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Note

Separation and quantitation of plasma lipoproteins by high-performance liquid chromatography

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The identification and follow-up of individuals at risk for cardiovascular disease require a better evaluation of the distribution of lipid and protein components in the various lipoproteins. The quantitation of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, and of the apoproteins AI and B, requires a rapid separation of the lipoprotein classes and the preservation of their antigenic properties.

The separation and quantitation of plasma lipoproteins has mainly been performed by ultracentrifugal flotation, either stepwise or in a density gradient [1, 2]. Electrophoretic separation in a gel gradient and column chromatography have also been applied to the separation of the lipoprotein fractions [3, 4]. Precipitation techniques using polyanions and divalent cations have been used for the selective fractionation of VLDL, LDL, HDL and the subclasses HDL_2 and HDL_3 [5, 6].

Most of these techniques are time consuming, require 2—5 ml of serum and cannot be easily applied to the routine analysis of the lipid and protein constituents of plasma lipoproteins.

High-performance liquid chromatography (HPLC) on a gel exclusion column appears to be a suitable alternative for lipoprotein separation [7, 8]. In this

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paper we report on the application of this technique to the separation of lipoproteins from purified mixtures, from newborn sera and from normal and pathological adult sera. The isolated lipoprotein fractions were subsequently analyzed for their cholesterol and apoprotein content and the results evaluated by comparison with those obtained by density gradient ultracentrifugation of the same samples.

EXPERIMENTAL

Samples

In adults, fresh serum was drawn after 16 h fasting from individuals with normal lipids, Fredrickson type IIB and IV patients and from a patient with nephrotic syndrome. Cord blood was obtained immediately after delivery and blood was drawn by venipuncture after 6 h fasting in 7- and 30-day-old infants [9].

Separation of lipoproteins by ultracentrifugation

VLDL, LDL and HDL were obtained by stepwise ultracentrifugation using the method of Havel et al. [1]. For the density-gradient ultracentrifugation, 0.3 and 0.5 ml plasma was spun in an SB 283 swinging-bucket rotor of an International B 60 ultracentrifuge, for 66 h at 180,000 *g* [10].

The gradient consisted of sucrose, NaBr and NaCl, spanning the density range 1.02–1.20 g/ml. Fractions of 0.5 ml were collected after reading the absorbance at 280 nm through the 8-μl flow-cell of a Pye-Unicam spectrophotometer.

Total lipoproteins were isolated by flotation of 170 μl serum in an air-driven ultracentrifuge (Beckman Airfuge) after spinning at 100,000 *g* at d = 1.21 g/ml for 4 h [11].

Lipid and apoprotein quantitation

Cholesterol was assayed in total plasma and in lipoprotein fractions using an enzymatic—fluorimetric assay [12], at a sensitivity of 1 μg of cholesterol, with a precision of ± 4%.

Aproproteins AI and B were assayed by immunonephelometry, using either 50 μl of serum diluted 150-fold or 100 μl of the ultracentrifugal or chromatographic fractions, diluted two- to ten-fold [13, 14]. The precision of these assays was 7%.

Separation of lipoproteins by HPLC

The separation of the lipoproteins was performed on a Spectra-Physics SP 8000 liquid chromatograph equipped with a Schoeffel SF 770 variable-wavelength UV detector. For gel permeation an Ultro Pac TSK-G 4000 SW (600 × 7.5 mm I.D.) column (particle size 10 ± 2 μm) from LKB preceded by an Ultro Pac LKB TSK-G SWP (75 × 7.5 mm I.D.) guard column (particle size 10 ± 2 μm) was used. The column was equilibrated in 0.2 M Na_2HPO_4 containing NaN_3 (0.5 g/l). A 50-μl sample was injected and eluted at room temperature with the 0.2 M phosphate buffer at a flow-rate of 0.5 ml/min.

The lipoprotein fractions were monitored by the absorbance at 280 nm and collected in 0.5-ml fractions with a Gilson Microcol TDC 80 fraction collector.

