short communications

Quantification of apoC-II and apoC-III of human very low density lipoproteins by analytical isoelectric focusing

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Abstract ApoC-II and apoC-III of human very low density lipoproteins (VLDL) have been quantified by analytical isoelectric focusing (IEF) between pH 4 and 6 in polyacrylamide gels containing 8 M urea. The isoelectric point of apoC-III_0 is pH 4.93; apoC-II, pH 4.78; apoC-III_1, pH 4.72, and apoC-III_2, pH 4.54. ApoC-I is not found in the pH range between pH 4 and 6. Two minor peptides, apoC-IV and apoC-V, with isoelectric points of pH 4.61 and 4.44, respectively, are apoproteins not previously identified. The sensitivity (5–40 μg) and reproducibility (±8%) of this method allow quantitative analysis of apoC-II and apoC-III distribution in VLDL.

Supplementary key words VLDL subclasses · C-IV and C-V apoproteins

Human plasma very low density lipoproteins (VLDL) contain several proteins, or apoprotein components, apoC-I, apoC-II, apoC-III, apoB, and apoE, the arginine-rich protein (1). The complete amino acid sequence of apoC-II has recently been established by this laboratory (2). This apoprotein is required for the activation of human adipose tissue lipoprotein lipase (triglyceride hydrolase), a key enzyme in the catabolism of chylomicrons and VLDL (3). Several reports suggest that the activation of lipoprotein lipase by apoC-II is inhibited by apoC-III (4, 5). Carlson and Ballantyne (6) found that the ratio between apoC-II and apoC-III was decreased in subjects with Type IV or Type V hyperlipoproteinemia (HLP) compared to normolipemic individuals. However, in these experiments, apoC-II and apoC-III_0, the form of apoC-III without sialic acid, were not resolved. Kashyap et al. (7) have also reported a decreased amount of apoC-II as percentage of total VLDL protein in subjects with Type IV HLP. These studies raise the possibility that the optimal activation of lipoprotein lipase depends critically on the concentration of apoC-II and/or a certain ratio of apoC-II and apoC-III. The purpose of this report is to describe a rapid and sensitive method of analytical isoelectric focusing to quantify both apoC-II and apoC-III in human VLDL.

MATERIALS AND METHODS

Chemicals

The electrophoresis reagents were obtained from Bio-Rad (Richmond, CA) and ampholine solutions from LKB (Rockville, MD). All other chemicals used were reagent grade. Aqueous solutions of urea were deionized immediately before use by passage through Rexyn I-300.

Isolation of lipoproteins and apolipoproteins

Blood was collected in EDTA, 1 mg/ml, from fasting, male normolipemic subjects; plasma was prepared

Abbreviations: IEF, isoelectric focusing; pI, isoelectric point; HDL, high density lipoproteins; VLDL, very low density lipoproteins; apoC, the low molecular weight apoproteins of VLDL and HDL; apoE, the apoprotein of VLDL characterized by a high arginine content; TMU, tetramethylurea; SDS, sodium dodecyl sulfate; HLP, hyperlipoproteinemia.

by low speed centrifugation at 4°C. VLDL was isolated by ultracentrifugation at plasma density at 40,000 rpm for 18 hr at 14°C in a 60 Ti rotor (8). VLDL subfractions, $VLDL_1$ (S_f 100–400), $VLDL_2$ (S_f 60–100), and $VLDL_3$ (S_f 20–60), were obtained by density gradient centrifugation in a 40.1 SW rotor as described by Lindgren et al. (9). Separation of VLDL on the basis of size was validated by electron microscopy after negative staining with 2% potassium phosphotungstate (10). The mean diameters of $VLDL_1$, $VLDL_2$, and $VLDL_3$ ($n = 200$ for each fraction) were 66.2 ± 11.0, 46.5 ± 7.5, and 38.0 ± 6.0 nm, respectively, in two different preparations. After dialysis at 4°C against 0.15 M NaCl containing 0.1 mM EDTA and 0.01 M Tris, pH 7.4, the VLDL was lyophilized and the lipids were extracted with ether–ethanol 3:1 (v/v) at 4°C as previously described (11); apoproteins were dried under a stream of nitrogen and stored at −70°C until used. Apoprotein recovery after delipidation ranged between 95 and 101% on four preparations, determined by solubilization of the dried apoproteins in 0.1 M NaOH (12). Apoproteins were also prepared for IEF by delipidization of VLDL with tetramethylurea (TMU) (13, 14).

