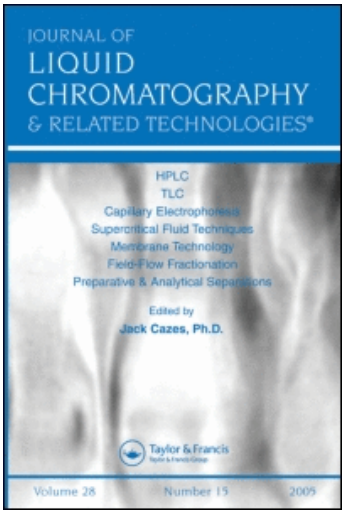

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Lipoproteins From Human Plasma by Flow Field-Flow Fractionation

Ping Li[a]; Marcia Hansen[a]; J. Calvin Giddings[b]
[a] FFFractionation, LLC 4797 South West Ridge Blvd, Salt Lake City, Utah [b] Department of Chemistry University of Utah, Field-Flow Fractionation Research Center, Salt Lake City, Utah

To cite this Article Li, Ping , Hansen, Marcia and Giddings, J. Calvin(1997) 'Separation of Lipoproteins From Human Plasma by Flow Field-Flow Fractionation', Journal of Liquid Chromatography & Related Technologies, 20: 16, 2777 — 2802

To link to this Article: DOI: 10.1080/10826079708005592

URL: http://dx.doi.org/10.1080/10826079708005592

J. LIQ. CHROM. & REL. TECHNOL., 20(16 & 17), 2777–2802 (1997)

SEPARATION OF LIPOPROTEINS FROM HUMAN PLASMA BY FLOW FIELD-FLOW FRACTIONATION

Ping Li,[1] Marcia Hansen,[1,*] J. Calvin Giddings[2,†]

[1] FFFractionation, LLC
4797 South West Ridge Blvd.
Salt Lake City, Utah 84118

[2] Field-Flow Fractionation Research Center
Department of Chemistry
University of Utah
Salt Lake City, Utah 84112

ABSTRACT

A flow field-flow fractionation (flow FFF) system was used in both isocratic and programmed-field procedures to rapidly analyze and characterize the HDL, LDL, and VLDL fractions of human blood plasma. In this paper, the general principles and theory of separation are briefly reviewed. The theoretically predicted retention values are shown to compare favorably with the experimental results. The sample recovery and system reproducibility were determined. The lipoprotein fractions were clearly separated into different peaks, although the peaks tended to be rather broad, predominantly due to the sample polydispersity and, to a smaller extent, due to systemic bandbroadening. Plasma samples were analyzed without sample pre-treatment and differences in lipoprotein profiles were observed for different individuals.

Not only could the HDL, LDL, and VLDL fractions be separated, but lipoprotein subspecies were also determined with the use of a programmed field. The hydrodynamic sizes and diffusion coefficients of plasma lipoproteins were deduced from their retention behavior based on FFF theory. The characterization of lipoprotein fractions, based on size or diffusion coefficient, provided additional information which may be useful for research or diagnostic purposes.

INTRODUCTION

Lipoproteins are complexes of lipids and proteins. These complexes have a heterogeneous distribution in density, size, protein composition, and charge. The outer surface of the lipoprotein particle is made up of polar groups of phospholipids, free cholesterol, and apolipoproteins. The interior of the particle includes neutral lipids, triglycerides, and cholesterol esters.[1]

Traditionally, plasma lipoproteins have been classified into high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL) according to their ultracentrifugation rate of flotation in a solution of sodium bromide. The further classification into α-lipoproteins, β-lipoproteins, and pre-β-lipoproteins is based on electrophoretic mobility.[2] In general, LDL and VLDL are most strongly correlated with human coronary heart disease (CHD).[3,4] The HDL component, in contrast, has been linked to both prevention and regression of this disease.[5] Analysis of the total lipoprotein profile is useful for assessing the risk of atherosclerosis and for monitoring the treatment of lipid abnormalities. Therefore, lipoprotein profile measurements have become one of the most popular methods to assess lipoprotein abnormalities and CHD risk in clinical diagnosis.

Several technologies have been successfully used to separate lipoproteins and determine the lipoprotein profile in plasma, including ultracentrifugation, chemical precipitation, electrophoresis, and chromatography. Additional characterization can be done by proton nuclear magnetic resonance spectroscopy,[6] and near-IR spectroscopy.[7] The advantages and disadvantages of some of the methods have been discussed in recent years.[8-10] Of these methods, ultracentrifugation, precipitation, and the Friedewald procedure[11] are considered to be the basic methods for both specialized research laboratories and routine clinical laboratories. Ultracentrifugation is the reference research technique for studying lipoproteins and has many advantages for preparative scale isolation of lipoprotein fractions. However, there are drawbacks to this technique. The cost, sample volume, and amount of time required for

ultracentrifugation analysis prohibit routine application in clinical work.[12] Also, structural changes of the lipoprotein complex may be induced due to shearing and ionic-strength effects during the centrifugation.[13] Although the ultracentrifugation method has been improved in recent years by use of a microcentrifuge in combination with a precipitation method to reduce sample volume and the amount of time required, these improved procedures are still difficult to use routinely to check the size or density distribution of plasma lipoproteins in clinical work.[10,14]

To avoid ultracentrifugation altogether, enzymatic reactions and the Friedewald calculation are often used in many clinical laboratories. In this procedure, the total plasma cholesterol (TC) and triglyceride (TG) levels are determined by enzymatic reactions. The VLDL-cholesterol (VLDL-C) is estimated to be a fixed fraction of TG (VLDL-C = TG/5). HDL-C is determined by precipitating out LDL and VLDL from plasma using 50 k Dalton dextran sulfate and magnesium chloride. LDL-C is then calculated from the Friedewald formula: (LDL-C) = TC - (HDL-C) - 0.2TG.[11]

Although this procedure has been commonly used for clinical measurement, there are serious drawbacks. One important consideration is its inaccuracy and incompleteness because of the assumptions and indirect measurements. Large relative inaccuracies have been reported for TC measurements using enzymatic reactions.[15] The procedure also assumes that the triglyceride level is highly correlated with the VLDL-C level. To enhance this correlation, the procedure requires a plasma sample from fasting individuals, an inconvenience for many patients.[16] Additionally, poor reproducibility of the precipitation reaction has led to large errors, with coefficients of variation in the range of 5 to 38%.[15,17,18] Another disadvantage is that this procedure estimates only TC, TG, HDL-C, LDL-C, and VLDL-C. Information about subspecies of the lipoproteins, which may have important health implications, cannot be determined by these methods.

Field-flow fractionation (FFF) is a family of chromatographic-like techniques that are capable of the rapid and highly efficient separation of macromolecules and colloids.[19-21] Because an open flow channel and simple physical forces are used in this technique, FFF is a widely applicable separation method for macromaterials with great flexibility in sample type, carrier liquid or solvent, pH, ionic strength, and so on. Since FFF separation takes place in a single phase without the participation of second phases or surfaces, there is minimal possibility that biological materials will be altered or denatured by interaction with a surface. Because there is no channel packing material, there is little tendency for shear degradation of fragile high-molecular-weight species.

