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Note

Automated gel permeation chromatography of plasma lipoproteins by preparative fast protein liquid chromatography

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Plasma lipoproteins are commonly separated by ultracentrifugal methods based on differential flotation in high-density solutions, using both sequential [1] and one-step gradient methods [2,3]. During the past decade it has become clear that the combination of high salt concentrations and high centrifugal forces may cause 'stripping' of lipoprotein components, e.g. apolipoproteins A-I, A-IV and E [4–10], lipid transfer protein (LTP) and the enzyme lecithin:cholesterol acyltransferase (LCAT) [11–15].

Plasma lipoproteins may also be separated according to size, using conventional column chromatography on soft gels [4–10, 16–20]. This method avoids the stripping mentioned above, but the procedure is very time-consuming. High-performance liquid chromatography (HPLC) is the obvious answer to this problem, and several papers on this methodology have been published recently [21–26]. However, in general the lipoproteins were first isolated by ultracentrifugation, before being separated according to size by HPLC. This seriously hampered the interpretation of the experiments with respect to the distribution of apolipoproteins and loosely bound enzymes or LTP. This paper describes the methodology for automated lipoprotein separation with gel permeation chromatography by HPLC of plasma, without a preliminary ultracentrifugal step.

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EXPERIMENTAL

Preparation of the plasma samples

Blood was obtained from human volunteers, after an overnight fasting period of 12 h. Blood was collected in tubes containing EDTA and placed on ice immediately. Plasma was prepared within 1 h by low-speed centrifugation at 4 °C for 15 min at 1500 g_{max}. Chylomicron-containing plasma was isolated from a subject who consumed a high-fat meal, 4 h prior to blood sampling. Plasma samples were filtered through 0.8-μm filter units (Type SLAA 025 BS, Millipore, Molsheim, France). These filters do not exclude plasma chylomicrons, as do the commonly used 0.22- or 0.45-μm filters.

Chromatographic conditions and configuration

The HPLC system consisted of a 2150 titaniumhead pump (LKB, Bromma, Sweden), a 2154-002 injection valve (LKB) equipped with a 2-ml loop, a fixed-wavelength monitor (Uvicord SD 2510, lightpath 0.25 cm), a 2212 Helirac fraction collector (LKB) and a 2210 dual-channel recorder (LKB). The configuration is controlled by a 2152 LC controller (LKB). When multiple automated separations are performed, the system is expanded with an E4 CSD 10 P ten-position sampling valve (Valco Instruments, Houston, TX, U.S.A.), a 2154-003 ten-port, two-position switching valve (LKB) and a 2132 low-pressure liquid chromatography pump (LKB) for filling the 2-ml loop (Fig. 1). The column was an HR 16/50 (Pharmacia, Uppsala, Sweden), packed with

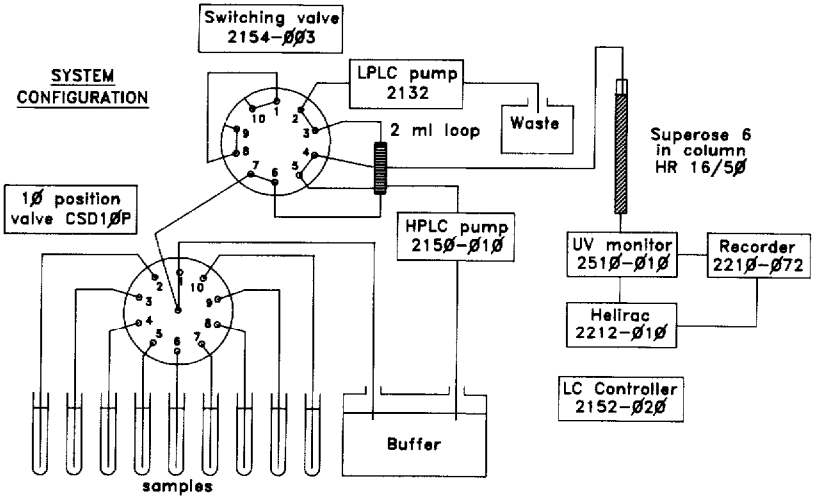

Fig. 1. Schematic representation of the HPLC system used for multiple automated separations of lipoproteins from serum or plasma. The recorder connections and the flow path of the elution buffer are indicated.

prep-grade Superose 6 (Pharmacia), using the packing equipment HR 16 (Pharmacia). The elution buffer consisted of 0.9% sodium chloride 2 m*M* sodium phosphate (pH 7.4), 0.02% sodium azide and 1 or 5 m*M* EDTA (serum or plasma separation, respectively). All operations were performed at 4 °C and monitored by absorbance at 280 nm.