The absence of apoproteins other than apoB in the TMU precipitate was confirmed by amino acid analysis of the TMU precipitate from VLDL and LDL after chloroform–methanol extraction and by the absence of other apoproteins when the TMU-insoluble material was subjected to SDS-gel electrophoresis. The individual apoC proteins were isolated by chromatography of delipidated VLDL (apoVLDL) on Sepharcryl S-200 and rechromatography of the apoC fraction on DEAE-cellulose (15). In a typical fractionation experiment, 200 mg dry weight of apoVLDL was dissolved in 10 ml of 0.01 M Tris-HCl, pH 7.5, containing 3 M guanidine-HCl. After stirring for 12 hr at 23°C, the insoluble apoB was removed by centrifugation for 15 min at 20,000 rpm. The soluble fraction was then chromatographed on a 2.5 × 150-cm column of Sephacryl S-200 equilibrated with the Tris–guanidine buffer. Two fractions were obtained; the second fraction containing apoC was dialyzed against 0.01 M NH_4HCO_3 at 4°C and lyophilized. After solubilization in 6 M urea containing 0.01 M Tris, pH 8.5, the apoproteins were fractionated at 4°C on a 1.5 × 30-cm column of DEAE-cellulose (Whatman), equilibrated with 0.01 M Tris, pH 8.5, containing 6 M urea, with 500 ml of a linear gradient from 0 to 0.125 M NaCl. The major components obtained in the order of elution were apoC-I, apoC-II, apoC-III_1, and apoC-III_2. The amino acid analysis and sialic acid determinations were in agreement with previously published values (15).

VLDL protein was determined by the method of Lowry et al. (12); 0.1% SDS, w/v, was added to clarify the samples. Concentrations of the individual apoproteins used as standards were determined by amino acid analysis.

IEF in polyacrylamide gels

Immediately before analysis, the apoproteins, 50–150 μg of protein, were incubated at room temperature for 45 min in 100 μl of 8 M urea containing 0.01 M Tris, pH 8.5. The sample density was increased with 20 μl of 40% sucrose. In some experiments, samples of VLDL were delipidated with 4.2 M TMU (14). Gels for IEF were prepared by the following procedure. A 3-ml portion of an aqueous solution of 30% acrylamide and 0.8% bisacrylamide was combined with 0.7 ml of ampholines in the desired pH range. To this mixture were added 5.12 g of urea, 10 μl of tetramethylethylenediamine, and 3.9 ml of distilled H_2O; the solution was degassed under vacuum for 15 min. $(NH_4)_2S_2O_6$, 0.4 ml of a 14 mg/ml solution prepared freshly for each experiment, was then added. Ten-cm gels were cast in 0.5 × 12-cm tubes. The anode solution was 0.01 M phosphoric acid; the cathode solution was 0.2 M NaOH. Excess $(NH_4)_2S_2O_6$ was removed, and the pH gradient was partially established by electrophoresis at 1.5 mA per tube for 1 hr. The sample, 120 μl, was applied by layering under the electrolyte solution. The IEF was performed at 400 V for 18 hr at 4°C. In our experience, the shorter separation time (4 hr) used by Pagnam et al. (16) does not produce a linear pH gradient. To determine the pH gradient after 18 hr, 2-mm segments of unfixed and unstained gels were transferred into a glass tube containing 1 ml of distilled water. After 24 hr at 4°C, the pH of each segment was determined.

The gels were simultaneously fixed and stained for 4 hr at 27°C with a subfraction of Coomassie blue as described by Malik and Berrie (17); they were stored at least 12 hr in a 7% aqueous acetic acid solution in the dark. By this procedure no destaining was required. Optical density at 560 nm was scanned on a Gilford spectrophotometer. The areas under the peaks were determined by planimetry. No differences between four different Coomassie blue staining solutions were found. This IEF procedure is similar to that developed for rat VLDL (18), except that 5% polyacrylamide gels are used.