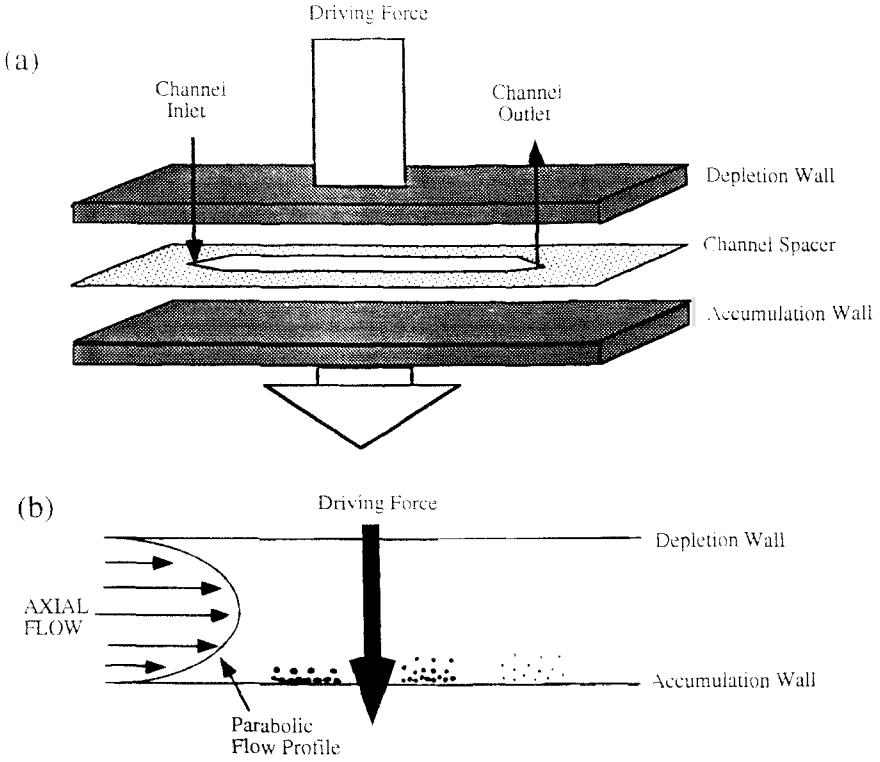

Figure 1. Schematic diagram of FFF channel (a) and separation process (b)

FFF has been successfully utilized for the separation of a variety of biological materials spanning a broad molecular weight and diameter range, including proteins,[20] protein aggregates,[21-24] protein-polymer conjugates,[25] DNA,[22,23,26] viruses,[23,27,28] bacteria,[21,29] and cells.[30] Preliminary results have also been shown for lipoproteins.[25] These results suggest that FFF should be examined at greater depth as a tool for the separation and characterization of lipoproteins.

The principles of FFF are shown in Figure 1. A stream of carrier liquid is introduced at one end of the channel and a small volume of sample is injected. The injected sample spreads out across the channel breadth and proceeds down the channel undergoing separation. The separation process originates in the

flow profile across the narrow dimension of the channel, which is parabolic in form. For parabolic flow, the flow velocity approaches zero at the walls (Figure 1b). An external driving force is applied on the contents of the channel in a direction perpendicular to the flow axis. The injected components are driven by the applied force toward one of the walls (the accumulation wall). They end up in different stream laminae near the accumulation wall, which causes the components to have different velocities and thus elute at different times.

Different driving forces lead to different FFF techniques, most notably sedimentation FFF, flow FFF, thermal FFF, and electrical FFF. In this work, flow FFF is used as the preferred method for characterizing human plasma lipoproteins. In flow FFF, a crossflow stream is applied as an external field.[19,21] The level of retention is determined by the flowrate of the crossflow stream and the sizes or diffusion coefficients of the components separated. The mechanism is as follows.

All species in the channel, large or small, are transported toward the accumulation wall at the same rate by the crossflow. They are driven away from the wall at different rates by diffusion. These opposing processes result in a steady-state exponential distribution of each component near the accumulation wall. The distributions have different thickness (as shown by component bands A, B, and C in Figure 1b) because of the unlike diffusion coefficients. Since smaller components have larger diffusion coefficients, they form thicker steady-state layers (for example, component band C in Figure 1b) and thus have a higher velocity in the parabolic flow stream. Consequently, smaller components are eluted first followed by larger ones (see theory section). The densities of lipoproteins vary from 1.063-1.210 g/mL for HDL, 1.019-1.063 g/mL for LDL, and <1.006 g/mL for VLDL. Their sizes are known to be inversely related to their densities; sizes vary from 5 nm up to 80 nm proceeding from HDL to VLDL.[31]

These large differences in size between the various lipoproteins result in differential retention and separation. The inverse correlation of size and density implies that the size-based separation of flow FFF also provides density fractionation. However, the subpopulations of lipoproteins may exhibit subtle variations in size and density that are meaningful but uncorrelated.

In the usual flow FFF procedure, small samples (a few tens of microliters) are injected into the flowstream entering the channel. Once the samples have entered the channel, the flow is stopped for a sufficient time (the stopflow time) to allow all sample components to reach the accumulation wall and become relaxed to their steady-state distributions. Flow is then resumed and the separation process begins. In this study, a modified flow FFF system was

utilized that employs hydrodynamic relaxation to avoid the flow disturbances and time delay of normal stopflow operation. The hydrodynamic relaxation was achieved using a special frit inlet channel.[32]

In flow FFF, the cross flowrate can be held constant or varied with time (programmed) during a run. Programmed operation has advantages for analysis, speed, and baseline enhancement, but the demands for flow control are more exacting.[33] The programming of a frit-inlet system has not previously been reported. In this study a programmed frit-inlet system was developed and used; a constant cross flowrate was also utilized for some experiments.

Using frit inlet flow FFF, the separation efficiency, recovery, and reproducibility of flow FFF were checked using proteins, purified lipoprotein fractions, and plasma samples. The hydrodynamic sizes and diffusion coefficients of the plasma lipoproteins were measured using flow FFF retention values and compared with literature values. Our goal is to achieve a direct and rapid measurement of lipoprotein fractions (especially LDL) that does not rely on the indirect techniques and assumptions of the usual clinical procedures.

THEORY

In flow FFF, the retention time is controlled by the crossflow. Since the geometry and flow profile of the channel are well defined, the retention times of the separated components can be theoretically predicted and can be related to the hydrodynamic diameters and diffusion coefficients of the components by mathematical expressions.[21] Below we give the limiting form of the equations valid for well-retained components.

The flow FFF retention time can be related to component diffusion coefficient D and to system operating parameters by[34]

$$t_R = \frac{w^2 \dot{V}_c}{6DV} \tag{1}$$

where w is channel thickness, $\dot{V}_c$ is the cross flowrate, and $\dot{V}$ is the channel flowrate. If D is replaced by the Stokes-Einstein equation,[35]

$$D = \frac{kT}{3\pi\eta d_h} \tag{2}$$

the retention time can also be related to the hydrodynamic diameter d_h

$$t_r = \frac{\pi \eta w^2 \dot{V}_c d_h}{2kT\dot{V}} \tag{3}$$

where η is the viscosity of the carrier, k is Boltzmann's constant, and T is temperature. When a programmed crossflow stream is applied in flow FFF, the retention time necessary to elute the sample through the entire particle size range can be greatly reduced. Using a linear programmed crossflow, the retention time t_r' can be approximated by[36]

$$t_r' = t_1 + t_p \left[1 - \exp\left(\frac{t_1}{t_p} - \frac{t_r}{tp} \right) \right] \tag{4}$$

where t_r is the retention time calculated using Equation (1) for the component at the initial crossflow, t_1 is the initial cross flowrate holding time, and t_p is the linear decay time of the cross flow rate.