The elution scheme consists of a low-flow separation of the lipoprotein complexes, followed by a high-flow washout of the remaining plasma proteins. Very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are separated during a single run.

Chemical and enzymic analyses

All chemicals used in solvents and buffers were obtained from E. Merck (Darmstadt, F.R.G.) and were analytical grade. The kits for the measurement of triglycerides (TG), unesterified cholesterol (UC) and total cholesterol (TC) were purchased from Boehringer (Mannheim, F.R.G.). The kit for the phospholipid (PL) assay was from BioMérieux (Craponne, France). Boehringer provided the ferritin, catalase and lactate dehydrogenase, and Sigma (St. Louis, MO, U.S.A.) the thyroglobulin, used together with the chylomicrons in the calibration procedure. Absorbances were measured in the U-2000 spectrophotometer, coupled with a 105 XY sampler (Hitachi, Tokyo, Japan). All concentrations are given in nmol/ml of plasma. The activity of LTP was measured using exogenous substrates ([^{3}H]cholesterylester-labelled LDL and unlabelled HDL), exactly as described by Groener et al. [27]. LCAT activity was measured according to Glomset and Wright [28]. Data were analysed with STATA (Computing Resource Center, Los Angeles, CA, U.S.A.).

RESULTS AND DISCUSSION

Column calibration and particle weight determination of VLDL, LDL and HDL

The column was calibrated with four reference proteins of known molecular weight. Chylomicrons, detected as the peak of TG after chromatography of postprandial plasma, were used as a marker of the void volume (particle weight $> 50 \cdot 10^6$). The reference proteins were thyroglobulin (MW 669 000), ferritin (MW 440 000), catalase (MW 232 000) and lactate dehydrogenase (MW 140 000). When the resulting K_{av} values were plotted against log MW, a regression line was obtained with a correlation coefficient of 0.978 (Fig. 2). Using this calibration it can be calculated that the phospholipid-rich lipoproteins (HDL) present in fasted plasma have an average particle weight of 205 000. Because the elution volumes of VLDL and LDL are outside the standard curve, their estimated particle weights can only be an approximation. As shown in Fig. 3, LDL has a slightly smaller elution volume than the immunoglobulin M fraction (MW 900 000). Therefore the molecular weight of LDL will be ca. 1000 000 daltons. VLDL, the TG-rich liprotein present in fasted plasma, con-

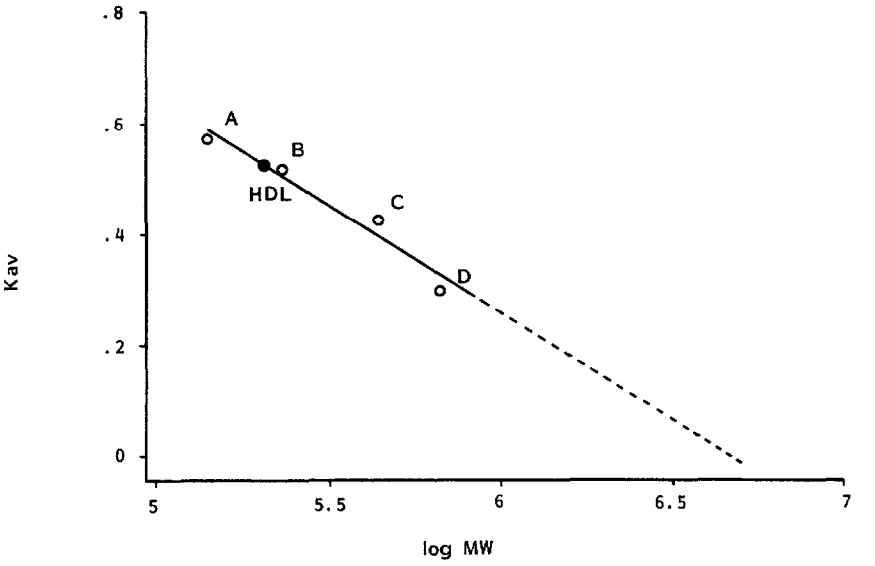

Fig. 2. Calibration plot of the Superose 6 (prep-grade) column. The K_{av} value is defined as [elution volume − void volume] / [total column volume − void volume]. Chylomicrons were used to mark the void volume. Lactate dehydrogenase (A), catalase (B), ferritin (C) and thyroglobulin (D) were used as reference proteins. The calculated regression line had a correlation coefficient (r) of 0.978. HDL marks the position of high-density lipoproteins. The average calculated particle weight of HDL is 205 000 daltons.

sistently starts eluting in fraction 1 and reaches a maximum in fraction 2. As fraction 1 corresponds to the void volume, we expect the VLDL particle weight to be between 40 000 000 (the exclusion limit of Superose 6 prep grade) and 5000 000 daltons (the upper limit of the theoretical separation range of Superose 6 prep grade).

