Two-Dimensional Electrophoresis of Human Plasma Apolipoproteins

Dennis L. Sprecher, Lila Tamm, and H. Bryan Brewer, Jr.

The two-dimensional electrophoretic method with silver staining describe better resolves plasma apolipoproteins (apo) than any procedure previously described. It can be used to screen for abnormalities in apoA-I, apoA-II, apoA-IV, apoC-II, apoC-III, apoD, apoE, and apoH. In addition, this is the first presentation of apoD and apoH on two-dimensional gels. This electrophoretic method will permit the quantification of plasma apolipoproteins by computer imaging. Such detailed analysis of the plasma apolipoproteins should provide a better understanding of apolipoprotein function and the role these apolipoproteins play in lipoprotein metabolism and the pathophysiology of dyslipoproteinemias and coronary artery disease.

Additional Keyphrases: heritable disorders • screening • atherosclerosis • heart disease • dyslipoproteinemia • apolipoproteins D and H

Apolipoproteins are the protein moieties of lipoprotein particles. Over the last several years apolipoproteins have been extensively investigated and have been shown to modulate the activity of enzymes that are important in lipid metabolism (e.g., lipoprotein lipase, lecithin-cholesterol acyltransferase) and to play a pivotal role, on lipoprotein particles, as the ligand for receptor-lipoprotein interactions (1). In addition, apolipoprotein concentrations both in plasma and in separated lipoprotein fractions have been found to correlate highly with the development of coronary artery disease in certain populations (2, 3).

Familial absence as well as structural defects in plasma apolipoproteins leading to clinical dyslipoproteinemias have now been recognized. Molecular defects in plasma apolipoproteins, therefore, are analogous to the hemoglobinopathies that have previously been shown to be ascribable to amino acid substitutions, insertions in the hemoglobin genes, and defects in post-translational modifications (4).

Structural defects in the plasma apolipoproteins may affect either the charge or the molecular mass of the protein. Two-dimensional electrophoresis, which characterizes proteins by both of these characteristics, may be used to identify up to 30% of the known amino acid substitutions (5).

The purpose of this study is to present a relatively simple two-dimensional electrophoretic method for evaluating plasma apolipoproteins A-I (apoA-I), A-II, A-IV, C-II, C-III, D, E, and H. In addition, apoC-I may be evaluated by using an extended pH range and apoB-48 as well as apoB-100 by use of 3% polyacrylamide SDS gel electrophoresis. We find this technique effective in screening for molecular defects in the plasma apolipoproteins in patients with dyslipoproteinemias and atherosclerosis.

Over the years, various nomenclatures have been used to identify the plasma lipoproteins that are resolved on one-dimensional and two-dimensional gel electrophoresis (6–8). Inconsistency and lack of a standardized nomenclature for the plasma apolipoproteins have resulted in confusion in the literature. Therefore, we have adopted a nomenclature for the apolipoproteins that is consistent with the nomenclature currently standardized for identification of proteins separated by two-dimensional electrophoresis (9–11).

Materials and Methods

Samples. Blood was collected in tubes containing Na2EDTA (final concentration, about 1 g/L) as anticoagulant. The erythrocytes were removed and the plasma was stored at 4°C for as long as two weeks before analysis.

For two-dimensional electrophoresis we used plasma or isolated lymph chylomicrons, VLDL, and HDL. Human thoracic duct lymph was collected and chylomicrons were separated by centrifugation in an SW 27 Beckman swinging-bucket rotor for 115 min at 25,000 rpm and 4°C. VLDL was isolated by ultracentrifugation (12) of 5 mL of plasma in a 40.3 Beckman rotor (Beckman Instrument Co., Fullerton, CA) at 39,000 rpm (= 110,000 × g) at 4°C for 18 h. HDL was isolated by ultracentrifugation between relative densities of 1.063 to 1.21 (4°C, 36 h, 39,000 rpm).

