Separation and isolation of human apolipoproteins C-II, C-III$_0$, C-III$_1$, and C-III$_2$ by chromatofocusing on the Fast Protein Liquid Chromatography system

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Summary Chromatofocusing, which separates proteins based on differences in isoelectric point, has been used on the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia) to separate the C apolipoproteins from human very low density lipoproteins (VLDL). Using a Mono P column (Pharmacia), a pH gradient between pH 6.2 and pH 4.0 was generated using buffers containing 6 M urea, at a flow rate of 0.5 ml/min. Typically, runs took approximately 45 min. Chromatofocusing of delipidated whole VLDL produced sharp, well-resolved peaks for the C apolipoproteins. However, as determined by analytical isoelectric focusing (IEF), the apolipoprotein E isoforms were not separated from apoC-II, and they contaminated the other apoC species to a variable extent. In addition, apoC-II was not resolved from apoC-III$_0$. Preliminary precipitation of VLDL with acetone prior to delipidation removed both apolipoproteins E and B. Using a start buffer of 25 mM histidine, pH 6.2, and a 1:30 dilution of the polybuffer exchanger (eluting buffer), apoC-II, C-III$_0$, C-III$_1$, and C-III$_2$ were well resolved in runtimes of approximately 60 min. The C apoproteins proved to be pure by analytical IEF and immunoassay with monospecific antisera against apoC-II and C-III. Recovery was over 90% of the protein chromatographed. In addition, a variant of apoC-II present in VLDL of a hypertriglyceridemic subject was clearly resolved from the other C apolipoproteins. This technique is superior to conventional methodology in terms of its time saving and high resolution. The application of this technique to the study of C apolipoprotein variants and C apolipoprotein specific radioactivity determinations is possible. —**Huff, M. W., and W. L. P. Strong.** Separation and isolation of human apolipoproteins C-II, C-III$_0$, C-III$_1$, and C-III$_2$ by chromatofocusing on the Fast Protein Liquid Chromatography system. *J. Lipid Res.* 1987. **28:** 1118–1123.

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The C apolipoproteins play an important role in the catabolism of human triglyceride-rich lipoproteins, very low density lipoproteins, and chylomicrons (1). Apolipoprotein C-II is required for maximal activation of lipoprotein lipase in vitro (2) and in vivo (3). The function of the sialated forms of apoC-III (C-III$_{0\text{-}2}$) is not yet fully understood. A low apoC-II/C-III ratio, in vitro, inhibited the C-II-activated lipoprotein lipase activity (4) and a low C-II/C-III ratio has been demonstrated in hypertriglyceridemic individuals (5). Excess apoC-III has been shown to inhibit the hepatic intake of triglyceride-rich lipoproteins by perfused rat livers (6). Kinetic studies have provided evidence that, in humans, the C apoproteins are metabolized as a group and probably share a common catabolic pathway (7, 8). Variants of apoC-II have been documented (9–12), some of which may prove to inhibit the activation of lipoprotein lipase by normal apoC-II. Recently, the major and minor plasma isoforms of apoC-II have been described (13).

Isolation of VLDL C apolipoproteins have conventionally been carried out by a combination of gel filtration chromatography and anion-exchange chromatography (14), gel filtration chromatography and preparative isoelectric focusing (7), and chromatofocusing (15–17). These methods are time-consuming and frequently fail to completely resolve apoC-II from C-III$_0$. Reverse-phase HPLC has been used to separate the C apolipoproteins following initial isolation by conventional gel filtration chromatography (18). However, complete resolution of the C apolipoproteins has proven difficult. Reverse-phase HPLC has also proven useful for the purification of apolipoprotein C-II to homogeneity, following initial partial purification by conventional ion exchange chromatography (19).

The present investigation was designed to combine the resolution of chromatofocusing with the advantages of the Fast Protein Liquid Chromatography system, to rapidly separate and isolate the C apolipoproteins from VLDL in order to facilitate further study.