Equations (1) and (3) show that the flow FFF retention time of a component is inversely proportional to the diffusion coefficient and directly proportional to the hydrodynamic diameter of the component.

EXPERIMENTAL

Equipment

The flow FFF system (Figure 2) consists of a channel whose breadth is 2.0 cm and length is 28.5 cm from tip to tip. A sheet of ultrafiltration membrane served as the accumulation wall in the channel, and the channel thickness is determined using the measured retention times of bovine serum albumin (BSA) and Equation (1). A 10 μL sample solution was injected using a septum injector placed near the inlet. Two Spectra-Physics Isochrom pumps (Spectra-Physics Inc., San Jose, CA, USA) were used to supply sample substream (flowrate $\dot{V}_s$) and frit inlet substream ($\dot{V}_f$) flows. A Kontron 410 HPLC pump (Kontron Electrolab, London, UK) was controlled by a microcomputer (designed and built in conjunction with the electronics shop at the Department of Chemistry, University of Utah) to supply either an isocratic or programmed crossflow substream ($\dot{V}_c$). A Shimadzu SPD-6A UV detector (Shimadzu

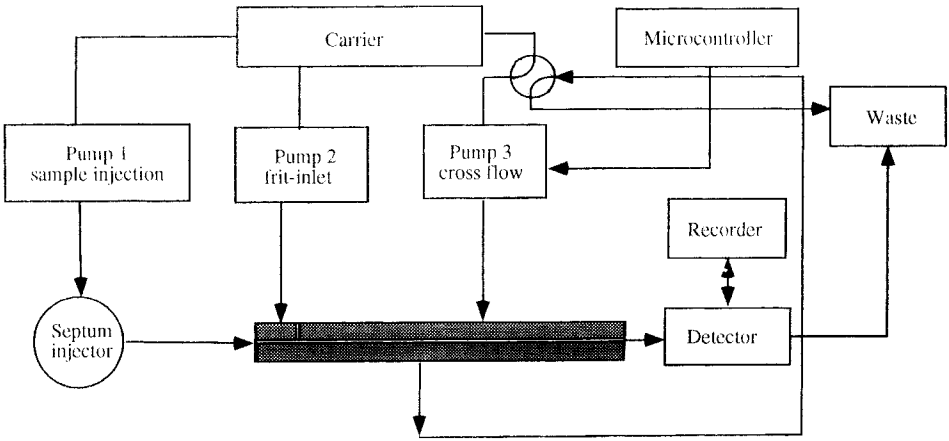

Figure 2. Schematic diagram of a frit-inlet flow FFF system.

Corporation, Kyoto, Japan) and a Linear UV-106 detector (Linear Instruments Corporation, Reno, Nevada, USA) along with a PC compatible computer were used to monitor and record the separation. The detector wavelength was set at 280 nm to monitor the separation. The experiments were carried out at room temperature (23 ± 1 °C).

Specimens

All protein samples were purchased from Sigma Chemical Company (St. Louis, MO). Doubly-distilled deionized water was used to prepare the carrier solutions. A phosphate buffer saline (PBS) solution (138 mM sodium chloride, 2.7 mM potassium chloride , and 10 mM phosphate buffer salts) at pH 7.4 was used as the carrier.

The plasma samples and the two batches, I and II, of purified lipoprotein fractions (HDL-I, LDL-I, and VLDL-I; HDL-II, LDL-II, and VLDL-II) were prepared by the following procedures. Plasma samples were obtained by collecting blood from different individuals after a 12 hour fast using an evacuated blood collection tube containing dry disodium EDTA (1mg/mL). The blood cells were spun out by centrifugation for about 30 minutes at 3000 rpm. The purified lipoprotein fractions were prepared by ultracentrifugation of the cell-free plasma. The plasma (density 1.006 g/mL) was ultracentrifuged at 40,000 rpm at 15°C for 24 hours. The VLDL components floated to the top in

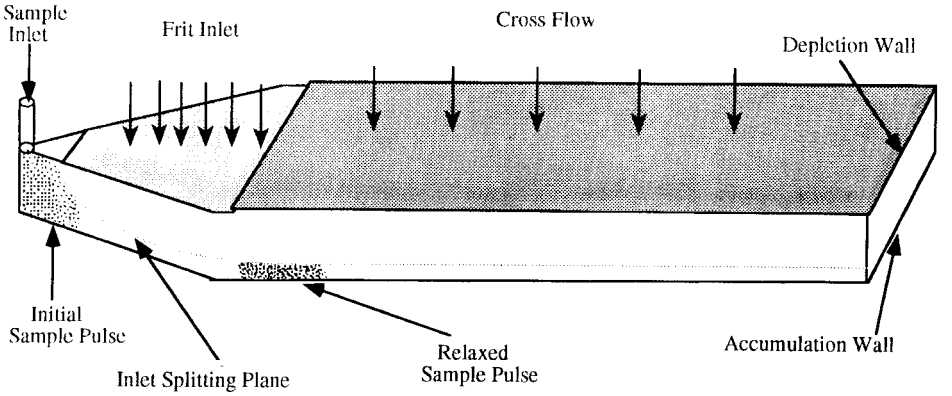

Figure 3. Illustration of hydrodynamic relaxation achieved in a frit-inlet flow FFF channel.

this step. The LDL and HDL components remained in the infranate. The centrifugal tube was sliced to separate the supernatant and infranatant fractions. The infranate density was adjusted to 1.063 g/mL with sodium bromide in order to float the LDL. Following this, the infranate was ultracentrifuged for another 24 hours. The HDL components were isolated from the infranate by spinning for 24 hours after further adjustment of the density from 1.063 to 1.210 g/mL.[37]

Procedures

Hydrodynamic relaxation

In typical FFF operation, a relaxation (or equilibration) step (known as the stopflow procedure) is carried out prior to separation.[19] As explained earlier, channel flow is halted during this step while the crossflow drives all components to the accumulation wall. This stopflow method has disadvantages that include increased analysis time, baseline instability, and increased probability of particle-membrane adhesion. A hydrodynamic relaxation technique using a frit inlet has been developed recently.[32] This procedure avoids the need for stopflow and thus minimizes the disadvantages listed above. The flow FFF system used in this work incorporates such a frit inlet system.

Frit-inlet hydrodynamic relaxation is a process in which sample material is rapidly driven close to its equilibrium position by the frit-inlet flow. The frit-inlet technique utilizes a special element of permeable wall material near the inlet of the channel through which a frit-inlet flowstream can be introduced into the channel. The sample enters the channel in a different substream that forms a thin lamina beneath the frit-inlet substream. The frit-inlet substream compresses the sample substream against the accumulation wall, thus achieving relaxation hydrodynamically (see Figure 3).

