molecular mass between 90 and 140 kDa (44). This correlates well with the results presented here, with the majority of apoJ in sHDL (Fig. 7), which also confirms previous ITP data (45).

Factor H-related proteins have been previously described to be associated with an HDL particle containing apoA-I, lipopolysaccharide-binding protein, and phospholipids (46). In addition, they have been found in triglyceride-rich lipoproteins (47, 48). The structural similarity of this protein family is so extensive that antibodies raised against complement factor H crossreact with the factor H-related proteins, precluding further differentiation. We found the majority of factor H immunoreactivity in the sHDL range. If factor H or related proteins are present in pre-β-HDL as described, this can only represent a very minor fraction of the protein. This apparent discrepancy could be caused by the use of one-dimensional separation which does not provide information on the particle size in previous work (46).

In summary, ITP is an alternative method to separate lipoproteins according to their electrophoretic mobility for analytical or preparative purposes. FS-ITP offers many advantages over the previously described preparative methods. In particular, FS-ITP is superior to agarose electrophoresis, as it has a larger capacity and does not need a step to elute lipoproteins from the gel matrix. The compositional differences between fHDL, iHDL, and sHDL particles suggest that they fulfill different tasks in lipoprotein metabolism and particularly in reverse cholesterol transport. As sHDL contain most of the cholesterol esterifying activity and fHDL are enriched in cholesteryl esters, one might speculate that there is a precursor–product relationship between these subfractions. However, this assumption can only be proved by metabolic studies. The association of several minor apolipoproteins with the sHDL fraction suggests that this fraction may serve specialized functions which are so far only poorly understood. It is important to note that ITP is the first method that permits a quantitative separation of the different α-migrating HDL particles. ITP may permit a more detailed analysis of lipoprotein subfractions in clinical routine, as it lends itself to automation and is currently already in use for serum or plasma protein electrophoresis. Further investigations in specific patient groups will be performed to evaluate the diagnostic implications of analytical ITP.

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