METHODS

VLDL preparation

Blood was collected from hypertriglyceridemic subjects, following an overnight fast, into tubes containing EDTA-Na$_2$ (1.5 mg/ml). Plasma was isolated by low speed centrifugation. VLDL ($S_f > 20$) was isolated by ultracentrifugation at $d < 1.006$ g/ml in a Beckman 60 Ti rotor (28 hr, 40,000 rpm at 12°C), and washed in a 50 Ti rotor (18 hr, 50,000 rpm at 12°C) through an equal volume of buffered saline (0.15 M NaCl, 1 mM EDTA, 1 mM Tris, pH 7.4, 3 mM NaN_3, 0.1 mM Merthiolate, and 10 μM phenylmethane sulphonyl fluoride). VLDL recovered by aspiration was dialyzed for 24 hr against 5 mM ammonium bicarbonate, pH 8.0, lyophylized, and delipidated with diethylether–ethanol 3:1, and dried under nitrogen. In some preparations, as indicated in results, prior to dialysis, VLDL was precipitated with acetone as described by Holmquist, Carlson, and Carlson (20). The supernatant was dialyzed, lyophylized, and delipidated as described above.

Abbreviations: VLDL, very low density lipoproteins; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; HPLC, high performance liquid chromatography.

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Chromatography

Chromatofocusing was carried out using a pre-packed (Mono P) column (Pharmacia, Montreal, Quebec) and the FPLC system (Fast Protein Liquid Chromatography system, Pharmacia, Montreal, Quebec). The column was equilibrated with 25 mM Bistris (pH 6.2) containing 6 M urea. In some experiments, as indicated, the equilibrating buffer was 25 mM histidine, pH 6.2, in 6 M urea. Urea solutions were deionized before use by passing them through a column of Rexyn I-300 (Fisher Scientific, Toronto, Ontario). The pH gradient was developed with Polybuffer 74 (Pharmacia), diluted 1:10, 1:20, or 1:30 (as indicated) with deionized 6 M urea and adjusted to pH 4 with HCl. Samples of whole VLDL apolipoproteins were resolubilized in 0.5 ml of the equilibrating buffer for 1–2 hr at room temperature, centrifuged at 2000 rpm for 5 min to remove insoluble apoB, and filtered through a 0.45-μm Millipore filter (Millipore, Mississauga, Ontario), prior to injection. Typically, 0.5–2.5 mg of protein was injected. A program was developed to facilitate reproducibility of each run, which consisted of 100% equilibrating buffer (pump A) for 2 min; the sample loaded through a 500-μl injecting loop; equilibrating buffer (pump A) for 1 min, through the injection loop; equilibrating buffer (pump A) for 5 min; 100% eluting buffer (polybuffer exchanger) for 45 min or 60 min (as indicated); and finally, 100% equilibrating buffer (pump A) for 15 min to re-equilibrate the column for the next run. Flow rates were 0.5 ml/min. The absorbance at 280 nm and pH were recorded throughout each run. Column fractions were collected in volumes of 0.5 ml with the exception of peaks in the C apoprotein region of the chromatogram. Each peak was collected in one tube starting when the UV detector exceeded 20% of full-scale deflection and ending when 20% of full-scale deflection was again reached on the down slope of each peak.

Analyses

Protein determinations were performed on VLDL and VLDL apoproteins by a modification of the Lowry procedure (21) and protein determinations of the FPLC-chromatofocusing fractions were by the modified Lowry method of Bensadoun and Weinstein (22). The apoprotein fractions from column separations were analyzed by analytical isoelectric focusing polyacrylamide gel electrophoresis, (7.5% polyacrylamide, 2% ampholine pH 4–6, 6.8 M urea), as described in detail previously (23). Electroimmunoassay was carried out directly on column fractions from the FPLC chromatography, using monospecific antisera to human apolipoproteins C-II and C-III (24) to further determine the purity of the separated C apolipoproteins. Urea and Polybuffer, at the dilutions required for quantitation, did not interfere with the assay.