Lipoprotein fractions were recovered by tube slicing, desalted by chromatography on a 2 × 5.5 cm column of Sephadex G-25M (PD-10; Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with an 0.8 g/L solution of ammonium bicarbonate. Lipoproteins were lyophilized and delipidated with chloroform/methanol (2/1 by vol) (13). The protein pellet was dried under a stream of nitrogen, dissolved by stirring in freshly prepared 8 mol/L urea containing 1 mg of DTT per liter, and then incubated for 30 min at 37°C.

Plasma samples were prepared as reported by O'Farrell (14), with minor modifications. Two microliters of plasma were placed in a "detergent solution," containing, per liter, 20 g of SDS, 50 mL of β-mercaptoethanol, 200 mL of glycerol, 20 mL of Nonidet-P40 detergent, 60 g of CHAPS, and 50 mL of ampholytes (pH 5–7 and 4–6, 1/2 by vol; Serva, Heidelberg, F.R.G.). Stored at −20°C, this solution was stable for as long as three months. For analysis, we added 10 μL of detergent solution to 2 μL of plasma, then 8 mg of solid urea, and followed this with intermittent vortex-mixing for the next 20 min, at room temperature.

For treatment with neuraminidase (EC 3.2.1.18) (15) we mixed 100 μL of plasma with 100 μL of a buffer containing 1 U of neuraminidase (from Clostridia perfringens; Sigma Chemical Co., St. Louis, MO), and, per liter, 0.1 mmol of potassium monophosphate (pH 4.5) and 1 mmol of calcium chloride. The pH was adjusted to 5.0 with HCl and the mixture was incubated at 37°C for 4 h. We then added 4 μL of this solution to 10 μL of the detergent solution, followed by 9 mg of solid urea.
Two-dimensional gel electrophoresis was performed similarly to the methods described previously (9, 16) with modifications. Polyacrylamide gels (3% of acrylamide and 88 mg of bisacrylamide dissolved in 38.5 mL of freshly prepared 8.6 mol/L urea) for isoelectrofocusing were prepared in 1.75 x 95 mm glass tubes with 1.5 mL of the above-mentioned ampholytes. Ammonium persulfate (75 μL of an 180 mL/L stock solution) and 40 μL of N,N,N',N'-tetramethylethylendiamine (Bio-Rad Lab., Richmond, CA) were used as polymerization catalysts. Gels were polymerized for 30 min, and either the plasma mixture described above or delipidated lymph chylomicrons (35 μg), VLDL (30 μg), or HDL (50 μg) dissolved in urea and DTT was placed on the gel and carefully overlaid with NaOH, 20 mmol/L. Solutions in the upper tray and the lower tray were 20 mmol/L NaOH and 10 mmol/L H3PO4, respectively. For plasma, the gels were isoelectrofocused (at 4°C) at 250 V for the first 2 h, 500 V for the next 15 h, and 1000 V for the last 2 h, a total of 10,000 V · h. Isolated delipidated lipoprotein fractions were electrophoresed at 250 V for 16 h at 4°C.

After isoelectrofocusing, gels were either rapidly frozen on solid CO2 and stored (as long as a month) or placed for 10 min at room temperature in an equilibration solution containing (per liter) 0.1 mol of Tris (pH 6.8), 10 g of SDS, and 25 mL of β-mercaptoethanol. The gels were then loaded on the top of a 11 cm x 12 cm x 0.75-mm-thick polyacrylamide slab gel (per liter, 150 g of acrylamide, 1 g of bisacrylamide, and 1 g of SDS). A stacking gel (4.0% T, 2.7% C) was used for the analysis of plasma samples. The gels were electrophoresed until the dye front was 5 mm from the bottom of the gel (approximately 3.5 h, 10 mA per gel for stacking; 20 mA per gel for running gel; 4°C) and then stained in Coomassie Blue or silver stain. We stained with Coomassie Brilliant Blue R250 (1 g/L) after fixing the gels in acetic acid/methanol/water (5/60/45 by vol). We destained them in 50 mL/L methanol and 75 mL/L acetic acid until a clear background was obtained. The silver stain used for the plasma gels was similar to that reported by Merrill et al. (17) and Morrissey (18).

