ELSEVIER

Journal of Chromatography B, 671 (1995) 237–253

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Chromatographic techniques for the isolation and purification of lipoproteins

Tanya Tadey, William C. Purdy*

Department of Chemistry, McGill University, 801 Sherbrooke St. W., Montreal, PQ, H3A 2K6, Canada

Abstract

Various modes of chromatography are available for lipoprotein separation. Gel permeation and affinity chromatography are used for preparative purposes and to separate lipoproteins according to size and apolipoprotein content, respectively. Development of rigid supports for gel permeation has led to large improvements in speed and resolution. Reversed-phase high-performance liquid chromatography (HPLC) of apolipoproteins offers the best performance in terms of speed and resolution of structural variants. Due to its high speed and superior resolving power, the recently developed technique of capillary electrophoresis should emerge as an important method for lipoprotein analysis.

Contents

* Corresponding author.

0378-4347/95/$29.00
SSDI 0378-4347(95)00051-8

Abbreviations

apoA	Apolipoprotein A
apoB	Apolipoprotein B
apoC	Apolipoprotein C
apoE	Apolipoprotein E
CDAP	1-Cyano-4-dimethylaminopyridium tetrafluoroborate
CE	Capillary electrophoresis
CET	Cholesterol ester transferase
CHD	Coronary heart disease
CNBr	Cyanogen bromide
con A	Concanavalin A
CPG	Controlled pore glass
CTAB	Cetyltrimethylammonium bromide
d	Density
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid
GPC	Gel permeation chromatography
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
ITP	Isotachophoresis
LDL	Low-density lipoprotein
PA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
tris	Tris(hydroxymethyl)aminomethane
VLDL	Very low-density lipoprotein

1. Introduction

Chromatographic techniques represent the newest addition to the list of techniques for the separation and characterization of plasma lipoproteins. In 1929 Macheboef [1] isolated the first lipoprotein fraction from horse serum by precipitation with half-saturated ammonium sulphate. The development of electrophoresis for the study of plasma proteins by Tiselius [2] was of great importance for characterization of lipoproteins. In 1941, Blix et al. [3] reported two major groups of lipoproteins with electrophoretic mobility corresponding to α- and ß-globulin. With the introduction of the analytical centrifuge, the major work of isolating and identifying the various lipoprotein fractions according to differences in their hydrated densities could be carried out [4,5].

Lipoprotein particles are heterogeneous with respect to size, hydrated density and composition. All are made up of cholesterol, cholesterol esters, triglycerides, phospholipids and proteins in varying proportions. As illustrated in Fig. 1, lipoproteins have a globular structure. Within the interior core of the lipoprotein are the hydrophobic lipids such as triglycerides and cholesterol esters. More polar lipids, phospholipids and cholesterol, as well as proteins, are found on the periphery of the lipoprotein molecule.

Table 1 lists the various physical and chemical characteristics used to classify lipoprotein particles [6]. Hydrated density remains the most common form of lipoprotein classification. Thus, lipoproteins are separated into very low-density (VLDL), low-density (LDL) and high-density (HDL) lipoprotein fractions. These correspond roughly to pre-ß, ß- and α-lipoproteins, which are separated by electrophoresis. As more information regarding the importance of apolipoproteins in determining lipoprotein structure and function was gained, a new system for classifying lipoproteins was introduced. In this system, introduced by Alaupovic [7], apolipoprotein content is the only criterion for naming of lipoproteins. Thus, lipoproteins are viewed as particles which are heterogeneous with respect to their physical properties but homogeneous with respect to apolipoprotein composition.

The main function of the lipoprotein system is

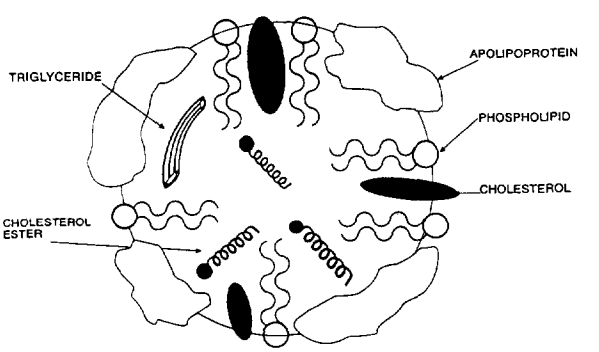

Fig. 1. Schematic of lipoprotein particle.

Table 1
Physical and chemical characteristics of apolipoproteins[a]

Lipoprotein class	Density (g/ml)	Diameter (nm)	μ_e	Major apolipoproteins
VLDL	0.95–1.006	30–70	pre-β	apoC, apoB, apoE
LDL	1.019–1.063	17–26	β	apoB
HDL	1.063–1.21	7.4–9	α	apoA-I, apoA-II, apoE

[a] Data for compilation of Table 1 were taken from Ref. [6].

to transport lipids to surrounding tissues. Triglyceride-rich VLDL are secreted by the liver and function to supply triglycerides and cholesterol to tissues. Stepwise hydrolysis of VLDL triglycerides by lipoprotein lipase results in conversion of VLDL to LDL. HDL are secreted from both the liver and intestines. The role of HDL is different than that of other lipoproteins since they are involved in reverse-cholesterol transport. That is, HDL transport cholesterol from tissues to the liver for secretion. This difference in function of lipoprotein groups results in their having different properties with respect to atherosclerosis. It is therefore very important to have methods available for the separation of the various lipoprotein classes. In this review, liquid chromatographic methods for lipoprotein analysis are discussed. Chromatography of apolipoproteins, the protein components of lipoprotein particles, is also presented since apolipoprotein distribution is important in diagnosing lipoprotein abnormalities. Finally, the potential of the relatively new technique of capillary electrophoresis (CE) is also discussed.

2. Preparation of samples for lipoprotein chromatography

The extent of sample preparation prior to chromatographic separation is dependent on the type of analysis sought. However, in all cases the success of a chromatographic separation will depend on collection and storage of blood samples. For a detailed discussion of collection of blood for lipoprotein analysis, the reader is referred to a review by Bachorik [8] and to the manual from the Lipid Research Clinics Program of the NIH [9].

Plasma or serum can be used for lipoprotein analysis. However, plasma is usually preferred since it can be kept cold in order to slow down most enzymatic processes which degrade lipoproteins. Blood for lipoprotein analysis is collected in tubes leading to 1.2 mg/ml of EDTA in the final solution. Plasma is then immediately isolated by centrifuging for 30 min at 1500 *g* in a centrifuge refrigerated at 4°C.

Some chromatographic separations use whole plasma as a starting material for lipoprotein separation. Many separations, however, subfractionate the lipoprotein fraction of plasma. For these analyses lipoprotein fractions are prepared by ultracentrifugation according to the method reported by Havel et al. [5]. If the whole plasma lipoprotein fraction is required, plasma is adjusted to $d = 1.21$ and centrifuged at 105 000 *g* for 24 h. Otherwise, individual lipoprotein fractions, VLDL, LDL and HDL are prepared by sequential ultracentrifugation.

3. Chromatography of lipoproteins

3.1. Separation of lipoproteins in whole serum

Differences in particle size can be used to separate lipoproteins. As illustrated in Table 1, lipoprotein size is inversely proportional to density so that lipoproteins in the VLDL fraction have the largest diameter, while those in the HDL fraction are smallest in diameter. Traditionally, fractionation of lipoproteins in whole plasma has been performed in agarose gel sup-

ports. It should be noted, however, that the fractions obtained also contain plasma protein contaminants. Thus, gel permeation chromatography of whole plasma is primarily used for analytical rather than preparative separation of plasma lipoproteins.

Gel permeation chromatography of plasma lipoproteins was initially performed on cross-linked dextran supports [10,11]. Unfortunately, the relatively small pore size of these gels yielded poor separations. Agarose gels, which have a larger pore size, have been found to yield better lipoprotein separations [12,13].

The three major lipoprotein families, VLDL, LDL and HDL, can be separated from whole plasma on 4% or 6% agarose gels using 0.9% NaCl containing 0.01% EDTA, pH 7.4 as the eluent [14]. The main advantage of using gel permeation is that compared to ultracentrifugation, it is relatively gentle and non-destructive to lipoprotein particles. There is evidence that the combination of high salt concentration and repeated high-speed ultracentrifugation causes modifications in lipoprotein structure and stripping of apolipoproteins [15–17]. Another advantage of gel permeation chromatography is that it can be performed in most labs since it does not require the purchase of expensive ultracentrifugation instrumentation.