RESULTS

The elution profile of a chromatofocusing run of apolipoproteins from whole VLDL is shown in **Fig. 1a.** The Bistris equilibrating buffer and a 1:10 dilution of the Polybuffer 74 were used. The C apolipoproteins appeared to be resolved into sharp peaks, between pH 4.9 and 4.0. Fig. 1b shows the analytical isoelectric focusing gels of the major peaks from this chromatogram. The starting material is shown and gel 2 corresponds to the material from peak 2. It contains the E apolipoproteins as well as apoC-III_0 and apoC-II as the most prominent band. Gel 3 corresponds to the material in peak 3. ApoC-III_1 is the major band; however, significant amounts of apoC-II and apoE are present. Gel 4 corresponds to peak 4, which contains predominantly apoC-III_2 but is contaminated with apoC-III_1 and apoE. A gel corresponding to peak 1 contained no detectable material in the apoC region and faint apoE bands were detected (not shown). A gel corresponding to the peak eluting prior to the start of the gradient showed only a faint band corresponding to apoC-I (not shown). In an initial effort to improve resolution, the equilibrating buffer was changed to 25 mM histidine, pH 6.2. This buffer produced a shallower pH gradient and better resolution compared to the recommended Bistris buffer (not shown). As assessed by analytical isoelectric focusing, apoE continued to contaminate all the apoC fractions, and apoC-II was not well resolved from C-III_0. Pure apoC-III_1 was obtained but C-III_2 was contaminated by C-III_1 (not shown).

In order to resolve the contamination of the C apolipoprotein peaks by apolipoprotein E, an acetone precipitation was carried out on the starting VLDL (as described in Methods), which precipitates apolipoproteins B and E and leaves the C apoproteins in solution. In addition, in an attempt to gain a better resolution of the C apolipoproteins, a 1:20 dilution of polybuffer was used in the eluting buffer, which has the effect of producing a shallower pH gradient (which took approximately 50 min to complete). Analytical isoelectric focusing of the column fractions demonstrated the complete absence of the E apolipoproteins; however, apoC-II was slightly contaminated by apoC-III_0. ApoC-III_1 and C-III_2 were found to be homogeneous.

In an effort to improve the separation of apoC-II from C-III_0, a further dilution of the polybuffer (1:30) was made which resulted in a pH gradient that required approximately 60 min to complete. **Fig. 2a** shows a run under these conditions, which clearly shows better resolution of the C apolipoproteins. Peak 2 now had a smaller peak (denoted as 2a) eluting slightly before the major peak (denoted as 2b). IEF gels corresponding to peaks 2a, 2b, 3, and 4 are shown in Fig. 2b. These gels demonstrate that these peaks correspond to pure apolipoproteins

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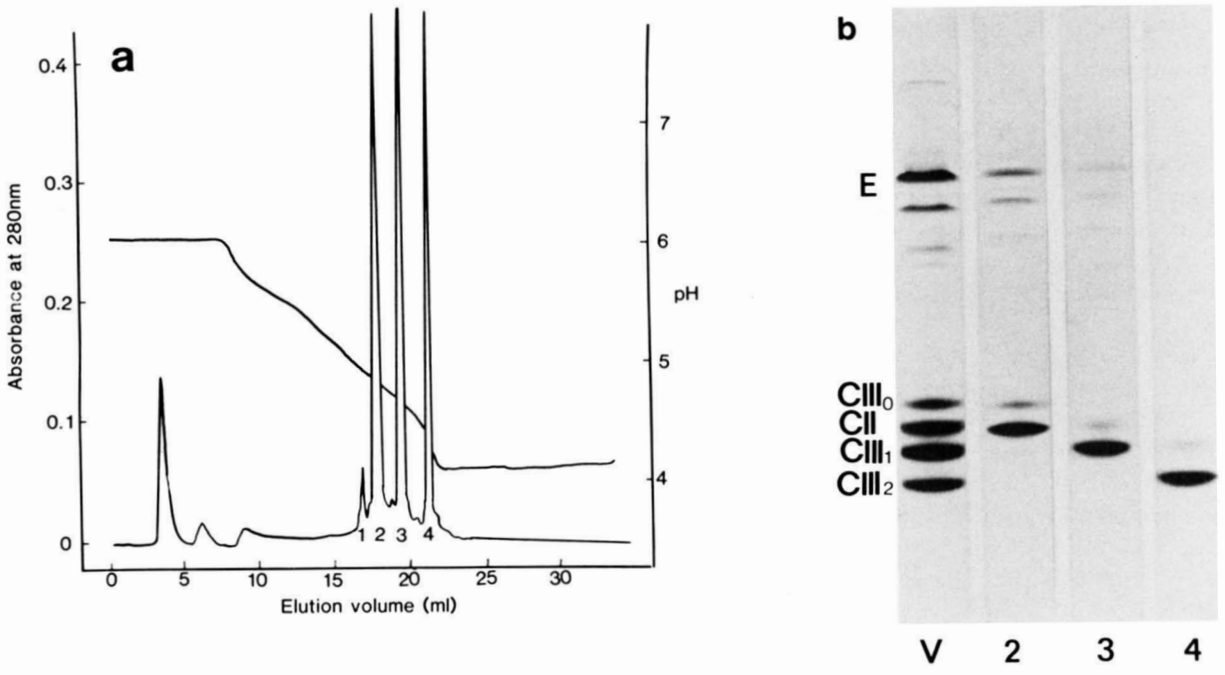