RESULTS

A mixture of VLDL, intermediate density lipoprotein (IDL), LDL and HDL prepared by stepwise ultracentrifugation was applied to a single TSK-G 4000 SW column. The elution profile depicted in Fig. 1 shows a baseline resolution for the major lipoprotein classes. The first peak which eluted at the exclusion volume of the column, contains the VLDL fraction and the chylomicrons. IDL (fraction 3) elutes in a well-separated peak between VLDL and LDL (fraction 4). The HDL peak (fractions 5 + 6) could not be subfractionated into HDL_2 and HDL_3 using the TSK-G 4000 SW column. Under the experimental conditions used all lipoproteins were eluted within 50 min.

The sensitivity of the fluorimetric and immunonephelometric assay used for cholesterol and apoprotein analysis is sufficient to analyze the lipoprotein fractions isolated from 20 μl of serum. The composition of the lipoprotein fractions was not altered by fractionation through the TSK-G 4000 SW column. Due to protein adsorption to the matrix, the recovery of the lipids and apoproteins lies between 70% and 80% and is similar for all fractions.

In Fig. 2, the UV pattern (dotted lines) corresponding to the total serum is compared to the UV pattern (full line) of the $d > 1.21$ supernatant of the same serum after flotation in the Beckman Airfuge. The flotation step in the Airfuge is sufficient to separate the lipoproteins from most of the plasma proteins, though some albumin is still present in the supernatant.

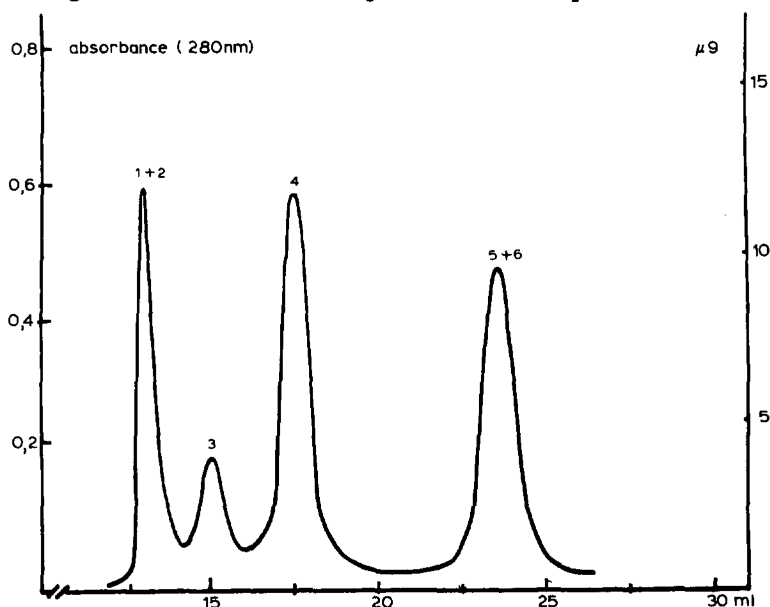

Fig. 1. Elution curve of a mixture of lipoprotein fractions on an Ultro Pac TSK-G 4000 SW column (600 mm × 7.5 mm I.D.). Eluent, sodium phosphate buffer, 0.2 *M*, pH 6.8; flow-rate, 0.5 ml/min; T, 25°C; load volume, 50 μl. Peaks: 1 + 2 = chylomicrons + VLDL; 3 = IDL; 4 = LDL; 5 + 6 = HDL_2 + HDL_3.