Conventional gel permeation chromatography has not, however, gained wide acceptance as a routine technique for lipoprotein separation. The main reasons for this are very long analysis times, in excess of 20 h, and excessive dilution of samples during separation.

The advent of high-performance liquid chromatography (HPLC) resulted in renewed interest in chromatographic separations of lipoproteins. The use of rigid supports which were able to withstand high pressures decreased separation time dramatically. Furthermore, the separation was easily automated to allow for unattended analysis of multiple samples. Automation also allowed for on-line monitoring of cholesterol in the separated LDL and HDL fractions.

Rigid, fast-flow agarose gels, such as Superose 6, available from Pharmacia, can be used to separate plasma lipoproteins by HPLC. Van Gent and Van Tol [18] used an HPLC system, illustrated in Fig. 2, for multiple automated separations of lipoproteins from whole plasma. The prep-grade Superose 6 column is loaded with 2 ml plasma and eluted with 0.9% sodium chloride, 2 m*M* sodium phosphate (pH 7.4). The

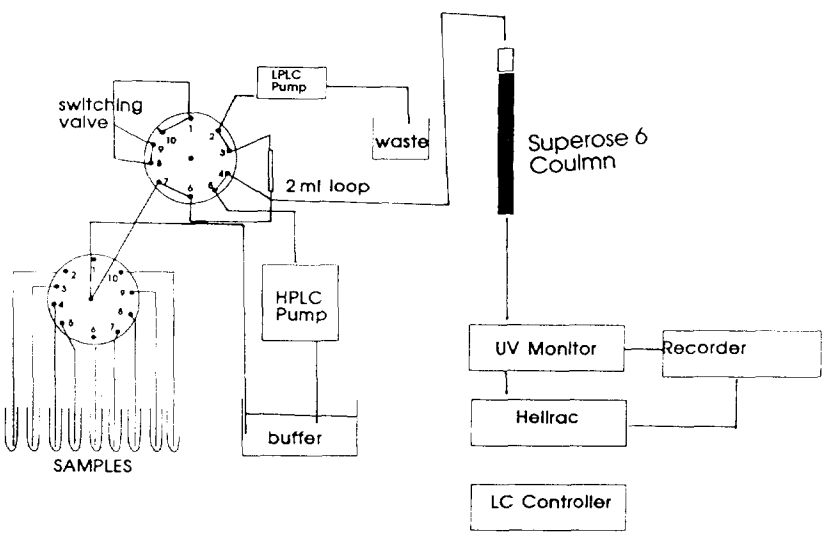

Fig. 2. Schematic representation of the HPLC system used for multiple automated separations of lipoproteins from serum or plasma.

eluent also contains 0.02% sodium azide and 1 or 5 m*M* EDTA for serum or plasma separation, respectively. VLDL, LDL and HDL can be separated in a single run. The system can be used to analyze up to six different plasma samples (2 ml per sample) overnight. A single separation can be completed in about 160 min, with an average overall recovery for lipoprotein lipids of 101%.

März et al. [19] reported the fast-flow separation of plasma in which lipoproteins are detected on-line at 500 nm after post-column derivatization with an enzymatic cholesterol reagent (CHOD-PAP, Boehringer Mannheim). The eluting lipoproteins and the cholesterol reagent were mixed in a chamber attached to the column outlet. The mixture was then passed through a reaction capillary and detected. Complete separation of lipoprotein fractions was usually achieved in less than 80 min. The chromatographic profiles of normal and several types of hyperlipoproteinemic serum samples is illustrated in Fig. 3. The three peaks correspond to, in order, VLDL, LDL and finally, HDL. Immunochemical characterization of the three fractions showed complete resolution of apoB-containing lipoproteins (VLDL and LDL) from apoA-I lipoproteins (HDL). As expected, all of the hyperlipoproteinemic samples exhibited decreased levels of anti-atherogenic HDL and increased levels of VLDL and LDL.

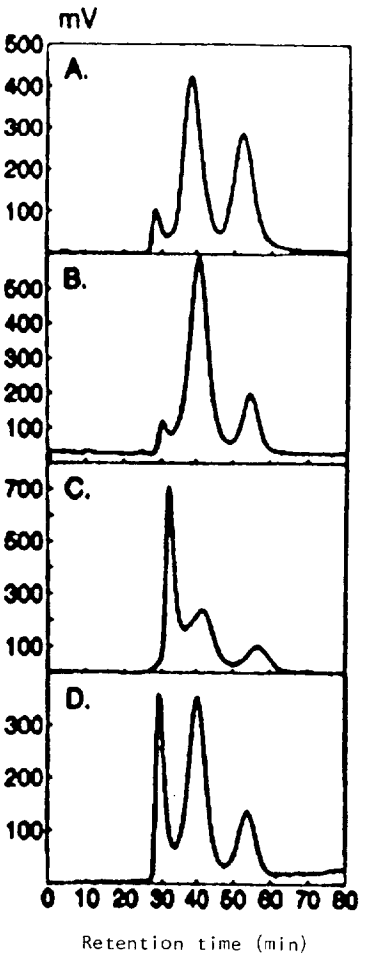

Fig. 3. Chromatographic profile of normal and hyperlipoproteinemic (HLP) sera. Conditions: 300-mm Superose 6 column eluted with 100 m*M* Na_2HPO_4, 200 m*M* NaCl, pH 7.4, 300 μl/min. (A) Normolipidimic sample. (B) Type IIa HLP. (C) Type III HLP. (D) Type IV HLP. Peaks from left to right: VLDL; LDL; HDL. (Reprinted from Ref. [19].)

Lipoproteins can also be separated by HPLC on TSK-type gel permeation columns. Hara and co-workers [20,21] described the separation and on-line determination of lipids using a combination of two TSK columns and on-line enzymatic reaction (Fig. 4). A variety of column systems was studied and it was found that a combination of G4000 SW and G3000 SW eluted with 0.15 *M* NaCl, pH 7, was best at separating serum lipoproteins into VLDL, LDL, HDL_2 and HDL_3. The system used two detectors. The first was placed immediately after the column and monitored protein absorbance at 280 nm. The second detector was placed after the enzymatic reactor and detected lipid absorbance at 500–600 nm. It should be noted that serum instead of plasma is preferred for TSK-gel HPLC of lipoproteins. With plasma there is a danger of fibrin forming during the analysis.

Although the TSK-based methods are generally faster than those that use Superose 6 columns, there are some disadvantages which limit their usefulness. TSK columns are generally more prone to clogging than Superose 6. Adsorption of lipoproteins to the support has also been noted by some workers [22]. A further disadvantage of the TSK supports is their limitation to relatively small sample volumes (<300 μl).

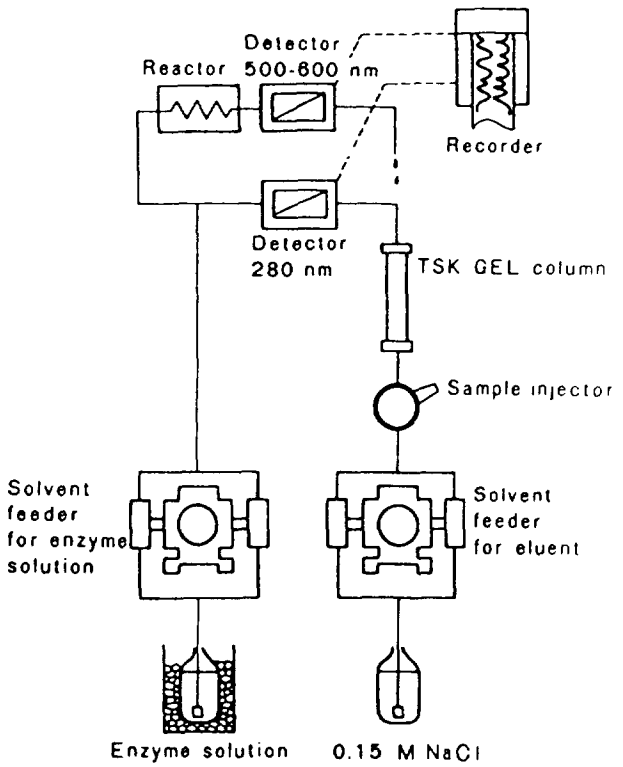

Fig. 4. Flow diagram of lipoprotein analysis developed by Hara and Okazaki. (Reprinted from Ref. [20].)