Immunoblotting was performed as previously described (19). Plasma gels were blotted at 80 V for 1 h onto nitrocellulose sheets. Isolation of antibodies to apoA-II and apoD has been previously reported (20, 21). Second antibody was used as described in manufacturer’s (Bio-Rad Lab.) instructions.

Results

Table 1 lists the major human plasma apolipoproteins and some of their physico-chemical properties. Figure 1 shows a gel on which the human plasma apolipoproteins are resolved by use of a narrow pH range (4.0-7.0). Figure 2 illustrates an electrophoretogram for a sample preincubated with neuraminidase (EC 3.2.1.18). ApoB does not appear on these gels, owing to its high molecular mass, nor does apoC-I, whose pl is outside the 4-7 pH range.

Figures 3, 4, and 5 illustrate two-dimensional electrophoretograms of the apolipoproteins present in isolated lymph

![Image](file)

**Fig. 1. Two-dimensional electrophoretogram of human plasma, pH 4-7, with specific apolipoproteins circled**

![Image](file)

**Fig. 2. Two-dimensional electrophoretogram of human plasma treated with neuraminidase, allowing easier identification of apoA-IV, apoE, and the desialylated form of apoD**

ApoH migrates off the basic end of the electrophoretogram.
plasma have been correlated with increased risk of premature coronary artery disease (30).

Mature apoA-I is polymorphic in plasma (Figure 1). The major isofrom in plasma, designated apoA-I₀, has been previously designated in the literature as apoA-I₉ (26), apoA-I₇ (7), or apoA-I₁ (31). ApoA-I₁ in plasma and lymph is proapoA-I (Figure 6) (26). The minor isofroms at apoA-I₁ and apoA-I₀ are primarily de-amidated proapoA-I and mature apoA-I, respectively.

Several apoA-I variants have now been recognized. The most extensively studied is apoA-I/Tangier (32, 33), characterized by a marked increase in apoA-I₁₋₁ or proapoA-I/Tangier (Figure 6). Several additional apoA-I variants identified by two-dimensional electrophoresis are summarized in Table 2 (31, 34–36). ApoA-I variants may be associated with subnormal concentrations of HDL and with premature cardiovascular disease.

ApoA-II

ApoA-II, the second major protein of HDL, is biosynthesized as a 100-residue precursor protein, preapoA-II (37). PreproapoA-II is co-translationally cleaved to proapoA-II, which contains a pentapeptide attached to mature apoA-II (38). Mature apoA-II exists as a 77-residue monomer (apoA-II/monomer) and a 154-residue dimer (apoA-II/dimer) in which two monomers are linked by a disulfide bridge at position 6 in the sequence (20). ApoA-II has been localized to the p21-qter region of human chromosome 1 (39). No definitive function of apoA-II has been established. ApoA-II activates hepatic lipase in vitro (40), but the physiological importance of this observation remains to be established. ApoA-II concentrations in plasma reportedly are inversely associated with the development of premature cardiovascular disease (41).

Mature apoA-II is also polymorphic in plasma (Figure 1) (42), and the major isofrom is designated apoA-II₀. The modifications of apoA-II responsible for the isofroms seen immediately to the acidic and basic sides of apoA-II₀ (Figure 7) are unknown. Analysis of the apoA-II isofroms separated on two-dimensional gel electrophoresis at pH 3.0–10.0 by the immunoblot technique revealed an additional isofrom of apoA-II, designated apoA-II₁₋₁ (Figure 7), now known to be the proform, proapoA-II (43).