Neuraminidase treatment of apoC

VLDL apoproteins, 1.4 mg in 0.5 ml, were dialyzed against 100 volumes of 0.2 M acetate buffer, pH 5, for 4 hr and then combined with 20 μg of neuraminidase (from *C. perfringens*, Sigma), in 0.5 ml of water for 3 hr at 37°C. After dialysis against water at 4°C,

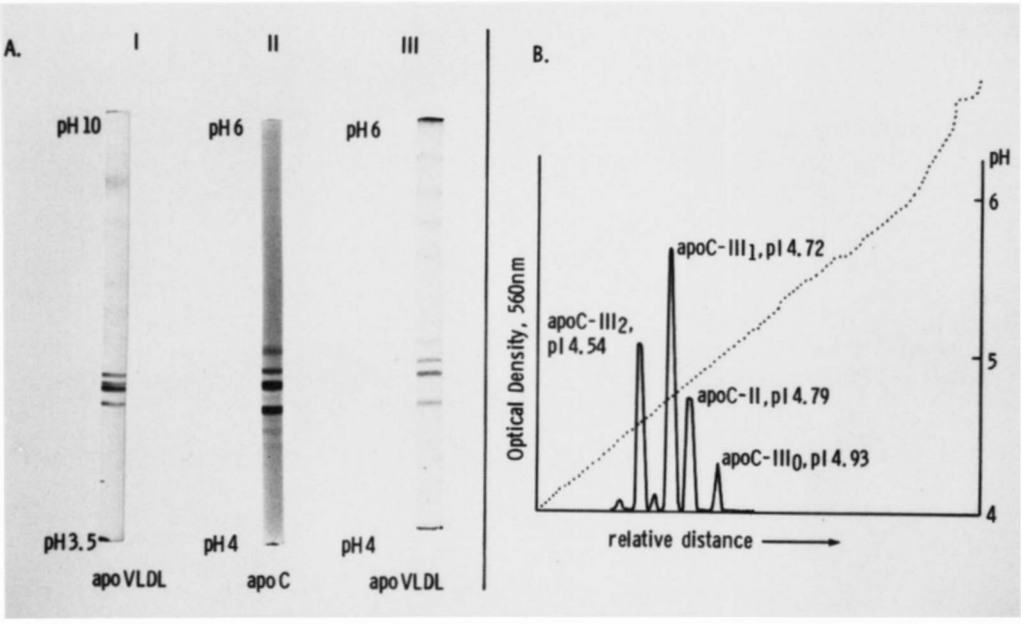

Fig. 1. Analytical isoelectric focusing of apoC. Panel *A*. (*I*) ApoVLDL, pH 3.5–10; (*II*) apoC, Sephacryl pool, pH 4–6; and (*III*) apoVLDL, pH 4–6. Panel *B*. Densitometric profile of apoC. The pH profile obtained from unstained gels is included.

the samples, and control samples without enzyme were lyophilized and analyzed by IEF.

RESULTS

The assignment of peptide bands obseved in IEF (**Fig. 1**) was made by comparison with purified apoproteins in the pH range 4–6. Separation of apoC by IEF in the pH range 4–6 gives four major bands and two minor bands (Fig. 1, *A-II*). ApoC-II migrated to pH 4.79 ± 0.03 (SD of four determinations); apoC-III_1 to pH 4.72 ± 0.03; and apoC-III_2 to pH 4.54 ± 0.04. ApoC-I did not appear in this range.

In the pH range 4–6, another major band is evident with an apparent isoelectric point of 4.93 ± 0.04 (SD) (Fig. 1*B*). The identity of this band as apoC-III_0 was suggested by comparison with apoVLDL after neuraminidase treatment. The distribution of apoC-III_0, apoC-II, apoC-III_1, and apoC-III_2 in the original sample was 9.2, 14.3, 49.1, and 27.4%, respectively, of the total densitometric area. After treatment with neuraminidase, the proportion of the band at pH 4.93 increased sevenfold and was associated with the corresponding decreases in the amounts of apoC-III_1 and apoC-III_2. The proportion of apoC-II was unchanged, as expected, since this peptide does not have sialic acid (15). No changes in pI for the apoproteins at pH 4.61 and pH 4.44 were observed.

Quantitation of apoC-II and apoC-III in VLDL

The densitometric areas for apoC-II, apoC-III_1, and apoC-III_2 increased linearly between concentrations of 5 and 40 μg protein per gel (**Fig. 2**). The plots were not linear at protein concentrations greater than 50 μg. ApoC-III_1 and apoC-III_2 showed no differences in dye binding. The chromogenicity factor for apoC-II was 1.25 that of apoC-III. The reproducibility, as the percent deviation from the mean of four analyses of the same sample ranged between 7.2 and 9.4 and was the same for all the apoproteins.