Therefore, prep-scale Superose 6 columns, which can accept large sample volumes (up to 5 ml) are preferred for preparation of larger quantities of lipoproteins.

3.1.1. *Affinity chromatography*

As mentioned in the introduction, lipoproteins may also be classified according to their apolipoprotein content. The system recognizes that lipoprotein families defined by hydrated density or electrophoretic mobility may contain different apolipoprotein compositions. It also recognizes the growing evidence that apolipoproteins are better markers of coronary heart disease (CHD) than serum cholesterol levels [23,24].

Much of the work of characterizing the discrete lipoprotein families according to apolipoprotein content has been performed by Alaupovic and co-workers [25–30]. Separation of lipoproteins according to apolipoprotein content requires selectivity not easily attainable by gel permeation chromatography. Affinity chromatography, which employs specific and reversible interactions between lipoproteins and bound ligands is capable of separating lipoproteins on the basis of their apolipoprotein content.

Affinity chromatography of lipoproteins can be performed using group-specific or biospecific ligands. Group-specific ligands include concanavalin A, which binds to apoB-containing lipoproteins [31,32], and heparin, which interacts specifically to apoB and apoE [33]. In immunoaffinity chromatography, antibodies to specific apolipoproteins serve as biospecific ligands.

The ability of heparin-Sepharose gels to selectively bind ß-lipoproteins (apoB) was demonstrated in 1972 by Iverius [34]. The exact mechanism of interaction between heparin and lipoproteins is not completely understood. Interaction between positive groups of apolipoproteins and anionic groups of heparin has been suggested as the mechanism that leads to selective binding [34,35]. Also, lipoprotein binding capacity of the heparin-Sepharose gel is generally increased by the presence of divalent anions such as Mn^{2+}.

Heparin-Sepharose gels used for lipoprotein separation lack the mechanical strength necessary for use in HPLC systems. In an effort to produce a more robust medium, Carpenter and Purdy [36] immobilized heparin onto glyceryl controlled-pore glass (CPG). Furthermore, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was used for activation of the glyceryl-CPG. Activation with CDAP was expected to lead to increased yields of immobilized heparin since it can be performed under less basic conditions than those used for cyanogen bromide activation. Serum samples applied to heparin-CPG were separated into two fractions. The unretained fraction was washed through the column with 0.1 *M* NaCl while the retained fraction was eluted with 1 *M* NaCl. Radial immunodiffusion studies confirmed complete resolution of apoA- and apoB-containing lipoproteins. However, attempts at incorporating the heparin-CPG column into a high-performance affinity chromatography system were unsuccessful [37].

Dextran sulphate, which is a synthetic ana-

logue of heparin, also binds preferentially to apoB-containing lipoproteins. Its usefulness in affinity chromatography is limited since it lacks residual amino groups necessary for direct coupling to a polysaccharide matrix via activation with cyanogen bromide. However, sulphated dextran beads (available from Sigma) which consist of sulphated cross-linked dextran [38], can be used as stable medium for affinity chromatography. They are unique since the ligand itself is cross-linked to form an insoluble gel.

A rapid separation of apoA- and apoB-containing lipoproteins on sulphated dextran beads has been achieved [39]. The concentration of sodium chloride solutions used to elute the fractions was found to strongly influence lipoprotein separation. At low ionic strengths, the gel swells, leading to exclusion of lipoproteins. At higher ionic strengths, ions may compete for binding sites. A strength of 80 m*M* NaCl was found to be optimal, and resulted in complete separation of lipoprotein fractions in less than 15 min.

Immunoaffinity chromatography provides the highest specificity for separation and isolation of lipoproteins on the basis of their apolipoprotein content. The technique can be used to separate relatively large volumes (1 to 10 ml) of whole plasma or lipoproteins. The experimental considerations in applying immunoaffinity chromatography for lipoprotein separation were recently reviewed by Alaupovic and Koren [40].

Antibodies to apolipoproteins coupled to cross-linked dextran (Sephadex) or agarose derivatives (Sepharose) serve as selective ligands for separation of lipoproteins by immunoaffinity chromatography. Generally, purified delipidized apolipoproteins are used as antigens for the production of antibodies. Preparation of both polyclonal [41–44] and monoclonal [45–47] antibodies to apolipoproteins have been described.

Flow diagrams for the separation and isolation of apoA- and apoB-containing lipoproteins in whole plasma by sequential immunoaffinity chromatography, developed by Alaupovic and Koren [40], are illustrated in Fig. 5. In this procedure, whole plasma is first fractionated into apoB and apoA lipoproteins by affinity chromatography on concanavalin A (con A). This first step can also be performed on an anti-apoB immunoaffinity column. However, con A affinity chromatography is preferred due to its simplicity and

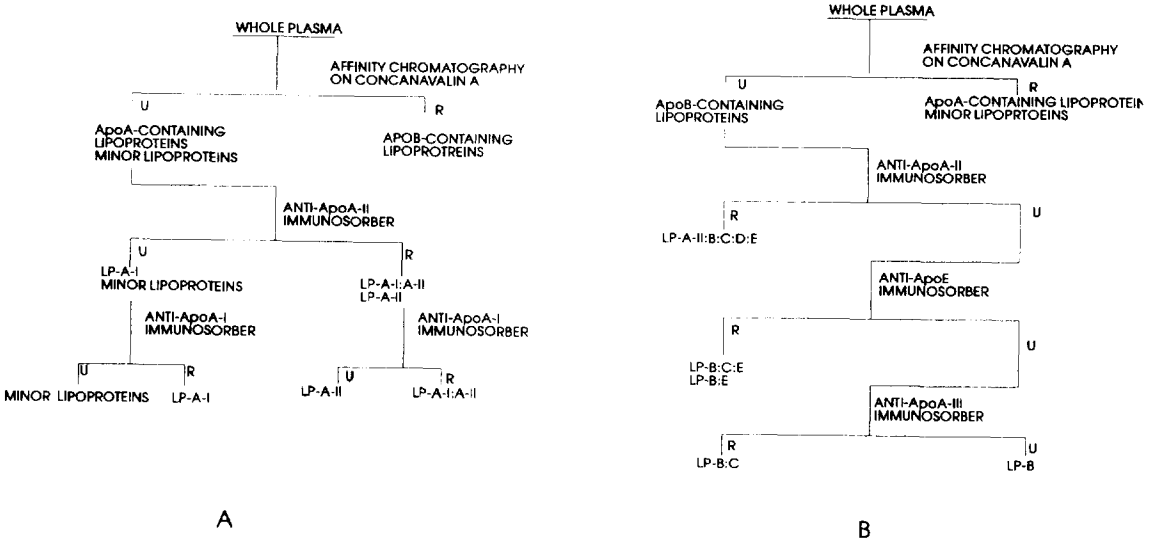

Fig. 5. Fractionation of plasma lipoproteins by sequential immunoaffinity chromatography as developed by Alaupovic and Koren [40]. (A) Fractionation of apoA-containing lipoproteins. (B) Fractionation of apoB-containing lipoproteins. R = retained fraction, U = unretained fraction.

greater capacity of con A for apoB lipoproteins than anti-apoB immunosorbers [48]. Further fractionation proceeds by immunoaffinity chromatography. In order to minimize contact between lipoproteins and elution agents the immunoaffinity column is usually placed between two Sephadex-25 desalting columns [49,50]. In this way lipoproteins are quickly dissociated from potentially destructive ions in the elution buffer.

ApoA-containing lipoproteins can be subfractionated by immunoaffinity chromatography on anti-apoA-II immunosorbent. This retained fraction contains lipoprotein (A-I + A-II) and lipoprotein A-II. Further, separation of this fraction on an anti-apoA-I immunosorber leads to the isolation of three types of apoA lipoproteins: lipoprotein A-I, lipoprotein A-II and lipoprotein (A-I + A-II). Either thiocyanate or acetic acid solutions can be used to dissociate lipoproteins from the immunosorbent. However, cholesterol ester transferase (CET) activity is completely diminished when acetic acid is the eluent [51].