ApoA-IV

ApoA-IV, a 393-residue protein, Mₐ 46,000, is present on triglyceride-rich lipoproteins and may be involved in their metabolism (44, 45). ApoA-IV has been provisionally assigned to chromosome 11 (46). The major apoA-IV isofrom,
Table 2. ApoA-I Variants

<table>
<thead>
<tr>
<th>(Acidic)</th>
<th>ApoA-I</th>
<th>ApoA-I \text{Milano} (Arg_{173} \rightarrow \text{Cys})</th>
<th>ApoA-I \text{Munster-2} (Lys_{107} \text{ deletion})</th>
<th>ApoA-I \text{Marburg} (Lys_{107} \text{ deletion})</th>
<th>ApoA-I \text{Giessen} (Pro_{143} \rightarrow \text{Arg})</th>
<th>ApoA-I \text{Munster-3} { (Asp_{103} \rightarrow \text{Asn}) }</th>
<th>ApoA-I \text{Tangier}</th>
<th>ApoA-I + \text{ApoC-III Deficiency}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-2</td>
<td>-1</td>
<td>0</td>
<td>+1</td>
<td>+2</td>
<td>+3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I \text{Milano} (Arg_{173} \rightarrow \text{Cys})</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I \text{Munster-2} (Lys_{107} \text{ deletion})</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I \text{Marburg} (Lys_{107} \text{ deletion})</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I \text{Giessen} (Pro_{143} \rightarrow \text{Arg})</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I \text{Munster-3} { (Asp_{103} \rightarrow \text{Asn}) }</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I \text{Tangier}</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-I + \text{ApoC-III Deficiency}</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ApoA-I variants, indicated above with amino acid substitutions, are all represented symbolically in the table as the reported heterozygote, i.e., products of one normal and one abnormal allele. Isoforms represented by dots to the basic side of the mature form(s), are the proforms, two positive charge units away from the corresponding mature form(s).

Fig. 7. Immunoblot of apoA-II, separated on two-dimensional electrophoresis (pH 3–10), revealing multiple isoforms of apoA-II in human plasma.

ApoA-IV₈, has a pI of 5.50. Two minor isoforms, apoA-IV₋ and apoA-IV₊ (number of charge units away from the major form unknown), are present in plasma. In approximately 10% of the German population, the major isoform of apoA-IV migrates as a doublet, with an additional isoform presumably one charge unit to the basic side (Figure 8), and has been designated apoA-IV_{Marburg} (31). Several structural apoA-IV variants have been reported (8), but the significance of these isoforms on apoA-IV function is as yet unknown (Table 3).

ApoB

Human apoB can be separated into two forms, apoB-48 and apoB-100, which are synthesized by the intestine and liver, respectively (47–49). ApoB-100 interacts with the LDL or apoB,E receptor (50). ApoB-48, a major protein of chylomicrons, may function as a structural protein for lipoprotein particles secreted by the intestine. Deficiency of apoB-100 and apoB-48 results in abetalipoproteinemia and homozygous hypobetalipoproteinemia, a condition in which the only lipoprotein in plasma is HDL (51). ApoB-100 and apoB-48 are not visible on two-dimensional electrophoreograms of plasma, owing to their large size. Rather, they are most readily separated and identified on SDS-agarose gel electrophoresis (Figure 9) (52).

ApoC-I

ApoC-I is synthesized as a 83-residue precursor protein, preapoC-I (53). Mature apoC-I, which contains 57 amino
ApoC-II and ApoC-III

**ApoC-II Deficiency**

Plasma ApoC-II and ApoC-III

(Neuraminidase treated)

Fig. 11. Two-dimensional electrophoreograms (left) of apoC-II and apoC-III with and without neuraminidase treatment; right, results of similar analysis and treatment plasma from a patient with apoC-II deficiency

Note that the –1 and –2 isoforms of apoC-III disappear with desialylation. In addition, the apoC-II deficient patient has a low concentration of apoC-II, which migrates to an abnormal electrophoretic position. The dashed circle illustrates the relative electrophoretic position of normal apoC-II.