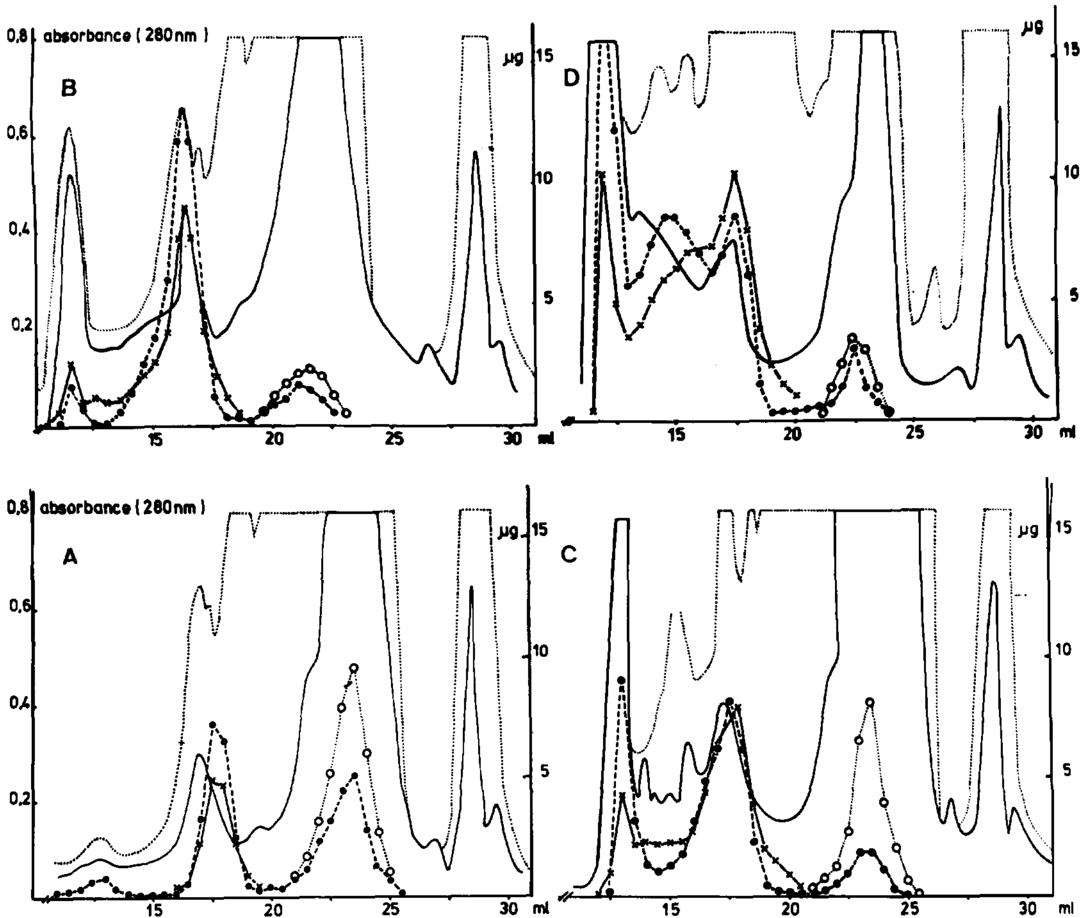

Fig. 2. Elution curves of lipoproteins in total serum (. . . . .) and ultracentrifugal Airfuge fractions (———): (A) normolipemic female; (B) Fredrickson type IIA patient; (C) Fredrickson type IV patient; (D) patient with nephrotic syndrome. HPLC conditions as in Fig. 1. (•), Cholesterol; (×), apo B; (○), apo AI.

The lipoprotein separation from serum by HPLC was completed in 60 min with a total elution volume of 30 ml. Fig. 2A is representative of the serum of a normal individual, whereas Fig. 2B and C represents the elution patterns obtained for sera of Fredrickson type IIA and type IV patients, respectively. Fig. 2D shows the results for a patient with nephrotic syndrome.

The cholesterol, apo AI and apo B contents of the eluted fractions are also plotted in Fig. 2A—D, showing that the UV maxima of the VLDL, LDL and HDL peaks coincide with those of cholesterol and apoproteins. The lipoprotein distribution in a normolipemic individual (Fig. 2A) is characterized by a low VLDL fraction and a comparable concentration of LDL and HDL. The type IIA individual has normal VLDL, an elevated LDL and a decreased HDL peak. The cholesterol/apo B ratio in the LDL fraction is decreased compared to that of the normal individual (Table I).