The scheme for separation and isolation of lipoproteins containing apoB as the sole apolipoprotein is illustrated in Fig. 5B. McConathy et al. [30] studied the effects of activation procedure and dissociating agent on apoB lipoprotein immunoaffinity chromatography. They found that immunosorbers of the highest capacity were obtained by cyanogen bromide activation of Sepharose. Comparison of several high- and low-pH dissociating agents found that 3 *M* sodium thiocyanate was the most effective desorbent for Separose-based immunosorbers.

3.2. Subfractionation of lipoprotein particles

Lipoprotein fractions prepared by ultracentrifugation are often used for chromatographic separation. The whole lipoprotein fraction ($d <$ 1.21) can be further fractionated into VLDL, LDL and HDL by gel permeation chromatography [53–55]. In this way, the procedure is both analytical and preparative since the isolated fractions contain no contamination from serum proteins. Procedures for gel permeation chromatography of the lipoprotein fraction of plasma are generally the same as those for whole plasma. If lipid concentration in individual lipoprotein classes is the only information required, whole plasma should be used in order to save time.

Subfractionation of HDL particles has been accomplished by gel permeation chromatography [56–58]. Clifton et al. [56] isolated five distinct HDL subfractions: HDL_{2a} (mean particle radius 4.58 nm), HDL_{2b} (5.28 nm), HDL_{3a} (4.22 nm), HDL_{3b} (3.98 nm), HDL_{3c} (3.81 nm), using a Superose 6B column. Analysis of the individual fractions revealed increasing protein content with particle size and a maximum in the apoA-I to apoA-II ratio in HDL_{2b}.

Ion-exchange chromatography has been used to study charge heterogeneity in LDL [59–61]. These types of separations are important since oxidatively modified LDL has been associated with atherosclerosis [62–64]. Chromatography of LDL on DEAE-Sepharose 6B separated two fractions [59]. The minor fraction (less than 1% LDL) possessed increased negative charge, higher flotation density and higher susceptibility to oxidation than native LDL.

LDL particles modified by Cu^{2+} oxidation were analyzed by anion-exchange chromatography on Mono Q HR 515 columns (available from Pharmacia) [60]. Five different fractions of oxidatively modified LDL were identified. The isolated fractions exhibited differences in density and lipid content. The authors also found that the protein component, apoB, was mainly responsible for the chromatographic behaviour of LDL since delipidated LDL exhibited a similar chromatographic pattern.

Heparin affinity chromatography can be used to subfractionate HDL particles according to apoE content [65–69]. In 1980, Weisgraber and Mahley [66] separated human HDL into apoE-rich and apoE-poor fractions on heparin-Sepharose columns. Gel electrophoretic analysis of the bound apoE-containing fraction suggested that it was composed of several distinct classes. Wilson and co-workers [67,68] further separated apoE-rich HDL into five subfractions using elution

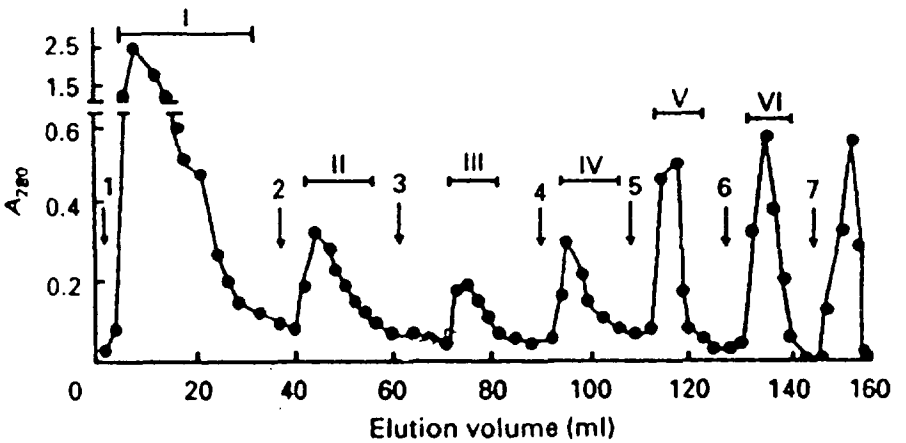

Fig. 6. Separation of HDL subfractions by heparin-Sepharose affinity chromatography. Conditions: 3 mg of HDL were applied to the column and eluted with the following buffers, all of which contained 5 m*M* tris–HCl, pH 7.4; 1 = 25 m*M* NaCl–80 m*M* $MnCl_2$; 2 = 50 m*M* NaCl–25 m*M* $MnCl_2$; 3 = 75 m*M* NaCl–25 m*M* $MnCl_2$; 4 = 50 m*M* NaCl–12.5 m*M* $MnCl_2$; 5 = 50 m*M* NaCl–6.2 m*M* $MnCl_2$; 6 = 0.1 *M* NaCl; 7 = 0.6 *M* NaCl. (Reprinted from Ref. [68].)

buffers of varying Mn^{2+} concentration. The resulting chromatogram and the buffers used for elution of apoE-containing HDL are illustrated in Fig. 6.

3.3. Liquid chromatography of apolipoproteins

The physical and chemical characteristics of the major apolipoproteins are listed in Table 2

Table 2
Physico-chemical characteristics of the major apolipoproteins[a]

Apolipoprotein	M_r	Polymorphs	Isoelectric point	Carbohydrates present
A-I	28 145	$A\text{-}I_1$	5.62	
		$A\text{-}I_2$	5.53	
		$A\text{-}I_3$	5.45	
		$A\text{-}I_4$	5.36	
A-II	17 400	–	5.10	
A-IV	46 000	–	5.05	
B	?	?	?	Man, Gal, $GluNH_2$ Fuc, sialic acid
C-I	6631	–	6.5	
C-II	8837	–	5.0	
C-III	8764	$C\text{-}III_0$	4.93	Gal, $GalNH_2$
		$C\text{-}III_1$	4.72	sialic acid
		$C\text{-}III_2$	4.54	
D	22 100	–	?	Gal, Glu, $GluNH_2$ Man, sialic acid
E	35 000–39 000	E-1	5.7	Gal, Glu, Man, $GluNH_2$, $GalNH_2$, sialic acid
		E-2	5.8	
		E-3	5.9	
		E-4	6.0	
		E-5	6.2	

[a] Data from Ref. [70].

[70]. Apolipoproteins, especially apoA and apoB, serve as markers of coronary heart disease (CHD) risk. Also, most dyslipoproteinemias can be attributed to apolipoprotein abnormalities. Consequently, there is growing interest in techniques for separation and characterization of apolipoproteins. Chromatography in the gel permeation, ion-exchange or reversed-phase mode can be used to separate apolipoproteins. Gel permeation chromatography is often used as a preparative technique for isolating apolipoproteins derived from HDL or VLDL. Separation of apolipoprotein isoforms can be achieved by either ion-exchange or reversed-phase HPLC.

Samples for apolipoprotein chromatography are obtained from delipidated lipoprotein fractions (HDL, LDL or VLDL). Preparation methods for apo-HDL [71,72] and apo-VLDL [73] by organic solvent delipidation have been described. With the exception of apoA-I and apoA-II, denaturing agents such as urea, guanidine chloride or sodium dodecyl sulphate (SDS) must be used to solubilize lipid-free apolipoproteins in aqueous solution [74].

Conventional gel permeation or ion-exchange chromatography on soft gel supports can be used for preparation of large quantities (10 to 50 mg) of pure apolipoproteins [75]. HDL apolipoproteins, apoA-I, apoA-II, apoC, can be separated on Sephadex G-200 columns using tris–HCl buffers (pH 8.6) containing 8 *M* urea [76]. Apolipoproteins C and E from VLDL can be isolated by Sepharyl S-200 (Pharmacia) chromatography [77].

Most HPLC separations of apolipoproteins are completed within 60 min. The main disadvantage of HPLC is that only small amounts of sample (<5 mg) can be applied to the column. However, the speed and ease of operation make HPLC ideal for analytical separation and characterization of apolipoproteins. Apolipoproteins from HDL and VLDL can be separated in less than 60 min on high-performance TSK gel permeation columns [78–80].