**ApoC-III**

ApoC-III is synthesized as a 99-residue protein, preapoC-III (68). This is co-translationally cleaved to yield mature apoC-III, which is present in plasma as three isoforms containing no, one, or two neuraminic acid residues. The major desialylated isoform, apoC-III0, is a minor isoform in plasma. The two predominant isoforms, apoC-III-1 and apoC-III-2, contain one and two sialic acid residues, respectively (69) (Figure 11). The pl of the apoC-III0, apoC-III-1, and apoC-III-2 isoforms are 4.9, 4.65, and 4.5, respectively. Figure 3 also illustrates additional minor apoC-III isoforms with lower isoelectric points. ApoC-III prevents lipoprotein uptake by the perfused rat liver, which suggests that it may modulate the metabolism of triglyceride-rich lipoproteins (70, 71).

Patients with a deficiency of apoA-I+apoC-III and severe atherosclerosis have been reported (36, 72). Patients with apoA-I+apoC-III deficiency have relatively normal concentrations of LDL in their plasma, but HDL are virtually absent. One kindred of patients with apoA-I+apoC-III deficiency is characterized by an insertion in the apoA-I gene (73). Restriction enzyme analysis of the genomic DNA of a second kindred with apoA-I+apoC-III deficiency has shown no major deletions or insertions in either the apoA-I or apoC-III gene (72).

**ApoD**

ApoD is a glycoprotein containing 18% carbohydrate by weight. Its Mr is approximately 32 000 (21, 74). ApoD is mostly present on HDL. It may function with LCAT and apoA-I in the esterification of cholesterol and the transfer of cholesterol esters from HDL to VLDL (75).

ApoD is polymorphic in plasma, the six isoforms being primarily ascribable to carbohydrate heterogeneity (Figure 12). The major isoform of desialylated apoD, apoD0, has a pl of 5.3 (Figure 2). The electrophoretic relationship of apoA-I, apoD, and apoA-I, as evaluated by the immunoblot technique, is illustrated in Figure 12.
ApoE

ApoE, a 299-residue glycoprotein, is synthesized as an amino acid precursor protein, preapoE (76, 77). PreapoE undergoes co-translational cleavage of the 18-residue pre-peptide to mature apoE, which is secreted into plasma as a sialylated apolipoprotein. Newly secreted sialylated apoE loses the sialic acid residues, and the predominant apoE isoforms in plasma contain no sialic acid (Figures 1 and 2).

ApoE is located at a single genetic locus, and there are three common alleles, designated e2, e3, e4 (Figure 13). As a result, six common apoE phenotypes are present in the population: E2/2, E3/3, E4/4, E2/3, E2/4, and E3/4 (6). The common apoE isoforms differ at two positions in the amino acid sequence (i.e., positions 112 and 158), apoE-2 containing Cys 112 and Cys 158, apoE-3 Cys 112 and Arg 158, and apoE-4 Arg 112 and Arg 158. The most common allele, e3, is considered to be a normal allele. The locus for apoE may be located on human chromosome 19 (79).

ApoE is also considered important in the metabolism of triglyceride-rich lipoproteins and to bind in vitro to a specific membrane receptor in the liver (79). The E2/2 phenotype is associated with type III hyperlipoproteinemia (80—82). Previous studies have established that the apoE-2 isoform isolated from type III patients has delayed catabolism (81) and defective binding to the cellular apoE receptor (82). The several additional structural variants of apoE reported are summarized in Table 4 (16, 83—90). A recently identified kindred with phenotype E1/3 is also presented (Figure 13).