Multiple, automated lipoprotein separations of human plasma

Up to six different plasma samples of fasted subjects can be chromatographed overnight in the automated system configuration (Fig. 1). Each separation (2 ml of plasma) starts with a flow-rate of 0.50 ml/min. The eluent is directed to the waste during the first 60 min, and is subsequently collected in fourteen fractions of 3.57 ml each, until all lipoproteins have eluted from the column (160 min after the start of the elution). After this time the eluent is either directed to the waste or recovered in one batch, and the plasma proteins are washed from the column with 40 ml of buffer at a flow-rate of 2.00 ml/min.

Fig. 3 shows a recording of the absorbance of the effluent at 280 nm in a typical experiment. The collected fractions are frozen and stored at −20°C. Samples of the original plasma (both before and after filtration through the

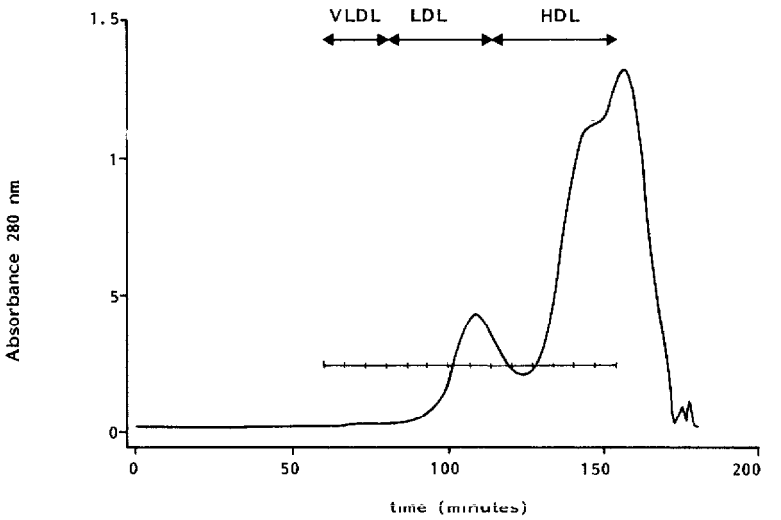

Fig. 3. Recorder tracing of the optical density at 280 nm of the effluent after gel permeation chromatography of 2 ml of plasma by HPLC. The component with a retention time of 110 min is immunoglobulin M. The increase in optical density at 160 min is caused by the programmed increase in flow-rate from 0.5 to 2.0 ml/min. The fourteen isolated fractions are indicated on the horizontal bar. VLDL is eluted in fractions 1–3, LDL in fractions 4–8 and HDL in fractions 9–14.

0.8-μm filter) are kept at 4°C during the lipoprotein separation and frozen together with the isolated column fractions.

The isolated fractions are assayed for TG, UC, TC and PL. Esterified cholesterol (EC) is calculated from the difference of TC and UC. In general, samples of 200–400 μl are necessary for analyses in the diluted column fractions. In the PL assay it is necessary to compensate for the EDTA present in the elution buffer, by adding an equivalent amount of calcium chloride.

Figs. 4 and 5 show typical examples (subjects A and B, respectively) of the elution profiles of the various plasma lipids. Plasma samples from different individuals have their own typical lipid profiles, depending on the relative amounts of the different lipoprotein classes. The highest individual variation is observed for the distribution of plasma TG (cf. Figs. 4A and 5A). A combination of the particle size and the relative amounts of the various lipids enables the characterization of the different lipoprotein classes. It can be concluded that VLDL, LDL and HDL are eluted in fractions 1–3, 4–8 and 9–14, respectively. This may be confirmed by separate gel permeation chromatography of the different lipoprotein classes, after prior isolation by ultracentrifugation.