Separation by high-performance gel permeation is strongly dependent on the completeness of the delipidation step. Incomplete delipidation of HDL causes poor resolution between apoA-I and apoA-II [74]. Apolipoprotein B has extremely low solubility in aqueous buffers and requires the presence of denaturing agents such as guanidine chloride for complete dissolution [96]. Possible sources of oxidation should be minimized during preparation of apo-VLDL and apo-LDL as oxidation of apoB drastically reduces its solubility [81]. Thus, delipidation should be performed using peroxide-free ether and the apolipoproteins should be stored under nitrogen.

Okazaki and co-workers [81,82] reported the high-performance GPC separation of HDL apolipoproteins without prior organic solvent delipidation of the HDL fraction. Samples were prepared by pre-incubating a mixture containing HDL and buffer (0.1 *M* sodium phosphate containing 0.1% SDS). Separations obtained using the incubation mixture of HDL and apo-HDL under three different eluent systems were compared. In eluents containing 0.1% SDS or 6 *M* urea at pH 3.15, the elution patterns for apoA-I, apoA-II and apoC of the incubation mixture and apo-HDL were very similar. Thus, the SDS procedure is very useful for rapid screening of HDL apolipoproteins without prior delipidation.

Separations of apolipoproteins by ion-exchange chromatography are based on differences in charge. Therefore, this mode of chromatography can separate apolipoproteins isomers with different isoelectric points. Chromatography of VLDL and HDL apolipoproteins is performed on anion-exchange columns such as SynChropak AX300 (SynChrom IUC) [83] and MonoQ-HR (Pharmacia) [84,85]. ApoC-III isoforms ($C\text{-}III_0$, $C\text{-}III_1$, or $C\text{-}III_2$) can be separated by FPLC on Mono HR 5/5 (Pharmacia) anion-exchange columns [86]. The C apolipoproteins are first isolated by gel permeation of apo-VLDL on Sephacryl S-200 media. Elution with tris-HCl 6 *M* urea (pH 8.2) using a linear gradient of 0 to 0.15 *M* NaCl results in complete resolution of C isoforms in less than 30 min.

Differences in hydrophobicity are the basis of separation by reversed-phase HPLC. There is growing interest in the use of RP-HPLC as a rapid and highly efficient technique for moni-

toring heterogeneity in apolipoprotein structure. Reversed-phase columns usually have a lower sample capacity than those used in gel permeation HPLC. RP-HPLC can nevertheless be used to prepare small amounts of apolipoprotein (< 100 μg). A drawback to the reversed-phase mode is the relatively short column lifetimes due to instability of silica-based stationary phases at pH extremes.

Hancock and co-workers first reported resolution of HDL (apoA-I + A-II) [87] and VLDL (apoC) [88] apolipoproteins by ion-paired RP-HPLC. Separations were performed using either μBondapak-alkylphenyl or Zorbax-C_8 columns and mobile phase containing 1% triethyl ammonium phosphate (pH 3.2). The ion-pairing reagent was necessary in order to minimize interaction between apolipoproteins and free silanols on the stationary phase.

Initial attempts to apply columns with more hydrophobic stationary phases such as C_{18}, resulted in poor separations. In fact, in some cases a normal rather than a reversed-phase separation was observed [88]. This was attributed to interaction of free underivatized silanol groups on the silica surface with basic residues of the apolipoproteins.

The development of more efficient derivatization techniques has led to successful separations of apoA and apoC apolipoproteins on C_{18} columns. Typically, these procedures require gradient elution with acetonitrile–water mixtures containing 0.1% trifluoroacetic acid [89,90]. A plot of retention time versus mean residence hydrophobicity reveals that a true reversed-phase separation is taking place on these C_{18} columns. Using a 47–55% gradient of acetonitrile–water, apolipoproteins A-I, A-II and A-IV as well as their isoforms are separated (Fig. 7). Following elution, the peaks were collected and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and isoelectric focusing in order to identify apolipoprotein isoforms. As expected, the two apoA-I isoforms had similar molecular mass but different isoelectric points. However, the apoA-II had a similar molecular mass and isoelectric point. This suggests a structural modification which affects hydrophobicity while leaving other physical characteristics of the protein unchanged.

The resolution of apoE from other HDL apolipoproteins has not been achieved on C_{18} columns. The apoE isoforms tend to coelute with apoA-II isoforms [90]. However, apoE can be

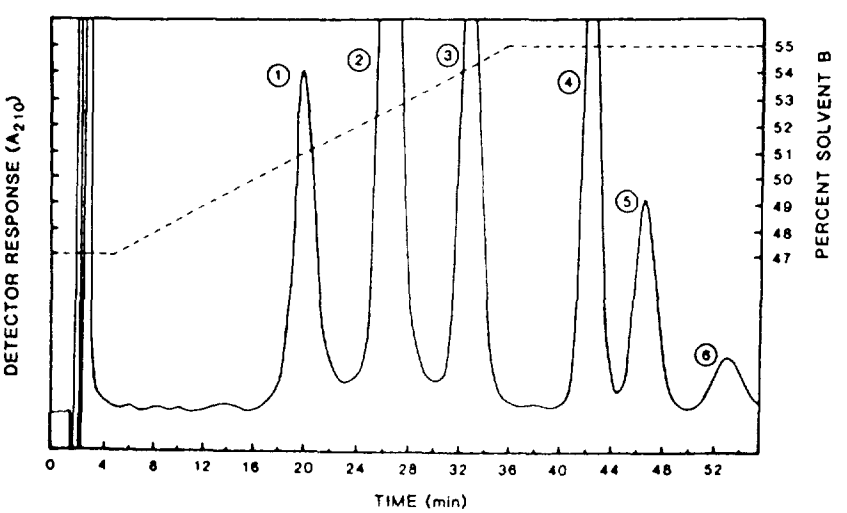

Fig. 7. Reversed-phase separation of apolipoproteins A-I, A-II and A-IV. Conditions: 30 cm × 3.9 mm C_{18} reversed-phase column, 5-min isocratic elution with 0.1% TFA in 47% acetonitrile, followed by a 30-min linear gradient to 0.1% TFA–55% acetonitrile, UV detection at 210 nm. Peaks: 1 = apoA-IV; 2, 3 = apoA-I isoforms; 4–6 = apoA-II isoforms. (Reprinted from Ref. [89].)

separated from other apo-HDL by using a column of lower hydrophobicity such as TSK Phenyl-5PW [91]. These columns consist of a hydroxylated polyether support with a low density of bound surface phenyl groups. Other advantages of using a column with lower hydrophobicity include shorter retention times and lower concentrations of acetonitrile in the mobile phase. Both factors should lead to increased biological activity of the recovered apolipoprotein fractions.

4. Electrophoretic methods for lipoprotein separation

Electrophoretic techniques have made a tremendous contribution to the characterization of lipoproteins, apolipoproteins and the diagnosis of dyslipoproteinemias. Since chromatographic techniques are the subject of this review, electrophoretic techniques are discussed only briefly. The reader is referred to several recent reviews for details regarding lipoprotein electrophoresis in gel and paper media [92,93]. More attention is given, however, to newly established capillary electrophoretic (CE) techniques. The instrumental format of CE closely resembles that of HPLC in that a high-speed separation takes place on a column with on-line detection. However, separation is based on differences in mobility in an applied electric field. This combination of high resolving power in an easily automated instrumental format guarantees CE an important place in the future of lipoprotein separation.

4.1. Conventional electrophoresis

A major breakthrough in lipoprotein electrophoresis occurred when Frederickson et al. [94] reported that the Lees and Hatch [95] paper electrophoretic method gave the same information that had hitherto been available only from analytical ultracentrifugation. By this simple technique it became possible to compare electrophoretic bands of normal patients with those suffering from disorders of lipid metabolism and use this information to diagnose lipoprotein deficiency diseases [96] and hyperlipoproteinemias, which are related to CHD [97]. In the latter case six phenotypes (Types I, IIa, IIb, III, IV and V) of hyperlipoproteinemias were described on the basis of their electrophoretic patterns and the appearance of serum.

Currently, most electrophoretic separations of lipoproteins are performed in discontinuous polyacrylamide gel (PAGE) or agarose media. The PAGE results have been compared with those from agarose gel methodology [98]. The disc PAGE technique was found to give more information about the HDL and VHDL fractions, whereas the agarose gel is more useful in the VLDL region [99]. A main advantage of polyacrylamide media is that the pore size of the gel can be easily controlled. Thus, gradient gels (typically 4 to 30% acrylamide) can be produced by increasing the acrylamide concentration down the gel. This results in sharper bands and an increased range of particle sizes which can be studied in a single run.