A flow FFF system can be modified for frit-inlet hydrodynamic relaxation simply by isolating a small element of the depletion wall to serve as the frit inlet as shown in Figure 3.[32] A substream of the flow is then fed into this isolated area, through the permeable wall, and into the channel. Typically, the frit inlet flow velocity is 20-50 times greater than the crossflow velocity. The channel flowrate $\dot{V}$ is then equal to the sum of the flowrates of the frit inlet substream ($\dot{V}_f$) and the sample substream ($\dot{V}_s$). The important advantage of this relaxation technique is its simplicity of operation and potential for automation.

Programmed crossflow

In this work, a linear programmed crossflow was supplied by a loop recycling system in which the crossflow outlet stream feeds the inlet crossflow pump (Figure 2). This system rigidly equalizes the flowrates of the crossflow inlet and outlet, thus assuring that the channel flowrate $\dot{V}$ can be kept constant or is free of gradients during the programmed operation. The dependence of the cross flowrate on time is illustrated in Figure 4. During time t_1 the cross flowrate is held constant prior to the initiation of linear programming. The cross flowrate reaches a final flowrate value in time t_p after the linear cross flowrate decay begins.

Cholesterol analysis

The cholesterol concentrations of the purified lipoprotein samples and of the fractions collected from the flow FFF system were measured enzymatically using a modified method of Allain[38] provided by Sigma Chemical Company. Samples were incubated with Sigma diagnostics cholesterol reagent (Cat. No. 352) for ten minutes at 25 °C. The light absorption for each incubated sample was determined at 500 nm. The cholesterol content was then calculated using a blank sample and a 200 mg/dL Sigma "cholesterol calibrator" (Cat. No. C0284).

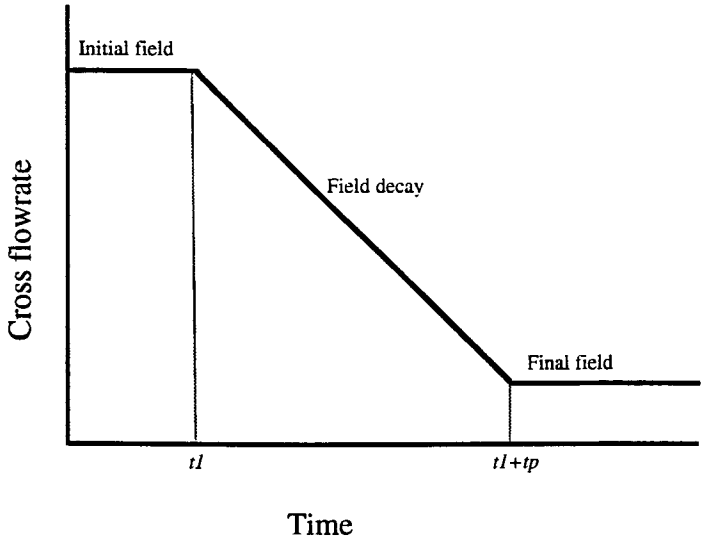

Figure 4. The profile of programmed field in the flow FFF channel.

Recovery determination

The system recovery for a component is defined as the amount of that component eluted from the channel outlet relative to the amount injected; the recovery is usually expressed as a percentage. Recoveries of various components were determined by measuring the amount of a specific component eluted and comparing this to the amount injected. For proteins, the initial sample was diluted into the same volume of carrier as the collected fraction and concentrations were determined using a UV-visible spectrophotometer at 280 nm. For lipoproteins, the initial sample and collected fractions were assayed for cholesterol concentration using the enzymatic method described above.

RESULTS AND DISCUSSION

HDL, LDL, and VLDL Separation and Size Distribution

Plasma lipoproteins are spherical lipid-protein particles with a heterogeneous density and size population. The average size of lipoprotein

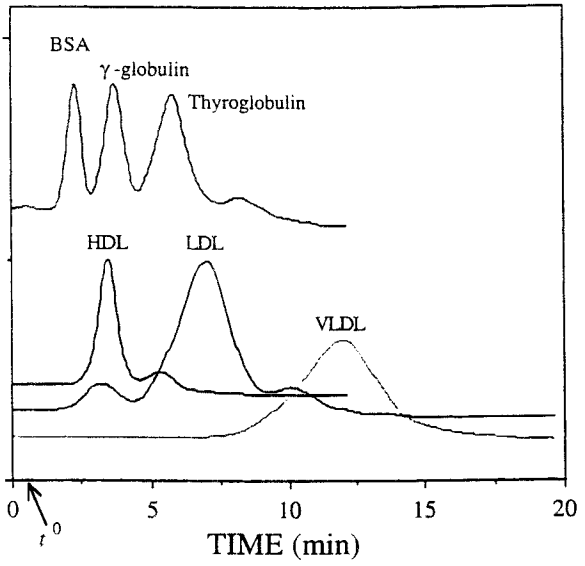

Figure 5. Flow FFF separation of proteins and lipoprotein fractions (HDL-I, LDL-I, and VLDL-I). Conditions are $\dot{V} = 2.2$ mL/min, $\dot{V}_c = 5.0$ mL/min, $\dot{V}_f / \dot{V}_s = 10$, UV 280 nm, $w = 153$ μm.

particles range from 5-12 nm for HDL, 20-30 nm for LDL, and 30-80 nm for VLDL fractions.[31] The significant difference in hydrodynamic sizes will cause differences in retention for the three lipoprotein fractions and, thus, their flow FFF elution sequence will be HDL, LDL and VLDL.

Individual lipoprotein fractions from the first batch of preparation (HDL-I, LDL-I, and VLDL-I) prepared as described in the Experimental Section, and three proteins, ranging from 7 to 17 nm in hydrodynamic diameter, were analyzed by a flow FFF system using an isocratic crossflow velocity to characterize their retention behavior. The elution times were also predicted according to Equation (1) or (3) based on diffusion coefficient or size of component and operation conditions, and the predicted results were compared with the experimental values. Figure 5 shows the retention characteristics of these components under channel and crossflow conditions of 2.2 and 5.0 mL/min, respectively. In the separation, the high concentrations of sodium bromide and other small molecular weight species in the purified lipoprotein fractions do not show any signal in the fractograms because they were eliminated by passing through the ultrafiltration membrane which was used as the accumulation wall in the channel. In Figure 5, LDL and VLDL fractions

Table 1

Molecular Weights, Diffusion Coefficients, Hydrodynamic Sizes, and Flow FFF Retention Times of Proteins and Lipoproteins

Sample	MW (k Dalton)	Diffusion Coefficient $D_{w,20}x10^{-7}$	Diffusion Coefficient $D_{PBS,23}x10^{-7}$	Size (nm)	t_r (min)† Cal.††	t_r (min)† FFF†††
BSA[1]	66	6.15	6.61	6.6	2.23	2.21±0.02
γ-globulin[1]	158	4.00	4.30	10.1	3.44	3.37±0.03
Thyroglobulin[1]	669	2.61	2.81	15.4	5.27	5.23±0.09
HDL[2]	150-300	8.12-3.36	8.73-3.61	5-12	1.7-4.1	3.29±0.04
LDL[2]	3000-5000	2.02-1.34	2.16-1.44	20-30	6.8-10.2	7.00±0.13
VLDL[2]	5000-80,000	1.34-0.50	1.44-0.54	30-80	10.2-27.3	12.0±0.33