Fig. 1. a): Separation of whole VLDL apolipoproteins by chromatofocusing on a Mono P column using the FPLC system (Pharmacia). The flow rate was 0.5 ml/min and the equilibrating buffer was 25 mM Bistris, pH 6.2, in 6 M urea. The gradient was developed with a 1:10 dilution (with 6 M urea) of Polybuffer 74, pH 4.0. The sample, 1.6 mg of VLDL protein resolubilized in 500 μl of the equilibrating buffer, was loaded in a volume of 500 μl. Fractions were collected in 500 μl except for the C apolipoproteins. Each peak was collected in the same tube starting when the UV recorder reached 20% of full-scale deflection and ending when 20% full-scale deflection was again reached on the down slope of each peak. b): Analytical isoelectric focusing polyacrylamide gel electrophoresis (7.5% gels, pH 4–6) of whole VLDL (V, initial sample) and the corresponding peaks from the chromatofocusing run shown in Fig. 1a.

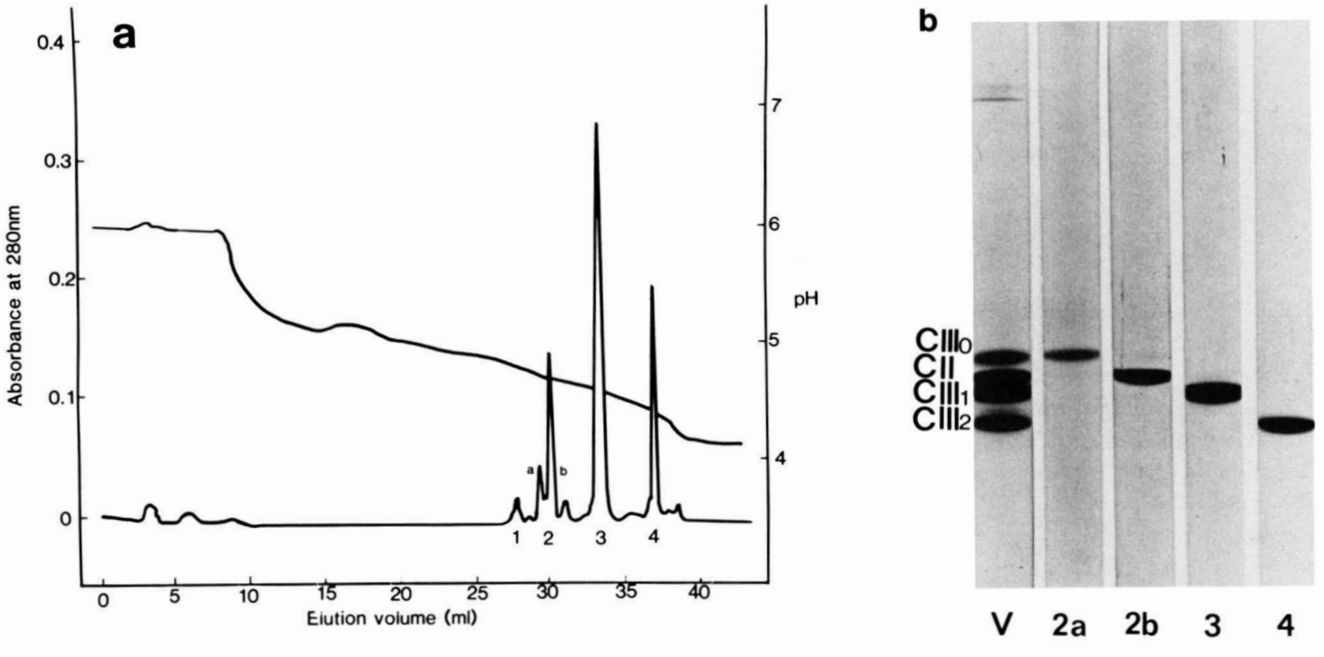

Fig. 2. a): Separation of acetone-soluble VLDL apolipoproteins by chromatofocusing on FPLC. The pH gradient was developed with a 1:30 dilution (with 6 M urea) of the Polybuffer 74, pH 4.0, at a flow rate of 0.5 ml/min. Acetone-soluble VLDL protein (2.4 mg) was loaded in 500 μl of equilibrating buffer (25 mM histidine, pH 6.2, in 6 M urea) and 500-μl fractions were collected except for the C apolipoproteins for which each peak was collected in one tube as described in the legend to Fig. 1a. b): Analytical isoelectric focusing gel electrophoresis (7.5% gels, pH 4–6) of acetone-soluble VLDL apolipoproteins and the corresponding peaks from Fig. 2a.

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$C\text{-}III_0$, C-II, $C\text{-}III_1$, and $C\text{-}III_2$, respectively, as shown in gels 2a, 2b, 3, and 4. Electroimmunoassay demonstrated that the apoC-II peak (2b) reacted against antisera to apoC-II, but not to antisera against apoC-III. Also, samples from peaks 2a, 3, and 4 reacted only against antisera to apoC-III (data not shown).