Apolipoprotein electrophoresis is typically performed using PAGE in the presence of sodium dodecyl sulphate (SDS-PAGE) or isoelectric focusing (IEF). Maguire et al. [100] reported a simple 3.5% SDS-glycerol PAGE system which allows separations of all plasma apolipoproteins (M_r 8800–550 000) on a single gel. The IEF mode of electrophoresis is useful for separating lipoprotein isoforms which differ in their isoelectric points. Two-dimensional electrophoresis which separates components first according to isoelectric point then according to size is one of the most powerful methods available for studying structural defects in plasma apolipoproteins. Sprecher et al. [101] have developed a high-resolution 2D-electrophoretic technique which effectively screens for abnormalities in apoA-I, apoA-II, apoA-IV, apoC-II, apoC-III, apoD, apoE and apoH.

4.2. Capillary electrophoretic techniques for lipoprotein analysis

In the last decade capillary electrophoresis has emerged as a powerful separation technique for biomolecules. The use of narrow-bore capillaries

and high voltages results in high separation efficiencies and short analysis times. The main advantage of CE over conventional electrophoresis is speed and instrumental format which eliminates the need for labor-intensive steps such as gel preparation and staining.

CE also exhibits advantages over HPLC for protein analysis. CE separations are faster and do not require the use of expensive columns. Furthermore, CE does not suffer from slow mass transfer rates which lead to band broadening in HPLC separations of apolipoproteins.

Recently, Tadey and Purdy [102,103] reported the use of CE to separate plasma apolipoproteins. They found that by adding the surfactant SDS to the separation buffer, the main apolipoproteins of HDL (apoA-I and apoA-II) and LDL (apo-B) could be separated in a single run in less than 12 min [102]. In the absence of detergent the apolipoproteins elute as a single peak. The effect of SDS was attributed to the presence of discrete, non-interacting detergent-binding sites on the apolipoproteins.

The CE separation of VLDL apolipoproteins was also studied [101]. Again, detergents were necessary to achieve any noticeable separation. The effect of various different detergents was studied and it was found that both SDS and the cationic surfactant cetyltrimethylammonium bromide (CTAB), significantly improved separation. Resolution and efficiency were also improved by using a capillary coated with linear polyacrylamide (PA). The PA coating provides a non-ionic hydrophillic layer on the capillary surface and therefore minimizes ionic interaction between apolipoproteins and the capillary wall. The separation of VLDL apolipoproteins in a PA-coated capillary filled with SDS and CTAB buffers is shown in Fig. 8.

High-performance capillary isotachophoresis (ITP) of lipoproteins has also been reported. In the procedure reported by Schmitz and co-workers [52,104–105], whole serum or lipoprotein subfractions were first stained by incubating with a lipophilic dye for 30 min. The sample was mixed with ampholyte spacers and injected into the capillary between the leading and terminating electrolyte. The capillary ITP profiles for patients with hyperlipoproteinemias are shown in Fig. 9. The differences in the profiles indicate

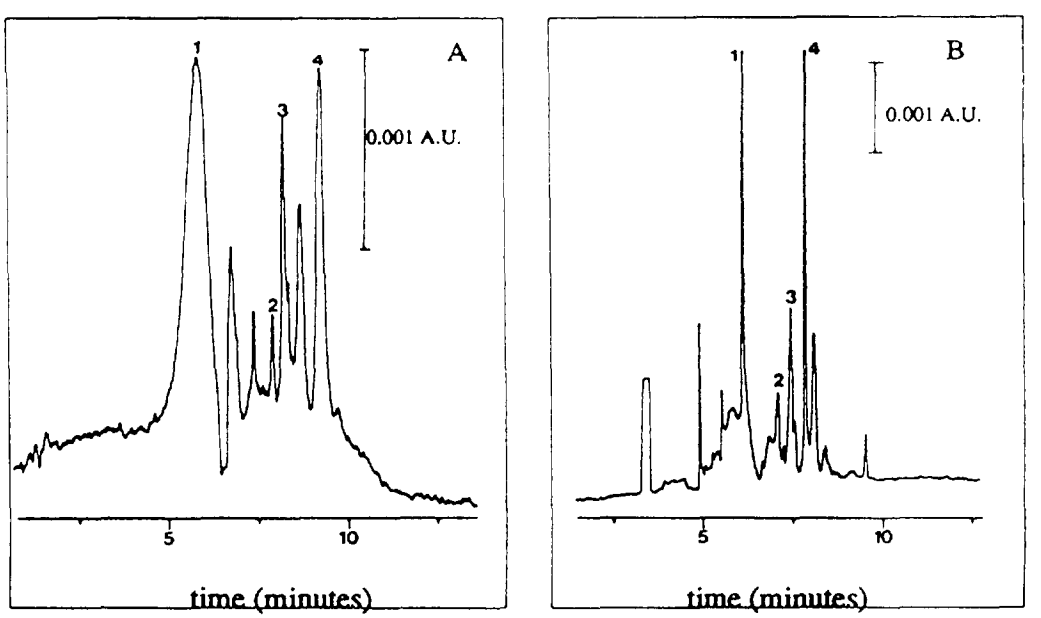

Fig. 8. Separation of VLDL apolipoproteins in polyacrylamide capillaries. Conditions: 30 m*M* borate, pH 9, UV detection at 214 nm. (A) 3.0 m*M* CTAB; (B) 3.5 m*M* SDS. Peaks: 1 = apoB; 2, 3 = apoC variants; 4 = albumin.

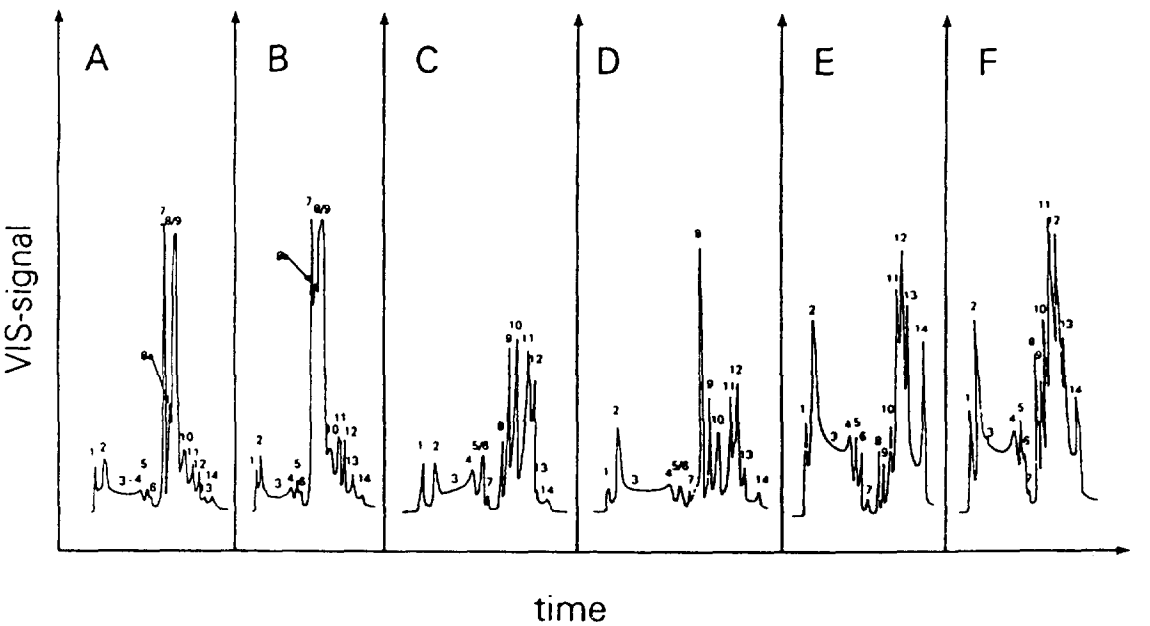

Fig. 9. ITP profiles for patients with HLP classified according to Frederickson phenotypes; HDL: fractions 1–6, VLDL + LDL: fractions 7–14. Conditions: 20-cm PTFE capillary, 500 μm I.D., 6 kV; leading electrolyte 5m*M* H_3PO_4–0.25% HPMC–20 m*M* 2-amino-2-methyl-1,3-propanediol (ammediol), pH 9.2; terminating electrolyte 100 m*M* valine–20 m*M* ammediol, pH 9.2. (A) Type 1 HLP; (B) type 5 HLP; (C) type III HLP; (D) type IV HLP; (E) type IIa HLP; (F) type IIb HLP. (Reprinted from Ref. [52].)

that capillary ITP provides for rapid screening of lipoprotein abnormalities.