We found that several normolipidemic individuals carry the majority of their plasma TG in LDL-sized lipoproteins and not in VLDL-sized particles in the fasted state (an example is shown in Fig. 4). The average lipid compositions of the separated lipoprotein classes are given in Table I. The values are vir-

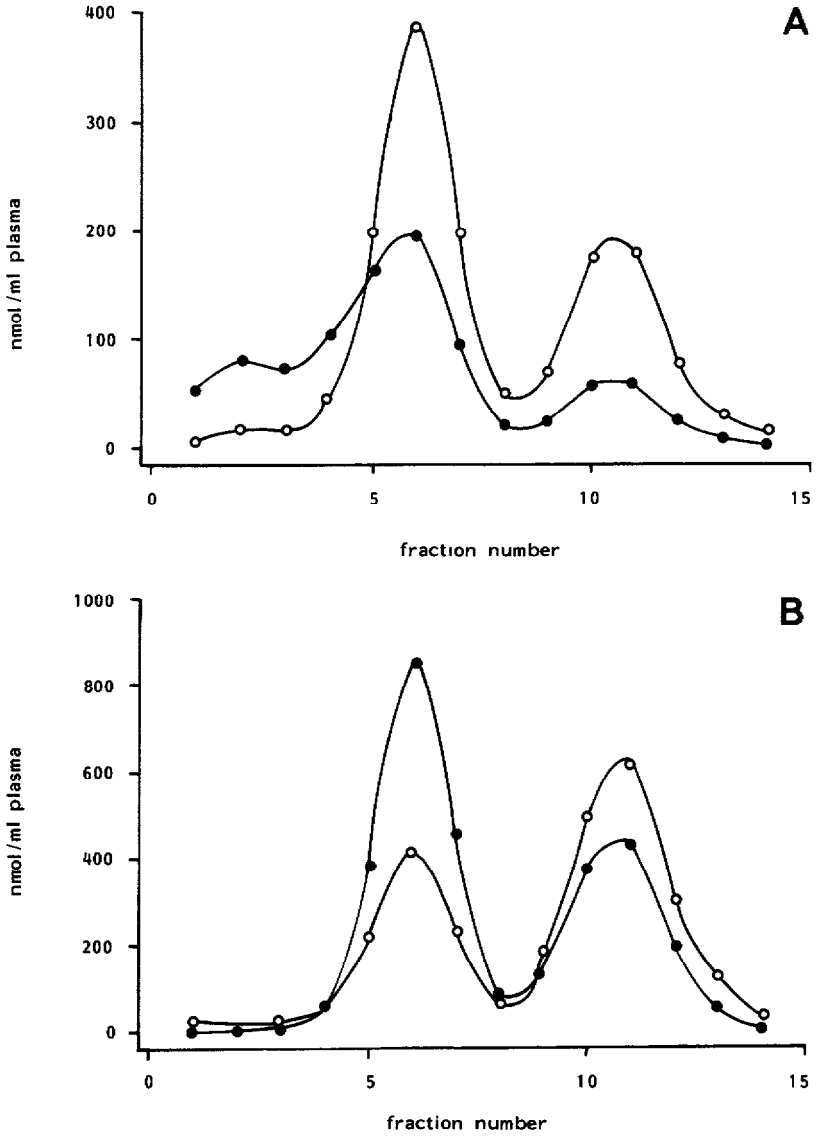

Fig. 4. Elution profiles of the lipids present in plasma lipoproteins after gel permeation chromatography of plasma from a fasted individual (subject A) by HPLC. Fourteen fractions are isolated. VLDL is present in fractions 1-3, LDL in fractions 4-8 and HDL in fractions 9-14. (A) (●) Triglycerides; (○) unesterified cholesterol. (B) (●) Cholesterylesters; (○) phospholipids. All lipid values are given in nmol/ml of plasma. Data points were connected using the cubic spline algorithm of the STATA program.