For five column runs, all column fractions in the C apolipoprotein region (25 through 40 ml) were assayed for protein using the modified Lowry procedure (19), which prevents interference from the Polybuffer 74. In each case the protein recovered was greater than 90% of the protein loaded on the column. Of the recovered protein, 16% was recovered as apoC-II, and 5%, 38%, and 31% were recovered as $apoC\text{-}III_0$, $C\text{-}III_1$, and $C\text{-}III_2$, respectively.

Figs. 3a and b show the chromatogram and corresponding IEF gels of a sample of VLDL C apolipoproteins, from a hypertriglyceridemic subject, that contains a variant of apoC-II. This variant elutes just prior to $apoC\text{-}III_1$ and is denoted as peak 3a on the chromatofocusing column. This variant is resolved from the other apoC subspecies, as seen on IEF (Fig. 3b, gel 3a). However, its position on IEF is intermediate between $C\text{-}III_1$ and $C\text{-}III_2$. By two-dimensional gel electrophoresis followed by immunoblotting against antisera to apoC-II and C-III, a reaction only to anti-C-II was observed (Breckenridge, W. C., M. W. Huff, and W. L. P. Strong, unpublished observations). Each fraction obtained from the chromatogram shown in Fig. 3a was subjected to electroimmunoassay against anti-apoC-II and C-III. **Table 1** shows that only fractions corresponding to apoC-II, peak 2b, and the variant, peak 3a, reacted to anti-apoC-II, whereas peaks 2a, 3b, and 4, corresponding to $apoC\text{-}III_0$, $C\text{-}III_1$, and $C\text{-}III_2$, respectively, reacted primarily with anti-C-III. $ApoC\text{-}III_0$ and $C\text{-}III_1$ contained 2.8% and 0.3% C-II, respectively. Fojo et al. (13) described an isoform of C-II, designated $C\text{-}II_{1/2}$, which has an isoelectric point identical to $C\text{-}III_1$. This was not detected in our samples since no C-II-reacting material was detected in the major $C\text{-}III_1$ peak (Table 1).

For this study the sample size loaded on the column ranged between 0.5 and 2.5 mg. As seen in Figs. 2 and 3, excellent resolution was obtained with a sample size of approximately 2.5 mg. In each of two column runs, 5.0 mg of VLDL acetone-soluble protein was loaded with no apparent loss of resolution as assessed by analytical isoelectric focusing gel electrophoresis. Higher sample loads have not been tested.