† PBS pH 7.4, $\dot{V} = 2.2$, $\dot{V}_c = 5.0$ mL/min, w = 0.0153 cm, $V^0 = 0.81$ mL. †† Calculated from $D_{PBS,23}x10^{-7}$ using Equation (1). ††† At peak maximum, ± x.xx is the standard deviation, n = 5. [1] H. A. Sober, ed., **CRC Handbook of Biochemistry**, 2nd ed., CRC Press, Cleveland, OH, 1970, pp. C3-C9. [2] Average value from **Biochemistry of Atherosclerosis**, A. M. Scanu, R. W. Wissler, G. S. Getz, eds., Marcel Dekker, Inc., New York, 1979, pp. 3-8, assuming that lipoproteins have the same size distributions in PBS buffer as in plasma. The average diffusion coefficients of lipoproteins were calculated from their average sizes using the Stokes-Einstein equation (Equation (2)).

showed partial peak overlap, which indicates that either these fractions are not purified by the ultracentrifugation procedure or that particles of similar size exist in different density fractions. However, the major components of each fraction were separated to a sufficient degree for analytical determination. Table 1 provides molecular weights, hydrodynamic sizes, diffusion coefficients, and retention times of the proteins and lipoproteins. The retention times were obtained from both theoretical prediction and flow FFF experiments. Because the experiment was done at 23 °C using a PBS buffer as carrier, the flow FFF retention times should be calculated using Equation (1) with the diffusion coefficients of proteins and lipoproteins in PBS buffer at 23 °C ($D_{PBS,23}$). The diffusion coefficient $D_{PBS,23}$ can be calculated from the diffusion coefficient of component in water at 20 °C ($D_{w,20}$) by the following equation that corrects for viscosity differences

$$D_{PBS,23} = D_{w,20} \frac{296}{293} \frac{\eta_{w,20}}{\eta_{PBS,23}} \qquad (5)$$

in which $\eta_{PBS,23}$ is the viscosity of PBS buffer at 23 °C and $\eta_{w,20}$ is the viscosity of water at 20 °C. Assuming that, $\eta_{w,20}/\eta_{PBS,20} = \eta_{w,23}/\eta_{PBS,23}$, thus $D_{PBS,23}$ can be calculated as

$$D_{PBS,23} = D_{w,20} \frac{296}{293} \frac{\mu_{w,20}}{\eta_{PBS,20}} \frac{\eta_{w,20}}{\eta_{w,23}} = 1.075 D_{w,20} \quad (6)$$

The retention results listed in Table 1 show that the flow FFF measured values are in very good agreement with the calculated values.

On the other hand, when unknown samples are analyzed using a flow FFF system, the diffusion coefficients or particle sizes of the eluted components can also be calculated from Equation (1) or (3) according to their retention times. Therefore, flow FFF fractograms do not simply provide general separation information as does chromatography. The diffusion coefficients or particle sizes can be deduced from retention times. Consequently, these results show that an isocratic crossflow field used in flow FFF is appropriate for the separation and size determination of the plasma HDL, LDL, and VLDL fractions, which demonstrates the potential of flow FFF for profiling lipoprotein contents.

System Recovery

As shown in Figure 3, a crossflow drives the sample to the accumulation wall of the channel. An ultrafiltration membrane acts as the accumulation wall to allow the flow across the wall while retaining sample components in the channel. Normally, the sample loss in flow FFF is contributed by sample adsorption on the membrane surface and sample loss across the membrane. Therefore, membrane properties such as polarity and pore size become two crucial factors affecting system separation efficiency and recovery. Since hydrophobic membranes have strong interactions with proteins, the hydrophilic membranes were chosen as the accumulation wall for the lipoprotein separation.

Several commercial ultrafiltration membranes have been used as the accumulation wall and their characteristics and sources are listed in Table 2. The study of FFF recovery (Table 3) indicated that hydrophilic membranes have less membrane adsorption with protein and lipoprotein probes. YM-type membranes as well as the PLGC membrane have negligible surface interactions with lipoproteins and are suitable for measuring lipoprotein profiles. Another important consideration is that the molecular weight cut-off of the membrane is a more significant factor for the system recovery for low molecular weight components; therefore, the pore size of the ultrafiltation membrane should be small enough to ensure that there is no sample loss across the accumulation

Table 2

Characteristics and Sources of Membranes

Materials	Membranes	MW/Size Cut-Off	Properties	Resources
Regenerated cellulose	YM-1	1,000	Hydrophilic	Amicon
"	YM-5	5,000	"	"
"	YM-10	10,000	"	"
"	YM-30	30,000	"	"
"	YM-100	100,000	"	"
Acrylic copolymer	XM-300	300,000	"	"
Regenerated cellulose	PLGC	10,000	"	Millipore
Polyethylene terephthalate	PETP(thick 12μm)	1μm	"	Cyclopore
"	PETP(thick 23 μm)	1μm	"	"
Polycarbonate	PC	1μm	"	"
Isotactic polypropylene	Celgard 2400	50 nm	Hydrophobic	Hoechst Celanese

Table 3

Flow FFF Recoveries

Membranes	Cytochrome C	BSA	γ-Globulin	Thyro-Globulin	HDL	LDL
YM-1	95	98	98	98	98	98
YM-5	85	96	95	97	99	98
YM-10	65	95	97	98	98	98
YM-30	5	95	96	97	98	98
YM-100	2	11	85	94	90	95
XM-300	2	5	18	87	40	95
PLGC	50	95	98	98	98	98
PETP(12μm)	2	3	5	6	2	5
PLGC(23μm)	2	4	5	5	2	5
PC	2	2	7	2	2	5
Celgard 2400		27	49			

wall. Since the molecular weight cut-off of an ultrafiltration membrane is affected by the flux (filtration flow per unit area), the real membrane cut-off in a flow FFF channel is related to the flowrate of crossflow and should be determined under the specific flow conditions employed. The results shown in Table 3 also indicate that YM membrane with nominal molecular weight cut-off 1000, 3000, 5000 and 10,000 shows good recoveries for all of the lipoprotein fractions.

The YM membrane with cut-off of 30k, 100k Dalton as well as XM membrane with cut-off 300k Dalton were determined as appropriate for large LDL and VLDL fractions.

For low MW species, the porosity or molecular weight cut-off of the membrane could lead to sample loss and low recoveries. In the case presented here and for other similar situations, the membrane cut-off feature can be applied to combine flow FFF and membrane separation to form the membrane-selective flow FFF.[39] The membrane-selective flow FFF can be used as a one-step purification and separation procedure to improve and simplify the lipoprotein analysis. The high concentration of sodium bromide in the ultracentrifuged lipoprotein fractions and the abundance of low molecular weight plasma proteins and albumin in a plasma sample can be removed easily by the flow FFF membrane with an appropriate MW cut-off. Without the bulk of these interfering small molecular weight components and proteins, the lipoprotein profile can be obtained directly from plasma samples. While the presence of these components does not affect flow FFF results, it does interfere with chromatographic and electrophoretic analyses.[40]

Reproducibility

The reproducibility of flow FFF determined diffusion coefficients and hydrodynamic size for lipoproteins was determined by (1) analyzing the same sample using different channel and crossflow conditions and calculating and comparing the flow FFF determined diffusion coefficients and hydrodynamic sizes, and (2) comparing the results of three different channels of the same dimensions.