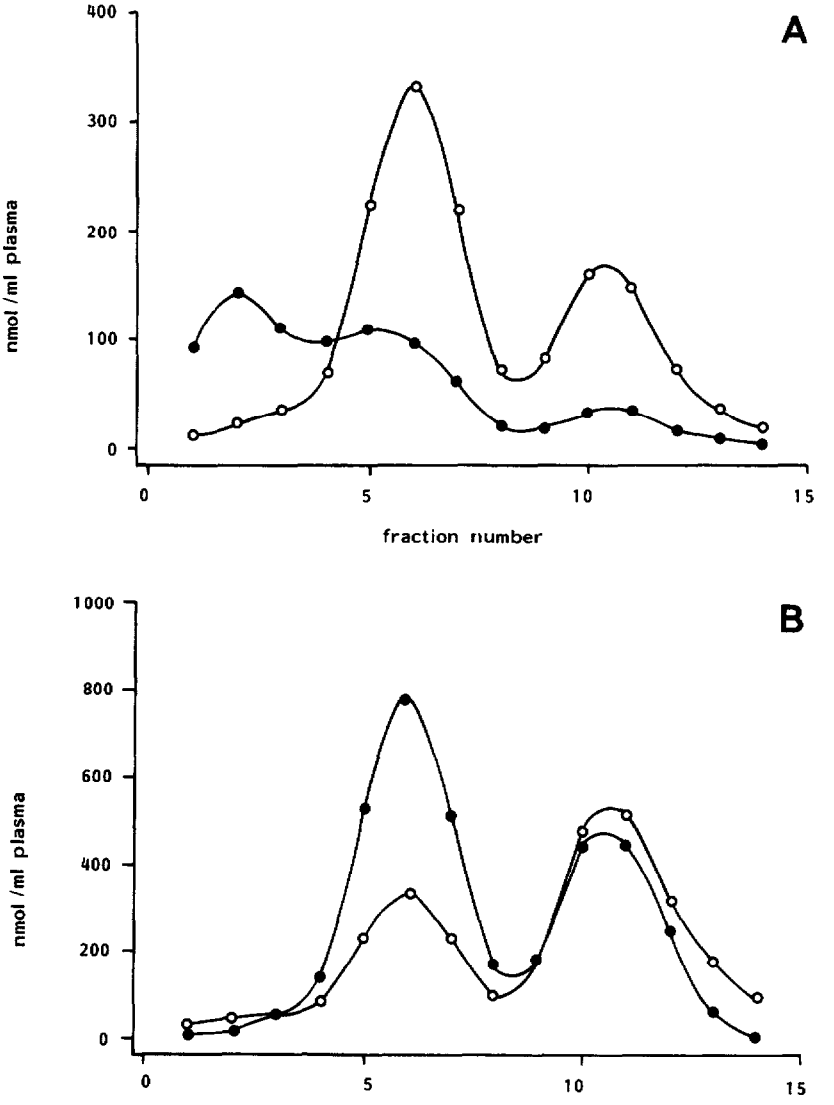

Fig. 5. Elution profiles of the lipids present in plasma lipoproteins after gel permeation chromatography of plasma from a fasted individual (subject B). For further explanation see the legend to Fig. 4.

tually identical with the lipid compositions reported for ultracentrifugally isolated VLDL, LDL and HDL [29,30].

On several occasions the column fractions were also assayed for LTP and LCAT activities. The overall profiles of these parameters were similar to that of HDL lipid. However, each activity had its specific size distribution. Both LCAT activity and LTP activity peaked at particle weights clearly above 125 000

TABLE I

LIPID COMPOSITION OF LIPOPROTEINS ISOLATED WITH GEL PERMEATION CHROMATOGRAPHY BY HPLC

Lipid component	Amount (mean ± S.D., $n=6$) (mol%)		
	VLDL (fractions 1-3)	LDL (fractions 4-8)	HDL (fractions 9-14)
Triglycerides	57.9 ± 3.5	11.7 ± 1.8	4.6 ± 1.0
Unesterified cholesterol	12.5 ± 1.8	20.5 ± 0.3	13.3 ± 1.2
Cholesteryl esters	11.1 ± 1.5	45.5 ± 2.6	36.4 ± 3.0
Phospholipids	18.5 ± 1.4	22.5 ± 0.7	45.6 ± 1.4

TABLE II

RECOVERIES OF LIPOPROTEIN LIPIDS AFTER GEL PERMEATION CHROMATOGRAPHY OF PLASMA BY HPLC

Lipid component	Recovery (mean ± S.D., $n=6$) (%)
Triglycerides	92.6 ± 2.3
Unesterified cholesterol	110.8 ± 2.7
Cholesteryl esters	100.3 ± 1.4
Phospholipids	103.3 ± 5.0

daltons, suggesting that these activities were bound to HDL-sized lipoprotein fractions and not present in plasma in an unbound, monomeric form (not shown).

The effect of storage of plasma was studied by comparing the lipid profiles obtained after gel permeation chromatography of freshly isolated plasma and plasma stored for 24 h at 4°C. The only effect is a slight broadening of the HDL peak. This small change does not have any consequences for the overall distribution of cholesterol between VLDL, LDL and HDL. It is concluded that the lipoprotein profiles obtained from the six plasma samples after the automated procedure, which includes storage of some samples at 4°C for up to 16 h, can be compared adequately.

Table II shows the recoveries of the various lipoprotein lipids after gel permeation chromatography of plasma by HPLC. The average overall recovery for all lipids is 101%, allowing reliable comparisons between different plasma samples.

Thus gel permeation chromatography of plasma by HPLC, using rigid gels, can be used for separation of the lipoprotein classes in plasma or serum of

fasted individuals. Six plasma or serum samples, of 2 ml each, can be handled overnight in an automated way.

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