5. Current trends in lipoprotein analysis

The development of novel chromatographic supports which offer increased speed and resolution has led to vast improvements in lipoprotein analysis. For example, the Superose 6 and Mono Q supports (from Pharmacia) will continue to play an important role in lipoprotein preparation and analysis. A particular advantage of these supports is that they are available in a variety of modes for analytical, semi-preparative and preparative purposes. Affinity chromatography should also play an increasing role in routine lipoprotein analysis once technologies for immobilization of ligands onto high-performance supports are established.

The speed and simplicity of CE should make it very useful for rapid screening of apolipoprotein profiles. However, since most CE studies have been carried out by analytical chemists, it may take some time before the technique is widely accepted in clinical laboratories. A further drawback to CE methods is their very low sample capacity which restricts CE to analytical applications.

6. Conclusions

The choice of method for separating lipoproteins depends very much on the need of the analyst. For separation of lipoproteins into apoA- and apoB-containing fractions, affinity chromatography on supports such as sulphated dextran beads is inexpensive and rapid. High-performance gel permeation chromatography with post-enzymatic reactors is more elaborate and expensive. However, it can provide very useful information regarding physical and chemical heterogeneity of lipoproteins. Finally, although they are not strictly chromatographic, capillary electrophoretic techniques should play

an increasingly important role in lipoprotein characterization.

Acknowledgements

The authors are indebted to the National Science and Engineering Council of Canada for financial support.

References

[1] M. Macheboef, *Bull. Soc. Chem. Biol.*, 11 (1929) 268.
[2] A. Tiselius, *Trans. Faraday Soc.*, 33 (1937) 524.
[3] G. Blix, A. Tiselius and H. Svensson, *J. Biol. Chem.*, 137 (1941) 485.
[4] O.F. Delalla and J.F. Gofman, *Methods Biochem. Anal.*, 1 (1954) 459.
[5] R.J. Havel, H.A. Eder and J.H. Bragdon, *J. Clin. Invest.*, 34 (1955) 1345.
[6] G.L. Mills, P.A. Lane and P.K. Weech, *A Guide Book to Lipoprotein Techniques: Techniques in Biochemistry and Molecular Biology*, Vol. 14, Elsevier, Amsterdam, 1984, Ch. 1.
[7] P. Alaupovic, *Protides Biol. Fluids Proc. Colloq.*, 19 (1972) 9.
[8] P.S. Bachorik, *Clin. Chem.*, 28 (1982) 1375.
[9] NIH, *Manual of Laboratory Operations, Lipid Research Clinics Program, Vol. 1: Lipid and Lipoprotein Analysis*, Publ. No. 75-628, US Dept. of Health, Education and Welfare, Bethesda, MA, 1974.
[10] P. Flodin and J. Killander, *Biochim. Biophys. Acta*, 63 (1962) 402.
[11] C. Franzini, *Clin. Chim. Acta*, 14 (1966) 576.
[12] T. Sata, D.L. Estrich, P.D.S. Wood and L.W. Kinsell, *J. Lipid Res.*, 11 (1970) 331.
[13] M. Werner, *J. Chromatogr.*, 28 (1967) 59.
[14] L.L. Rudel, C.A. Marzetta and F.L. Johnson, *Methods Enzymol.*, 129 (1986) 45.
[15] S.T. Kunitake and J.P. Kane, *J. Lipid Res.*, 23 (1982) 936.
[16] M. Fainaru, R.J. Havel and K. Imaizumi, *Biochim. Biophys. Acta*, 490 (1977) 144.
[17] M.C. Cheung and A.C. Wolf, *J. Lipid Res.*, 29 (1988) 15.
[18] T. van Gent and A. van Tol, *J. Chromatogr.*, 525 (1990) 433.
[19] W. März, R. Skeimer, H. Scharnagl, U.B. Seiffert and W. Gross, *Clin. Chem.*, 39 (1993) 2276.
[20] I. Hara and M. Okazaki, *Methods Enzymol.*, 129 (1986) 57.
[21] M. Okazaki, H. Itakura, K. Shiraiski and I. Hara, *Clin. Chem.*, 29 (1983) 768.
[22] R.M. Carrol and L.L. Rudel, *J. Lipid Res.*, 24 (1983) 200.
[23] H.K. Naito, *Clin. Chem.*, 34 (1988) B84.
[24] N. Rifai, J.F. Chapman, L.M. Silverman and J.T. Gwynnes, *Ann. Clin. Lab. Sci.*, 18 (1988) 429.
[25] G. Kostner and P. Alaupovic, *Biochemistry*, 22 (1972) 3419.
[26] W.J. McConathy and P. Alaupovic, *Biochemistry*, 15 (1976) 515.
[27] E. Koren, W.J. McConathy and P. Alaupovic, *Biochemistry*, 21 (1982) 5347.
[28] D.M. Lee and P. Alaupovic, *Biochem. J.*, 137 (1974) 155.
[29] P.A. Alaupovic, E.D. Bekaert and E. Koren, in C. Lenfant, A. Albertini, R. Paoletti and A.L. Catapano (Editors), *Biotechnology of Dyslipoproteinemias: Applications in Diagnosis and Control*, Raven Press, New York, 1990.
[30] W.J. McConathy, E. Koren, H. Wieland, E. Campos, D.M. Lee, H.U. Kloer and P. Alaupovic, *J. Chromatogr.*, 342 (1985) 47.
[31] J.A.K. Harmony and E.H. Cordes, *J. Biol. Chem.*, 250 (1975) 8614.
[32] V.G. Shore and B. Shore, *Biochemistry*, 12 (1976) 1516.
[33] K.H. Weisgraber and R.W. Mahley, *Methods Enzymol.*, 129 (1986) 145.
[34] P.H. Iverius, *J. Biol. Chem.*, 247 (1972) 2607.
[35] T. Nishida and U. Cogan, *J. Biol. Chem.*, 245 (1970) 4689.
[36] A. Carpenter and W.C. Purdy, *J. Chromatogr.*, 573 (1992) 132.
[37] A. Carpenter, *Ph.D. Thesis*, McGill University, Montreal, 1991.
[38] J.P. Miletich, G.J. Broze Jr. and P.W. Majerus, *Anal. Biochem.*, 105 (1980) 304.
[39] A. Carpenter and W.C. Purdy, *Clin. Biochem.*, 25 (1992) 89.
[40] P. Alaupovic and E. Koren, in E.G. Perkins (Editor) *Lipoproteins*, AOCS Press, Champaign, IL, 1993, p. 104.
[41] E. Koren, P. Puchois, P. Alaupovic, J. Fesmire, A. Kandoussi and J.-C. Fruchart, *Clin. Chem.*, 33 (1987) 38.
[42] R.W. James, A. Proudfoot and D. Pometta, *Biochim. Biophys. Acta*, 1002 (1989) 292.
[43] P.R. Bukbert, N.-A. Le, H.N. Ginsberg, J.C. Gibson, L.C. Goldman and V.W. Brown, *J. Lipid Res.*, 24 (1983) 1251.
[44] N. Yamada, D.M. Shames and R.J. Havel, *J. Clin. Invest.*, 80 (1987) 507.
[45] E. Koren, P. Allaupovic, D.M. Lee, N. Dashti, H.U. Kloer and G. Wen, *Biochemistry*, 26 (1987) 2734.
[46] E. Koren, D. Solter, D.M. Lee, Z. Reiner and W.J. McConathy, *Biochim. Biophys. Acta*, 876 (1986) 91.
[47] E. Koren, C. Knight-Gibson, G. Wen, L.E. DeBault and P. Alaupovic, *Biochim. Biophys. Acta*, 876 (1986) 101.