Reproducibility over a range of flow conditions

Equations (1) and (3) show that the flow FFF retention times depend on the crossflow rate $\dot{V}_c$ and channel flow rate $\dot{V}$. But the magnitude of crossflow and channel flow also strongly affect resolution.[26] Figure 6 shows flow FFF fractograms of lipoprotein fractions (HDL-II, LDL-II, and VLDL-II) that resulted from using different $\dot{V}_c$ with a constant $\dot{V}$. Obviously, retention time and separation efficiency are increased by changing the driving force from 5.0 to 8.5 mL/min as shown by comparing Figures 6a and 6b. However, hydrodynamic sizes deduced from retention times show good agreement between the two different separation conditions. Average particle diameters were 8.0, 22, and 40 for the HDL, LDL, and VLDL components using the

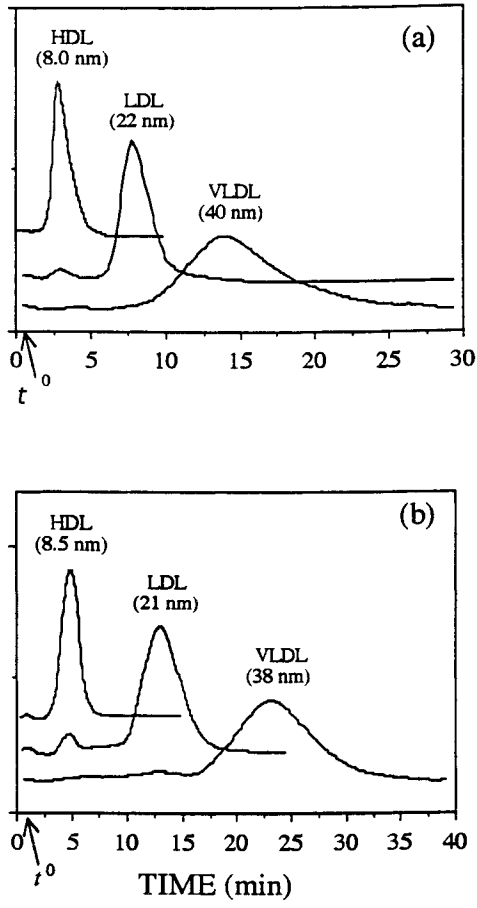

Figure 6. HDL-II, LDL-II, and VLDL-II fractions separation and size distribution using different crossflow field (a) $\dot{V}_c$ = 5.0 mL/min, (b) $\dot{V}_c$ = 8.5 mL/min. PBS pH 7.4, $\dot{V}$ =2.2 mL/min, $\dot{V}_f$ / $\dot{V}_s$ = 10, UV 280 nm, w = 158.

experimental conditions of Figure 6a versus 8.5, 21, and 38 nm for Figure 6b. The disadvantage of increased crossflow rate is also shown since the elution time of the last component, VLDL-II, increased from about 13 up to 23 minutes. The appropriate crossflow rate or channel flowrate would be chosen as the flowrate that adequately resolves the lipoprotein components within a reasonable elution time.

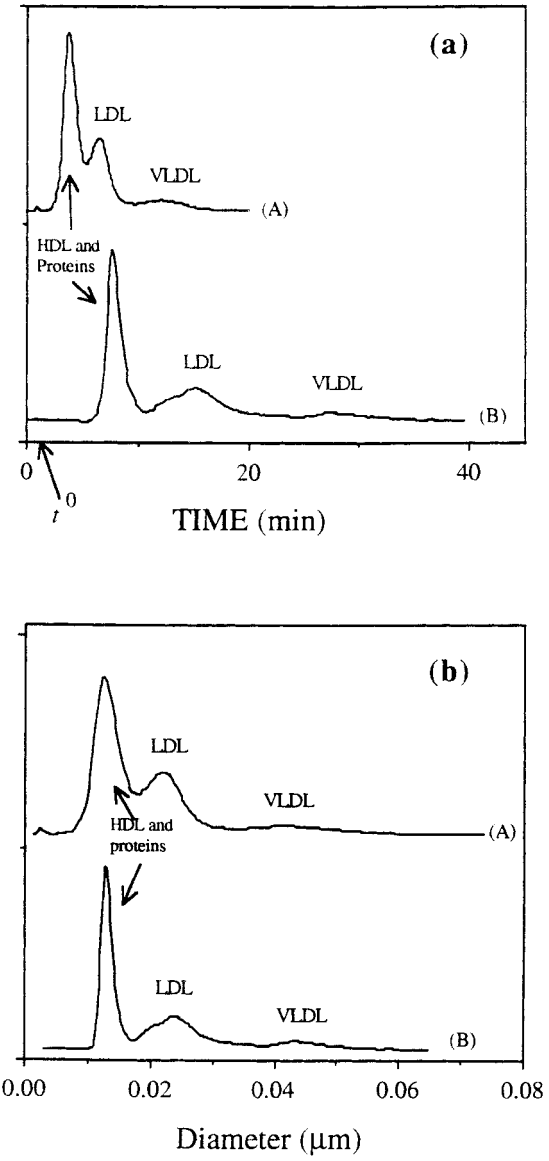

Figure 7. Flow FFF separation (a) and characterization (b) of human lipoproteins in plasma samples using different channel and crossflow conditions. Flow conditions are (A) $\dot{V}$ = 2.2 mL/min, $\dot{V}_c$ =5.0 mL/min, (B) $\dot{V}$ = 1.1 mL/min, $\dot{V}_c$ = 6.8 mL/min, $\dot{V}_f$ / $\dot{V}_s$ = 10, UV 280 nm, plasma sample No. 1.

The separation efficiency and reproducibility of lipoprotein analysis were checked using different flow conditions to analyze and characterize the lipoprotein fractions directly from blood plasma. Adjustments of the channel and crossflow rate values for different amounts of retention and resolution were made by increasing the ratio of $\dot{V}_c / \dot{V}$ from 2.3 to 6.2. A plasma sample of 10 μL was directly injected in the flow FFF channel without pretreatment. The lipoprotein profiles and size distributions are shown in Figure 7a and b, respectively. While the elution time and resolution are expected to differ due to the varying experimental conditions, the calculated sizes at peak maxima are found to be identical. The resolution was obviously improved when the ratio $\dot{V}_c / \dot{V}$ was increased to 6.2. The HDL and LDL fractions were baseline separated, and the LDL fraction was separated into a bimodal distribution. This result confirmed the good reproducibility of size determinations using a single channel at these different flow conditions.

Channel-to-channel reproducibility

The channel-to-channel reproducibility was measured using three channels to separate and characterize proteins and purified lipoprotein samples. The experimental results are compared in Table 4. Coefficients of variations between the channels were less than 4.5%. If the results were compared with the literature values in Table 1, good agreement was found.

It can be concluded that flow FFF has suitable reproducibility when using different operating conditions or different channels and can easily be used for determining diffusion coefficients or sizes of lipoprotein components.