The pattern of the congenital type IV patient (Fig. 2C) shows an elevated

TABLE I

DISTRIBUTION AND COMPOSITION OF PLASMA LIPOPROTEINS AFTER FRACTIONATION BY HPLC AND BY DENSITY-GRADIENT ULTRACENTRIFUGATION

Patient	Technique	VLDL-C* (%)	IDL-C (%)	LDL-C (%)	HDL-C (%)	VLDL-C/ apo B	LDL-C/ apo B	apo AI/ apo B
Baby, 0 days	HPLC	<3	<3	43	57	—	0.9	1.5
	UCF**	8.1 (VLDL-C + IDL-C)		37	56	1.2	1.4	1.7
Baby, 7 days	HPLC	3.2	5.0	50.0	42.0	—	1.2	1.4
	UCF	26.4 (VLDL-C + IDL-C)		42.6	30.9	1.5	0.9	1.4
Baby, 30 days	HPLC	2.2	9.9	51.5	36.4	1.6	1.0	1.3
	UCF	25.2 (VLDL-C + IDL-C)		47.6	27.5	—	1.1	1.2
Normolipemic individual	HPLC	<3	<3	61.1	38.9	—	1.5	0.8
	UCF	3.1	—	60.3	37.4	1.8	1.7	1.0
Type IIA patient	HPLC	6.9	—	79.6	13.4	1.3	1.2	0.33
	UCF	9.1	—	73.8	17.1	1.8	1.5	0.38
Type IV patient	HPLC	25.3	18.2	43.6	12.8	2.1	0.9	0.53
	UCF	29.0 (VLDL-C + IDL-C)		58.7	11.2	1.8	1.2	0.55
Patient with nephrotic syndrome	HPLC	24.2	41.2	27.0	7.6	1.8	1.2	0.14
	UCF	58.0 (VLDL-C + IDL-C)		35.0	7.0	2.0	1.5	0.18

*C = cholesterol.
**Density-gradient ultracentrifugation.

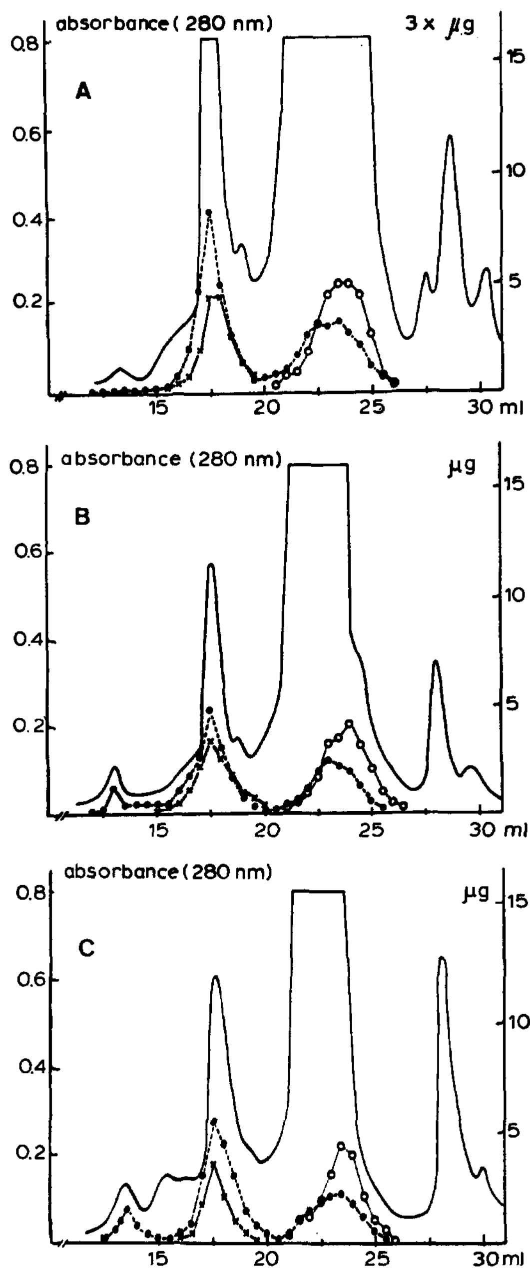

Fig. 3. Elution curves of lipoproteins in Airfuge fractions: (A) cord blood; (B) 7-day-old infant; (C) 30-day-old infant. HPLC conditions as in Fig. 1. (——), UV absorption at 280 nm; (•), cholesterol; (×), apo B; (○), apo AI.