DISCUSSION

A variety of methods have been used to isolate the C apolipoproteins from triglyceride-rich lipoproteins (7, 14–19). Most of these methods are time-consuming, some have poor resolution of all the apoC species, and frequently the yield of protein is low. High performance

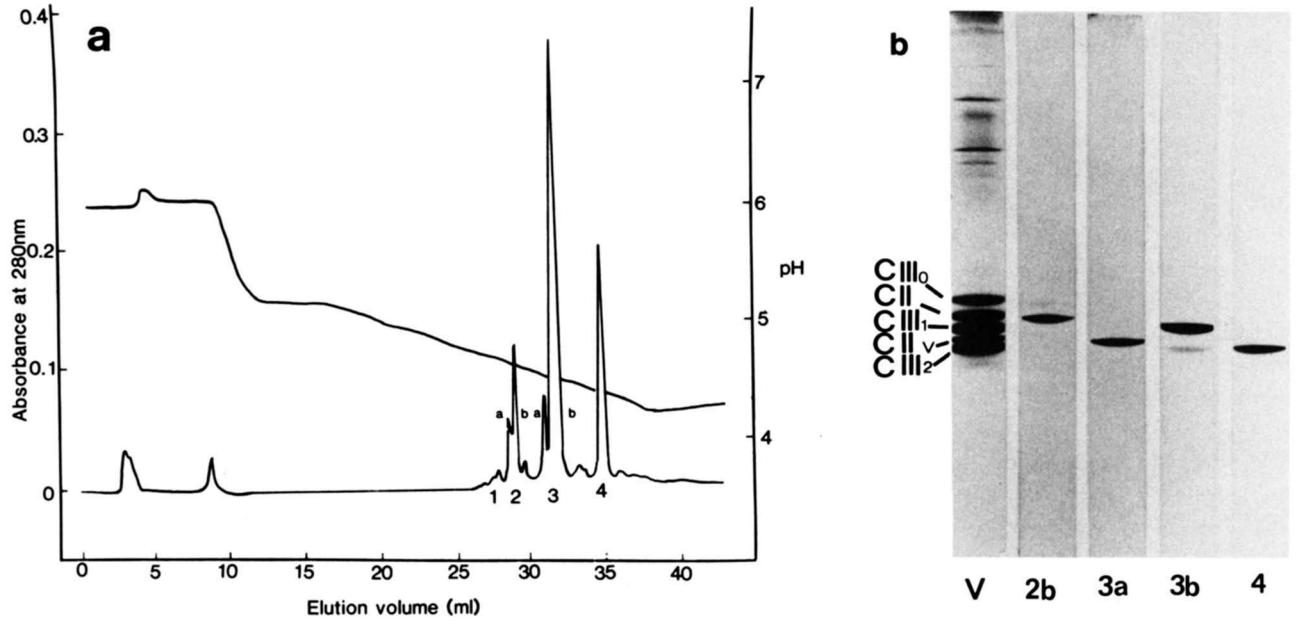

Fig. 3. a): Separation of acetone-soluble VLDL apolipoproteins from a hypertriglyceridemic subject, which contained a variant of apoC-II, by chromatofocusing on FPLC. The pH gradient was developed with a 1:30 dilution (with 6 M urea) of Polybuffer 74, pH 4.0, at a flow rate of 0.5 ml/min. Acetone-soluble VLDL protein (2.6 mg) was loaded in 500 μl of equilibrating buffer (25 mM histidine, pH 6.2, in 6 M urea) and 500-μl fractions were collected except for the C apolipoproteins for which each peak was collected in one tube as described in the legend to Fig. 1a. b): Analytical isoelectric focusing gel electrophoresis (7.5% gels, pH 4–6) of whole VLDL from a subject with a variant of apoC-II (C-IIv). The acetone-soluble VLDL apoproteins were chromatofocused and gels 2b–4 correspond to the peaks from Fig. 3a.