Programming Field to Separate Lipoproteins and Their Subspecies

Because lipoproteins vary widely in size and diffusion coefficient, different conditions are needed to achieve complete separation between the various species and subspecies. HDL particles have the smallest molecular weight and largest diffusion coefficients of the lipoprotein fractions and so require a larger field than the LDL or VLDL particles. When a high isocratic field is used to characterize the lipoprotein profile, the HDL subspecies can be distinctly separated with high resolution. However, the high field conditions can be problematic for the LDL and especially the VLDL components. For example, using a cross flowrate of 9.0 mL/min, two main subclasses of HDL (HDL_3 and HDL_2) can be separated and a broad peak (indicating increased resolution of the size-based subspecies) results for the LDL components. However, the VLDL components will be difficult to detect and their retention

Table 4

Diffusion Coefficients and Hydrodynamic Sizes of Proteins and Lipoproteins Determination by Flow FFF[1]

	Diffusion Coeff. ($D_{PBS,23}x10^{-7}$)			----- Size -----			
Proteins	**Channel I**	**Channel II**	**Channel III**	**Channel I**	**Channel II**	**Channel III**	**CV%**
BSA	6.65±0.06	6.67±0.04	6.65±0.07	6.5±0.06	6.4±0.04	6.5±0.07	0.2
γ-globulin	4.35±0.04	4.38±0.05	4.32±0.04	10.0±0.09	9.9±0.11	10.1±0.09	0.7
Thyro-globulin	2.60±0.06	2.71±0.05	2.72±0.03	16.7±0.38	16.0±0.30	15.9±0.18	2.5
HDL	4.65±0.08	4.84±0.07	4.85±0.04	9.3±0.16	9.0±0.13	8.9±0.07	3.8
LDL	1.95±0.04	2.10±0.03	1.94±0.04	22.2±0.53	20.6±0.29	22.4±0.46	4.5
VLDL	1.16±0.03	1.23±0.04	1.24±0.03	37.3±0.96	35.4±1.10	35.0±0.85	3.6

[1] With standard deviation and n = 5.

time will become unreasonably long. Additionally, under these experimental conditions, the VLDL components are forced to positions very close to the membrane and there is increased risk of adsorption onto the membrane. Therefore, the isocratic 9.0 mL/min crossflow field is useful for the subspecies separation of the HDL component only. A lower isocratic field is similarly useful for separating the subspecies of LDL and VLDL; however, the resolution of the smaller HDL components will suffer. In an attempt to achieve higher resolution without excessive analysis time or loss of resolution for the smaller components, programmed field conditions were used.

A linear field decay programming was used, that is, a constant but high initial field strength was applied for a predetermined period of time for the separation of HDL particles, after which the field strength was programmed downward to provide suitable conditions for the prompt elution of LDL and VLDL particles.

First of all, the crossflow pump was manipulated so that the initial flowrate was 9.0 mL/min. This crossflow field was then decreased by gradually slowing the cross flowrate to 1.0 mL/min with a constant channel flowrate of 2.0 mL/min. This procedure allowed for better resolution of the HDL components and was completed in 20 minutes using an initial t_1 = 1.7 minutes and then a decay time, t_p = 20.7 minutes (see Figure 4). In this case, the HDL_2 and HDL_3 components were separated, but better resolution of the LDL and VLDL was not achieved (Figure 8). This result illustrates that this programmed decay time is appropriate to the HDL subspecies, but a longer field

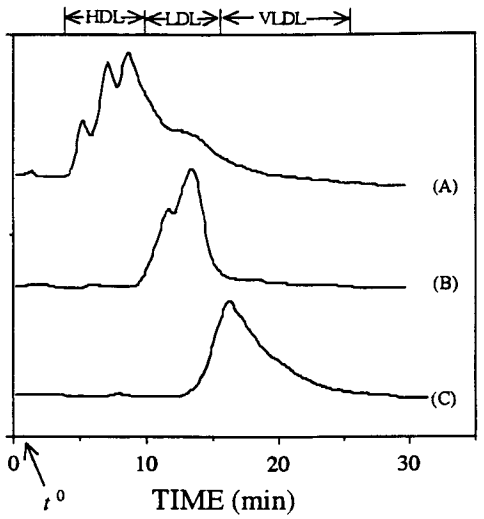

Figure 8. Flow FFF of human plasma lipoprotein fractions using a programmed field. (A) HDL-I, (B) LDL-I, and (C) VLDL-I fractions. Flow conditions are $\dot{V}$ = 2.0 mL/min, programmed $\dot{V}_c$ from 9.0 mL/min to 1.0 mL/min, t_1 = 1.7, t_p = 20.7 minute, $\dot{V}_f$ / $\dot{V}_s$ = 9, UV 280 nm, w = 174 μm.

decay time should be used to get better information regarding the LDL and VLDL subspecies. When the field decay time t_p is increased to 40 minutes, the LDL and VLDL fractions show longer retention times and broader size distributions. Figure 9 shows the separation of a mixture of the HDL-I, LDL-I, and VLDL-I standards and two blood plasma samples at these conditions. When the plasma fractograms (Figure 9 B and C) are compared with the corresponding lipoprotein fraction standards profile (Figure 9A), information regarding concentration and size distribution of lipoproteins is immediately available. Plasma 2 (Figure 9B) shows a large VLDL peak, which supplies information about the lipid abnormality of the individual.

It should be mentioned that overlap between LDL-I and VLDL-I fractions exists as shown in Figure 5 which is the result of isocratic field conditions; Figure 9a, which employed programmed field conditions, also showed this overlap. This is possibly due to overlapping size distribution which exists between the two density classed fractions since, even under varied field conditions, the aberration was noted. Since separation in normal mode flow FFF occurs according to the sample diffusion coefficient or hydrodynamic size, the overlap means that the same size particles exist in the different lipoprotein

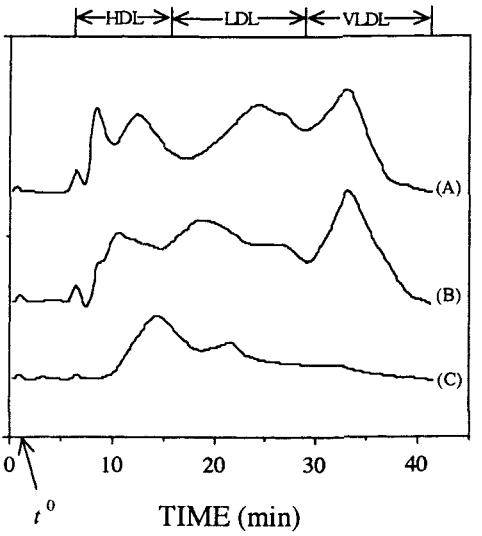

Figure 9. Lipoprotein fractions and human plasma lipoproteins separation by programmed flow FFF. (A) a mixture of HDL-I, LDL-I, and VLDL-I fractions, (B) human plasma No.2, (C) human plasma No.3. Conditions are same as Figure 8 except $t_1 = 8.3$, $t_p = 40$ minutes.

fractions. The reason for the overlap is not clear and a possible alternative reason is incomplete separation by the ultracentrifugation procedure. However, flow FFF is possibly the only convenient method capable to investigate this relationship between the density and hydrodynamic size. It should also be mentioned that the broad peaks and size distributions contain information regarding the lipoprotein subspecies, each of which has a distinct function for the development of coronary heart disease. Information about the subspecies in human plasma is important for both clinical and research work to assess the risk of atherosclerosis.