[48] M. Tavella, P. Alaupovic, C. Knight-Gibson, H. Tournier, G. Schinella and O. Mercuri, *Prog. Lipid Res.*, 30 (1991) 181.
[49] M.C. Cheung and A.C. Wolf, *J. Lipid Res.*, 30 (1989) 499.
[50] W.J. McConathy and P. Alaupovic, *Biochemistry*, 15 (1976) 515.
[51] M.C. Cheung, *Methods Enzymol.*, 129 (1986) 130.
[52] G. Schmitz and C. Mollers, *Electrophoresis*, 15 (1994) 31.
[53] M. Okazaki, K. Shiraiski, Y. Ohno and I. Hara, *J. Chromatogr.*, 223 (1981) 285.
[54] U. Matsumoto, H. Nakayama, Y. Shibusawa and T. Niimura, *J. Chromatogr.*, 566 (1991) 67.
[55] Y.C. Ha and P.J. Barter, *J. Chromatogr.*, 341 (1985) 154.
[56] P.M. Clifton, A.M. MacKinnon and P.J. Barter, *J. Chromatogr.*, 414 (1987) 25.
[57] M. Okazaki, H. Hagiwara and I. Hara, *J. Biochem.*, 92 (1982) 517.
[58] L. Holmquist and L. Carlson, *Lipids*, 20 (1985) 378.
[59] H. Shimano, N. Yamada, S. Ishibashi, H. Mokuno, N. Mori, T. Gotoda, K. Harada, Y. Akanuma, T. Murase, Y. Yazaki and F. Takaku, *J. Lipid Res.*, 32 (1991) 763.
[60] B. Vedie, I. Myara, M.A. Pech, J.C. Maziere, C. Maziere, A. Caprani and N. Moatti, *J. Lipid Res.*, 32 (1991) 1359.
[61] P. Avogaro, G. Bittolo Bon and G. Cazzolato, *Arteriosclerosis*, 8 (1988) 79.
[62] W. Palinski, M.E. Rosenfeld, S. Yla-Herttuala, G.C. Gurtner, S.S. Socher, S.W. Butler, S. Parthasathy, T.E. Carew, D. Steinberg and J.W. Witzum, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 1372.
[63] Yla-Herttula, S.W. Palinski, M.E. Rosenfeld, S. Parthasarathy, T.E. Carew, S. Butler, J. Witzum and D. Steinberg, *J. Clin. Invest.*, 84 (1989) 1086.
[64] D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo and J.L. Witzum, *N. Engl. J. Med.*, 320 (1989) 915.
[65] C.C. Lee and S.I. Koo, *Atherosclerosis*, 70 (1988) 205.
[66] K.H. Weisgraber and R.W. Mahley, *J. Lipid. Res.*, 21 (1980) 316.
[67] H.M. Wilson, B.A. Griffin and E.R. Skinner, *Biochem. Soc. Trans.*, 17 (1989) 152.
[68] H.M. Wilson, B.A. Griffin, C. Watt and E.R. Skinner, *Biochem. J.*, 284 (1992) 477.
[69] T. O'Brien, J. Buithieu, T.T. Nguyen, L. Klein, N. Bren, M. Wentworth, B.J. Hallaway and B.A. Kottke, *J. Chromatogr.*, 613 (1993) 239.
[70] G.L. Mills, P.A. Lane and P.K. Weech, *A Guide Book to Lipoprotein Techniques: Techniques in Biochemistry and Molecular Biology*, Vol. 14, Elsevier, Amsterdam, 1984, p. 244.
[71] A.M. Scanu and C. Edelstein, *Anal. Chem.*, 44 (1971) 576.
[72] V. Shore and B. Shore, *Biochemistry*, 7 (1968) 3396.
[73] J.P. Kane, T. Sta, R. Hamilton and R.J. Havel, *J. Clin. Invest.* 56 (1975) 1622.
[74] C. Edelstein and A.M. Scanu, *Methods Enzymol.*, 128 (1986) 339.
[75] C. Edelstein, in E.G. Perkins (Editor), *Analysis of Oils, Fats and Lipoproteins*, AOCS, Champaign, IL, 1991, p. 589.
[76] A. Scanu, J. Toth, C. Edelstein and E. Stiller, *Biochemistry*, 8 (1969) 3309.
[77] G.L. Mills, P.A. Lane and P.K. Weech, *A Guide Book to Lipoprotein Techniques: Techniques in Biochemistry and Molecular Biology*, Vol. 14, Elsevier, Amsterdam, 1984, Ch. 6.
[78] P.M. Young and T.M. Boehm, *J. Chromatogr.*, 311 (1984) 79.
[79] D. Polacek, C. Edelstein and A.M. Scanu, *J. Lipid Res.*, 24 (1983) 796.
[80] D.M. Lee, A.J. Valente, W.H. Kuo and H. Maeda, *Biochim. Biophys. Acta*, 66 (1981) 133.
[81] M. Okazaki, M. Kinoshita, C. Naito and I. Hara, *J. Chromatogr.*, 336 (1984) 151.
[82] M. Okazaki, M. Kinoshita and I. Hara, *J. Chromatogr.*, 430 (1988) 135.
[83] G.S. Ott and V.G. Shore, *J. Chromatogr.*, 23 (1982) 1.
[84] A. Steinmetz, V. Clavel, N. Vu-dac, H. Kaffarnik and J.C. Fruchart, *J. Chromatogr.*, 487 (1989) 154.
[85] G. Hocke, H. Kaffarnik, G. Minscher and A. Steinmetz, *J. Chromatogr.*, 526 (1990) 203.
[86] H. Mezdar, V. Clavey, I. Kora, M. Katfigan, A. Barkia and J.C. Fruchart, *J. Chromatogr.*, 363 (1987) 35.
[87] W.S. Hancock, H.J. Pownall, A.M. Gotto and J.T. Sparrow, *J. Chromatogr.*, 216 (1981) 285.
[88] W.S. Hancock, C.A. Bishop, A.M. Gotto, D.R.K. Harding, S.M. Lamplugh and J.T. Sparrow, *Lipids*, 16 (1981) 250.
[89] R. Weinberg, C. Patton and B. DaGue, *J. Lipid Res.*, 29 (1988) 819.
[90] T.A. Hughes, M.A. Moore, P. Neame, M.F. Medley and B.H. Cheung, *J. Lipid. Res.*, 29 (1988) 363.
[91] T. Tetaz, E. Kecruis, G. Grego and N. Fidge, *J. Chromatogr.*, 511 (1990) 147.
[92] L. Rosenfeld, *Arch. Pathol. Lab. Med.*, 113 (1989) 1101.
[93] V.G. Shore, in E.G. Perkins (Editor), *Analysis of Oils, Fats and Lipoproteins*, AOCS, Champaign, IL, 1991, pp. 573–588.
[94] D.S. Frederickson, R.I. Levy and F.T. Lindgren, *J. Clin. Invest.*, 47 (1968) 2446.
[95] R.S. Lees and F.T. Hatch, *J. Lab. Clin. Med.*, 61 (1963) 518.
[96] D.S. Frederickson, A.M. Gotto and R.I. Levy, in J.B. Stanbury, J.B. Wyngaarden and D.S. Frederickson (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill Book Co., New York, 3rd ed., 1972, p. 493.
[97] D.S. Frederickson and R.I. Levy, in J.B. Stanbury, J.B. Wyngaarden and D.S. Frederickson (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 3rd. ed., 1972, p. 545.

[98] J. Wollenweber and W. Kahle, *Clin. Chim. Acta*, 29 (1970) 411.
[99] P.N.M. Demacker, H.E. Vos-Janssen, A. Van't Laar and A.P. Jansen, *Clin. Chem.*, 24 (1987) 1439.
[100] G.F. Maguire, M. Lee and P.W. Connely, *J. Lipid Res.*, 30 (1989) 757.
[101] D.L. Sprecher, L. Taam and H.B. Brewer Jr., *Clin. Chem.*, 30 (1984) 2084.
[102] T. Tadey and W.C. Purdy, *J. Chromatogr.*, 583 (1992) 11.
[103] T. Tadey and W.C. Purdy, *J. Chromatogr.*, 652 (1993) 131.
[104] G. Schmitz, U. Borgmann and G. Assman, *J. Chromatogr.*, 320 (1985) 253.
[105] D. Josic, A. Bottcher and G. Schmitz, *Chromatographia*, 30 (1990) 703.