The nomenclature of apoE has been confusing and has recently been reviewed (6). In the present report, we locate apoE-3, the predominant normal plasma isoform, at the designated apoE0 position, for consistency with the nomenclature for proteins separated by two-dimensional electrophoresis. The other two major isoforms, apoE-2 and apoE-4 are located at positions designated apoE−1 and apoE−2, respectively. However, the previous apoE-2, apoE-3, and apoE-4 designations are also included in parentheses in the two-dimensional electrophoretograms in Figure 12. The importance of the standardized nomenclature for apolipoproteins separated by electrophoresis is particularly relevant to the categorization of apoE isoforms, which have charge differences based on amino acid substitutions as well as post-translational modifications. Many variants, therefore, may have a similar electrophoretic position on two-dimensional gel electrophoresis, but may not necessarily be the identical protein; e.g., an apoE−1 isoform may be apoE-2 or a de-amidated form of apoE-3. Therefore, two different structural apoE isoforms may be located at the same electrophoretic position, and amino acid sequence analysis may be required to differentiate the two different isoforms. The potential for different E apolipoproteins to be located at the same electrophoretic position illustrates the need for the
standardized designation of apoE isoforms based on unit charge with an accompanying designation in parentheses as the sequence and (or) post-translational modifications of the isoform become known.

**ApoH**

The glycoprotein ApoH ($M_r$ 46 000) is also designated $\beta_2$-glycoprotein-I ($91, 92$). Reportedly, it modulates the activity of lipoprotein lipase in vitro ($92$). Identification of sialylated apoH on the electrophoretogram of plasma is difficult because of the streaking of the albumin bands (Figure 1). Treatment of apoH with neuraminidase shifts the electrophoretic position of apoH to a $pI = 9.5$ (Figure 14). Desialylated apoH is not visible on the electrophoretogram in Figure 2 because of the narrow $pH$ range.

**Discussion**

The analytical system we describe is sensitive and reproducible for evaluating apolipoproteins in normal subjects and patients with dyslipoproteinemia. Variants, as well as deficiencies of individual apolipoproteins, can now be readily identified in human plasma. This procedure can also be used to study the biosynthesis of apolipoproteins by cells, in addition to intracellular and extracellular processing and modification of apolipoproteins. Of particular interest is an evaluation of the coordinated control of the biosynthesis of several different apolipoproteins and the factors that modulate lipoprotein biosynthesis.

By using a narrow $pH$ range ($pH$ 4–7) and a sensitive silver stain, we have enhanced the resolution of apolipoproteins. Complications induced by protein–protein interactions via disulfide bonds are eliminated by reducing the sample before the analysis. (Mixed disulfides are particularly important in apoE–apoA-II dimers and apoE–apoE dimers.) Reduction of the sample also significantly increases the quantity of monomer apoA-II and apoE.

In the present study, we identified two new proteins on the plasma two-dimensional gel electrophoregrams: apoD and apoH. Both are glycoproteins, with many polymorphic isoforms present. Most of the isoforms are eliminated by treatment with neuraminidase.

Use of computerized gel-image systems for apolipoprotein quantification will provide additional information on plasma apolipoprotein concentrations and isoform distribution in normal subjects and patients with dyslipoproteinemia. This quantification technique is currently under investigation.

We extend our gratitude to Rosemary Ronan for the isolation of the apolipoproteins, to the NIH graphic arts department and the photography department (particularly R. V. Dryfus) for their excellent technical assistance, and to Imogene Surrey for typing this manuscript.

**References**

10. Anderson NL, Hickman BJ. Analytical techniques for cell


52. Gabeli C, Stark D, Gregg RE, Brewer HB Jr. Separation of apolipoproteins B-100 and B-48 by agarose polyacrylamide gel electrophoresis (manuscript submitted).


57. LaRosa JC, Levy RI, Herbert PN, et al. A specific apoprotein...
84. Rall SC Jr, Weisgraber KH, Mahley RW. Human apolipoprotein E: The complete amino acid sequence. Ibid. 257, 4171–4178 (1982).