CONCLUSIONS

A frit-inlet hydrodynamic relaxation flow FFF system was successfully utilized for the separation and characterization of biological materials from small proteins to macromolecular lipoproteins. With improvements in instrumentation and techniques, flow FFF separations have become faster and more convenient. The separation of HDL, LDL, and VLDL fractions can be done in 10 to 20 minutes with only 10 μL of sample necessary. Plasma

lipoproteins can be separated directly from human plasma without sample pretreatment. The well developed FFF theory can be used to predict system operating conditions, and lipoprotein size distributions can be deduced from their retention times. The subspecies of the lipoprotein profile were determined when a programmed field was used. Distinctly different lipoprotein profiles were obtained for plasma samples from different individuals, providing a characteristic fingerprint for these important constituents and suggesting possible clinical and research applications.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant GM10851-39 and by SBIR Grant R43HL50213 from the National Institutes of Health.

We would like to thank Dr. L. Wu of Cardiovascular Genetics Research Clinic, University of Utah, for the gift of various lipoprotein samples and the technical discussions on the lipoprotein purification and cholesterol analysis.

REFERENCES

† Deceased: October 24, 1996.

1. N. Rifai, Arch. Pathol. Lab. Med., **110**, 694-701 (1986).

2. F. T. Lindgren, L. C. Jensen, F. T. Hatch, in **Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism**, G. L. Nelson, ed., R. E. Publishing, Huntingdon, New York, 1979, pp. 181-274.

3. W. A. Bradley, S.-L. C. Hwang, J. B. Karlin, A. H. Y. Lin, S. C. Prasad, A. M. Gotto, Jr., S. H. Gianturco, J. Biol. Chem., **259**, 14728-14737 (1984).

4. T. Gordon, W. B. Kannel, W. P. Castelli, T. R. Dawber, Arch. Intern. Med., **141**,1128-1131 (1981).

5. G. R. Warnick, J. J. Albers, Clin. Chem., **24**, 900-904 (1978).

6. J. D. Otvos, E. J. Jeyarajah, D.W. Bennett, Clin. Chem., **37**, 377-386 (1991).

7. L. A. Cassis, R. A. Lodder, Anal. Chem., **65**,1247-1256 (1993).

8. G. R. Warnick, M. H. Dominiczak, Current Opinion in Lipidology, **1**,493-499 (1990).

9. G. Warwick, C. J. Packard, J. Shepherd, Current Opinion in Lipidology, **1**, 500-507 (1990).

10. N. Rifai, G. R. Warnick, J. R. McNamara, J. D. Belcher, G. F. Grinstead, I. D. Frantz, Jr. Clin. Chem., **38**, 150-160 (1992).

11. W. T. Friedewald, R. I. Levy, D. S. Fredrickson, Clin. Chem., **18**, 499-502 (1972).

12. B. H. Chung, J. P. Segrest, M. J. Ray, J. D. Brunzell, J. E. Hokanson, R. M. Krauss, Ken Beaudrie, J. T. Cone. Methods Enzymol., **128**, 181-209 (1986).

13. C. J. Fielding, P. E. Fielding, in **Biochemistry of Lipids and Membranes**, D. E. Vance, J. E. Vance, eds., The Benjamin/Cummings Publ. Co., Menlo Park, California, 1985, pp. 424-425.

14. L. L. Wu, G. R. Warnick, J. T. Wu, R. R. Williams, J. M. Lalouel, Clin. Chem., **35**, 1486-1491 (1989).

15. Laboratory Standardization Panel, "NCEP: A Report from the Laboratory Standardization Panel of the National Cholesterol Education Program," Clin. Chem., **34**, 193-201 (1988).

16. J. R. McNamara, J. S. Cohn, P. W. F. Wilson, E. J. Schaefer, Clin. Chem., **36**, 36-42 (1990).

17. S. M. Grundy, D. W. Goodman, B. M. Rifkind, J. I. Cleeman, Arch. Int. Med., **149**, 505-510 (1989).

18. G. R. Warnick, J. J. Albers, E. Teng-Leary, Clin. Chem., **26**, 169-170 (1980).

19. J. C. Giddings, ed., **Unified Separation Science**, John Wiley, New York, 1991.

20. M.-K. Liu, P. Li, J. C. Giddings, Protein Science, **2**, 1520-1531 (1993).

21. J. C. Giddings, Science, **260**, 1456-1465 (1993).

22. K.-G. Wahlund, A. Litzen, J. Chromatogr., **461**, 73-87 (1989).

23. A. Litzen, K.-G. Wahlund, J. Chromatogr. **476**, 413-421 (1989).

24. A. Litzen, K.-G. Wahlund, Anal. Biochem., **212**, 469-480 (1993).

25. J. C. Giddings, M. N. Benincasa, M-K. Liu, P. Li, J. Liq. Chromatogr., **15**, 1729-1747 (1992).

26. M.-K. Liu, J. C. Giddings, Macromolecules, **26**, 3576-3588 (1993).

27. J. C. Giddings, F. J. Yang, M. N. Myers, J. Virol., **21**, 131-138 (1977).

28. A. Litzen, K.-G. Wahlund, Anal. Chem., **63**, 1001-1007 (1991).

29. J. J. Kirkland, C. H. Dilks, Jr., S. W. Rementer, W. W. Yau, J. Chromatogr., **593**, 339-355 (1992).

30. B. N. Barman, E. R. Ashwood, J. C. Giddings, Anal. Biochem., **12**, 35-42 (1993).

31. W. Stoffel, C. Bode, in **Selective Plasma Component Removal**, A. A. Pineda, ed., Futural Publishing, New York, 1984, p. 2.

32. J. C. Giddings, Anal. Chem., **62**, 2306-2312 (1990).

33. S. K. Ratanathanawongs, J. C. Giddings, Anal. Chem., **64**, 6-15 (1992).

34. J. C. Giddings, F. J. Yang, M. N. Myers, Anal. Chem., **48**, 1126-1132 (1976).

35. C. Tanford, ed., **Physical Chemistry of Macromolecules**, John Wiley, New York, 1961.

36. P. S. Williams, J. C. Giddings, R. Beckett, J. Liq. Chromatogr., **10**, 1961-1998 (1987).

37. **Lipid and Lipoprotein Analysis, Manual for Laboratory Operations**, Lipid Research Clinics Program, Vol. 1, DHEW Publication No.(NIH)75-628, Washington, DC. U.S. Dept. of HEW, 1974.

38. C. A. Allain, L. S. Poon, C. S. G. Chan, W. Richmond, P. C. Fu, Clin. Chem., **20**, 470-475 (1974).

39. P. Li, J. C. Giddings, J. Pharm. Sci., **85**, 895-898 (1996).

40. Y. C. Ha, P. J. Barter, J. Chromatogr., **341**, 154-159 (1985).

Received January 8, 1997
Accepted April 24, 1997
Manuscript 4437