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TABLE 1. Apolipoprotein C-II and C-III concentrations in column fractions following separation of acetone-soluble VLDL apolipoproteins by chromatofocusing on FPLC

Fraction Number[a]	C-II[b]	C-III[b]
	μg/fraction	
57 (C-III$_0$)	2.8	100.8
58	28.0	70.0
59 (C-II)	458.0	0.0
60	48.0	0.0
61	0.0	0.0
62	0.0	0.0
63 (C-IIv)	276.0	0.6
64	25.0	87.2
65 (C-III$_1$)	2.4	992.0
66–70	0.0	0.0
71	0.0	60.0
72 (C-III$_2$)	0.0	448.0

[a] Fractions 59, 63, 65, and 72 contained the samples that correspond to gel 2b, 3a, 3b, and 4, respectively, in Fig. 3b.
[b] Determined by electroimmunoassay (24).

liquid chromatography has been applied to the separation of the C apolipoproteins (18, 19). These methods are based mainly on reverse-phase columns and, although these are rapid methods, prior isolation of the C apoproteins by conventional gel filtration and ion-exchange chromatography is required. Conventional chromatofocusing has been used to separate the C apolipoproteins (15–17) according to their isoelectric points. Although good resolution is obtained, separation times can take 8–10 hr. In this report we describe a method for the complete separation of the C apolipoprotein species, free of other apolipoproteins, by chromatofocusing on a "high performance" chromatography system, FPLC. Separation times of 70 min are significantly faster than conventional techniques and protein recovery is over 90%.

To achieve this resolution, VLDL was initially precipitated with acetone to provide a sample of the C apolipoproteins free of apoproteins B and E. Without this preliminary step, apoE is found to a variable extent in the peaks containing C-II, C-III$_1$, and C-III$_2$. The reason for this is not known, since the isoelectric points of the E apoproteins are higher (> pH 5.0) than any of the C apoproteins (< pH 5.0). In addition, apolipoproteins C-II and C-III$_0$ were eluted in the same peak when the pH gradient was generated by the recommended dilution (1:10) of the Polybuffer 74 exchanger. We found that dilution of the Polybuffer to 1:30 produced a shallower pH gradient. Although the run time increased from 35 to 70 min, apoC-II was completely resolved from apoC-III$_0$.

While this study was being completed, Weisweiler, Friedl, and Schwandt (25) reported the use of chromatofocusing of VLDL apolipoproteins on FPLC. However, these authors used a 1:10 dilution of Polybuffer 74 and a higher flow rate of 1.0 ml/min. They were unable to resolve C-II from C-III$_0$, and C-III$_1$ and C-III$_2$ were not homogeneous. This was not related to flow rate or amount of protein loaded. Using similar buffers and sample preparation but a lower flow rate (0.5 ml/min, recommended by the manufacturer) and lower column loads (i.e., 1.6 vs. up to 10 mg) we obtained a similar degree of contamination and poor resolution (see Figs. 1a and 1b). These same authors have recently reported that a combination of gel filtration followed by ion exchange chromatography on FPLC could separate the individual C apoproteins free from contamination by other apoproteins (26). This method, however, requires two FPLC column procedures.

Several investigators have found variants of apoC-II in VLDL from human subjects, some of which are associated with hypertriglyceridemia. Maguire et al. (11) have identified C-II$_X$ and C-II$_Y$ from a hypertriglyceridemic kindred which have isoelectric points similar to apoE and do not activate lipoprotein lipase. Other C-II variants have recently been described which also do not activate lipoprotein lipase (12). Havel, Kotite, and Kane (9) and Menzel et al. (10) have reported the presence of a C-II variant from an African kindred designated C-II-2 which has glutamine substituted for lysine at residue 55, an isoelectric point intermediate between C-III$_1$ and C-III$_2$, yet activates lipoprotein lipase to the same extent as native C-II. In this study, we report the separation of a C-II variant from the VLDL of a hypertriglyceridemic Caucasian subject, using chromatofocusing on FPLC. This method clearly separates this variant from both native apoC-II and apoC-III$_1$ (see Fig. 3a and b). The position of elution on the FPLC chromatogram (i.e., more basic than C-III$_1$) differs from its apparent isoelectric point on IEF (i.e., more acidic than C-III$_1$). The reason for this is not known. Its position on IEF is similar to the C-II-2 reported by Havel et al. (9, 10). Other than its strong reaction against monospecific antibodies to apoC-II but not to anti-C-III, we do not as yet have any information regarding its primary structure or activation potential. We simply report here the use of the chromatofocusing FPLC technique as a means to quickly isolate quantities of C-II variants with high resolution to enable further characterization studies.

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