VLDL concentration and the presence of an IDL fraction, characterized by an cholesterol/apo B ratio intermediate between those of VLDL and LDL. The concentrations of HDL cholesterol and apo AI are decreased in this patient.

The patient with nephrotic syndrome (Fig. 2D) is also characterized by an elevated VLDL and shows an IDL fraction eluting between VLDL and LDL which is highly heterogeneous in its cholesterol and apo B content. The HDL fraction is also lower in this patient than in the normal individual. Similar patterns were observed by density-gradient ultracentrifugation [15].

HPLC was also applied to the study of lipoproteins in cord serum and in the serum of 7- and 30-day-old infants (Fig. 3A—C). The HPLC pattern obtained after injection of 50 μl of cord serum, concentrated three-fold using a Minicon concentrating system, is depicted in Fig. 3A. This chromatogram demonstrates that newborns have very low VLDL at birth, low LDL and relatively high HDL, in agreement with the patterns obtained by density-gradient ultracentrifugation [10]. At 7 and 30 days VLDL and especially LDL levels have increased to a value about half of that measured in adults. The HDL concentration remains elevated at both 7 and 30 days.

The measurement of the cholesterol and apoproteins in the various fractions enabled calculation of the lipoprotein composition and distribution. These data are summarized in Table I and compared to those obtained by density-gradient ultracentrifugation of the same samples. The results indicate that the percentages of the lipoprotein fractions obtained by the two techniques are comparable. The resolution of the density-gradient ultracentrifugation did not enable efficient separation of VLDL and IDL, while HDL_2 and HDL_3 were well resolved [10]. The sensitivity of the lipid and apoprotein assay was insufficient for accurate analysis of the low VLDL levels in cord blood.

DISCUSSION

The data obtained by HPLC separation of the lipoproteins, present either in a mixture or in total serum, illustrate the potentiality of the HPLC technique for this type of separation. In agreement with other authors [7], we observed a good separation of VLDL, LDL and HDL, and were also able to separate IDL in a well-resolved peak. HDL_2 and HDL_3 could not be resolved using the TSK-G 4000 SW column. After injection of 50 μl of either infant or adult serum, the cholesterol and the apoproteins AI and B assays in the various lipoproteins were satisfactory. The patterns were in good agreement with those obtained by density-gradient ultracentrifugation of the same samples and illustrate the variability of the lipoprotein distribution in normal and dyslipemic individuals.

The cholesterol/apoprotein ratio in the various lipoproteins was also calculated, as Sniderman et al. [16] have suggested that the apo B/cholesterol ratio in LDL is significantly increased in patients with coronary artery disease. The HPLC technique provides a new approach to a rapid and more exact quantitation of these parameters, which are of clinical relevance.

The resolution of the particular column in the VLDL—IDL—LDL range enables the detection and quantitation of several subclasses within this heterogeneous distribution of particles whose size and composition are correlated with their atherogenicity index [17]. The advantage of HPLC for

analyzing the lipoprotein distribution and composition in infants appears clearly from the above data. The use of a minimal sample volume (50 μl), if necessary after concentration, and a reduced separation time, shows its applicability to the screening and follow-up of newborns at risk for atherosclerosis. Such a separation could be combined with the existing screening procedure based on apoprotein quantitation in plasma [18].

Previous authors [8] had proposed the use of a pre-staining procedure for the quantitation of cholesterol in the various lipoprotein fractions. In our experience this approach has several disadvantages. The lifespan of the column can be drastically shortened due to the absorption of the dye; the recovery of the fractions is decreased, and finally pre-staining does not enable a subsequent immunological quantitation of the apo AI and B proteins.

In conclusion, HPLC represents a potentially useful technique for the separation and isolation of lipoproteins from serum, and the further analysis of their lipid and apoprotein components. It should be applicable to the detection and follow-up of individuals at risk for atherosclerosis and the monitoring of patients with primary and secondary dyslipoproteinemia during diet and drug therapy.

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