Apoprotein distribution in VLDL and VLDL subfractions

The distribution of apoC-II and apoC-III was measured in total VLDL and VLDL subfractions from plasma of normal subjects (**Table 1**). ApoC-III_1 accounts for approximately 45% of the total apoC protein; apoC-III_2, 31%; apoC-II, 16.8%; and apoC-III_0, 7.1%. The data are expressed also as percent of the total VLDL protein for comparative purposes. The other minor components, apoC-IV and apoC-V, were present as 1.4% of the total protein in a ratio of 1.2:1. When VLDL was analyzed within 48 hr of plasma separation or when the VLDL samples remained at 4°C for 1 week the pattern was identical. The total amount of apoC as a proportion of the protein in VLDL subfractions decreased as the size of the VLDL decreased (Table 1). However, no differences were

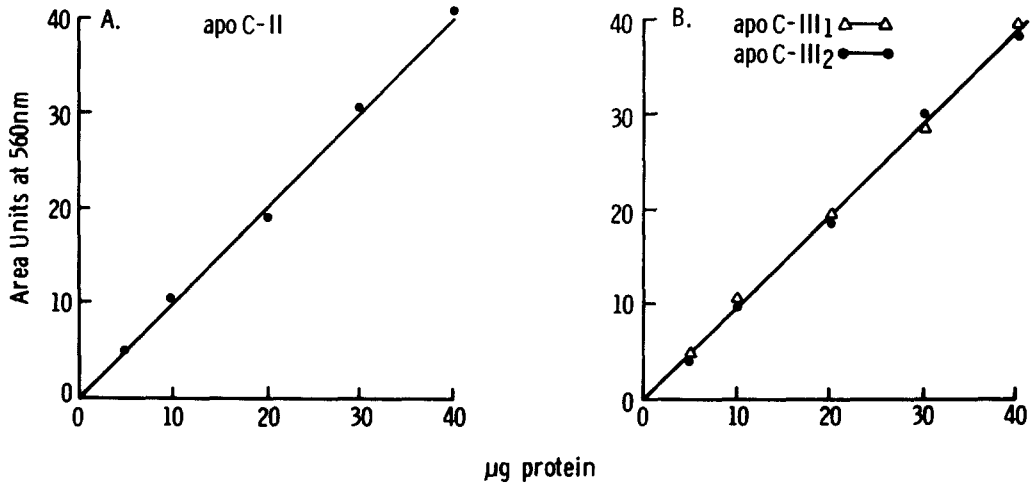

Fig. 2. Linear relationship of densitometric response and amount of apoC-II and apoC-III. Each point is the mean of three determinations. The chromogeneity for apoC-II was 1.25. Panel *A*, apoC-II (● —— ●); Panel *B*; apoC-III_1 (△ —— △) and apoC-III_2 (● —— ●).

observed in the proportions of the apoproteins. ApoC-II:apoC-III ratios were 0.2 for total VLDL and 0.18 for $VLDL_1$, $VLDL_2$, and $VLDL_3$.

DISCUSSION

Because analytical methods of polyacrylamide gel electrophoresis in either SDS or 8 M urea give unsatisfactory resolution of apoC-II and apoC-III_0 or do not separate the apoC-III, as seen by inspection of published densitometric gel scans (6, 14, 20), a method of analytical isoelectric focusing was developed. Using this technique we showed that apoC-II and apoC-III have isoelectric points in agreement with those previously established by preparative IEF (19). Unambiguous identification of the apoprotein with pI of 4.93 was accomplished by treatment of VLDL apoproteins with neuraminidase to remove sialic acid residues of the apoC-III_1 and apoC-III_2. ApoC-I does not appear in the pH 4–6 range; no systematic attempts to determine the isoelectric point of apoC-I at higher pH were made. In addition to apoC-II and apoC-III forms, two other apoproteins with isoelectric points of 4.61 and 4.44 were identified. The possibility that these bands were produced by carbamylation was excluded by isoelectric focusing apoC in 0.1% Triton instead of 8 M urea. Neuraminidase treatment produced no change in the isoelectric points of these apoproteins. The characteristics of these two apoproteins are being studied. The relationship, if any, of these bands with two apoproteins present in alkaline urea gels (14) is not known.

Under the conditions described in this paper, an apoC-III specie containing three residues of sialic acid, which have a pI of approximately 4.33, was not found. Kane et al. (14) have described an apoC-III protein, identified as Ala-4 or apoC-III_3, which accounted for

TABLE 1. Distribution of apoC-II and apoC-III in VLDL and VLDL subfractions

Apoprotein	VLDL[a]	$VLDL_1$[b]	$VLDL_2$[b]	$VLDL_3$[b]
			% of apoC	
C-II	16.8 ± 1.9 (8.7)[c]	15.3 (9.9)	14.9 (9.2)	15.1 (7.1)
C-III_0	7.1 ± 2.1 (3.7)	6.0 (3.9)	6.0 (3.7)	6.6 (3.1)
C-III_1	45.0 ± 3.6 (23.3)	48.7 (31.4)	49.1 (30.4)	48.9 (23.1)
C-III_2	31.1 ± 3.8 (16.1)	30.0 (19.4)	29.9 (18.5)	29.4 (13.9)
C-IV + C-V	1.4[c]	1.8	1.4	1.0
C-II/C-III	0.20 ± 0.02	0.18	0.18	0.18

[a] Mean value ± SD of duplicate determinations performed separately on VLDL from nine normal subjects.

[b] Mean values of duplicate determinations of three samples from normal subjects.

[c] As percentage of the total VLDL protein. No correction for the different chromogenicity (14) of apoB was made.

about 10% of the apoC mass and disappeared after treatment of the apoVLDL with neuraminidase. We found no differences in the apoprotein distribution when VLDL was isolated and analyzed immediately after the blood was drawn (n = 5) or after storage at 4°C for 1 week. The methods of isolation of VLDL are different; all our samples were dialyzed 24 hr prior to delipidation. A complete explanation requires further study.

Our measurements of apoC-II as total protein (Table 1) are comparable to those reported for apoC-II determined by radioimmunological techniques, 8.7% and 9.1%, respectively (7). Although delipidation with ethanol–ether has been reported to decrease significantly the apoC-II content of VLDL (7), the amounts of apoC-II in our system were the same after delipidation with either TMU or ethanol–ether. The amount of apoC-II as percent of the total apoC is also in good agreement with the data reported by Kane et al. (14).

The distribution of apoC-III species is the same as that found by preparative isoelectric focusing (19) but does not agree with other published data (14, 20). We found that apoC-III_0 is present in every sample tested and the amount of apoC-III_1 is somewhat higher, 45% vs. 38.4% (14). These differences are probably related to the presence of a peptide, Ala-4, which accounts for about 10% of the apoC, as determined by Kane et al. (14).

The amount of apoC-II and apoC-III as percentage of total VLDL protein decreases with decreasing size, although there is no change in the relative proportions of apoC-II, apoC-III_0, apoC-III_1, and apoC-III_2. Rapid exchange of apoC between HDL and triglyceride-rich lipoproteins (21) would account for this finding. By contrast, Kane et al. (14) reported different data for VLDL subfractions from four patients with endogenous hyperlipidemia. ApoC-II decreased from 15.4 to 11.5% between $VLDL_1$ and $VLDL_3$ as percent of the total apoC. VLDL subclasses are heterogeneous because they contain partially degraded VLDL arising in VLDL catabolism (22). Different tissue secretion of triglyceride-rich lipoproteins (23), in addition to the genetic heterogeneity associated with hypertriglyceridemia (23), must also be considered in studies of apoprotein content. Whether a decreased amount of apoC-II or a decreased ratio of apoC-II/apoC-III is related to hypertriglyceridemia will be determined in future studies. In conclusion, IEF has adequate sensitivity and reproducibility for quantitative determination of apoC-II and apoC-III in VLDL from normal and hyperlipidemic patients.

This work was supported in part by USPHS Grants HL-15648 and HL-17269 and NHLBI Lipid Research Clinic Contract 71-2156. The authors thank Dr. W. Bradley for the amino acid analysis and Mrs. Phyllis Gutierrez for typing the manuscript.

Manuscript received 17 January 1978; accepted 16 May 1978.

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