

**LIPOPROTEIN SUBCLASS ANALYSIS BY IMMUNOSPECIFIC DENSITY
GRADIENT ULTRACENTRIFUGATION**

A Thesis

by

SANDY M. LESTER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Chemistry

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Approved by:

Chair of Committee,	Ronald D. Macfarlane
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ABSTRACT

Lipoprotein Subclass Analysis by Immunospecific Density Gradient Ultracentrifugation.

(December 2008)

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Chair of Advisory Committee: Dr. Ronald D. Macfarlane

Apolipoprotein C-1 (apo C-1) enriched HDL has been described as an atherogenic form of HDL associated with an increased risk for cardiovascular disease (CVD). The objective of the present study was to develop a rapid method for the separation, purification, and characterization of Apo C-1 from serum. We isolated and characterize HDL subclasses from individuals with and without angiographically-proven CVD who have elevated and normal-to-low HDL-C levels.

Ultracentrifugation was linked with immunoaffinity separations for the specific separation of Apo C-1 enriched HDL from other lipoproteins. A 50 μ L sample of serum is diluted in TRIS HCl buffer (pH 7.5) and incubated with CNBr-activated Sepharose (Amersham) containing antibodies to apo C-1 (Academy Bio-medical Company). The apo C-1-depleted serum is removed by centrifugation and all apo C-1-containing lipoproteins are released from the Sepharose beads at pH 2. The apo C-1-depleted sample and the apo C-1-containing sample were ultracentrifuged to obtain a lipoprotein density profile in the absence and presence of apo C-1. Density Lipoprotein Profiling (DLP) gives relevant information of lipoproteins, such as density and subclass

characterization, and is a novel approach to purify apo C-1-enriched HDL. An additional advantage of this approach is that lipoprotein-a (Lp(a)), which is often an interfering component in the HDL density region, is eliminated.

Results show feasibility that these methods could be used in a clinical setting, was achieved. This measurement may yield a precise and quantitative profile of the distribution of apo C-1 for all lipoprotein particles including HDL.

DEDICATION

To my husband and family, whose continued love and support made all of this possible.

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I would like to thank Dr. Ronald D. Macfarlane for allowing me the opportunity to work on this exciting project. His constant guidance and advice were extremely helpful in completing my research.

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Finally, thank you to my mother and father for their encouragement and to my husband, Michael, for his patience and love.

LIST OF ABBREVIATIONS

ACN	acetonitrile
Apo	apolipoproteins
AR	antigen retrieval
BHT	butylated hydroxytoluene
CE	capillary electrophoresis
CVD	cardiovascular disease
CETP	cholesteryl ester transfer protein
CsBiY	cesium-bismuth-EDTA
Cs ₂ CdY	cesium-cadmium-EDTA
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetracetic acid
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
M	molar
mM	milli molar
MW	molecular weight
MWCO	molecular weight cut-off
NaBiY	sodium-bismuth-EDTA

NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl ceramide
OD ₂₈₀	optical density @ 280m
PB	phosphate buffer
TFA	trifluoroacetic acid
UC	ultracentrifuge
UV	ultraviolet
UV/Vis	ultraviolet/visible
VLDL	very low density lipoprotein

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CHAPTER I

INTRODUCTION

What Is Cardiovascular Disease?

The heart consists of muscle, called myocardium that pumps blood; arteries that supply blood to the heart muscle; and valves to ensure that the blood is pumped in the correct direction. At any point in the pumping process, or in any part of the heart, something can go wrong. The diseases and conditions affecting the heart are collectively known as heart disease. [1]

Coronary heart disease means that blood flow through the coronary arteries has become impaired. A more general term that includes non-coronary arteries is cardiovascular disease (CVD). The most common way such obstructions develop is through a condition called atherosclerosis, a largely preventable type of CVD. The actively contracting heart muscle needs a steady supply of oxygen and nutrients, carried through blood vessels or coronary arteries, to function. Over the course of a person's lifetime — usually starting in early childhood — arteries, whose inner lining is normally smooth, can slowly become clogged with clumps of fats, cholesterol, and other material, called atherosclerotic plaques. The inner walls of arteries become slowly narrower because of a buildup of these plaques, importantly, rupture of a plaque can occur suddenly and the subsequent formation of a blood clot around the ruptured plaque may

This thesis follows the style of *Biochimica et Biophysica Acta*.

completely occlude the artery. As a result, the blood containing oxygen and nutrients traveling to the heart muscle is blocked off and causes myocardial ischemia. As less blood and oxygen reaches the heart, it cannot function normally, and provokes overt physical consequences. [1, 2]

Lipoproteins and Cardiovascular Disease

The 2006 report on CVD by the American Heart Association (AHA) confirmed CVD as the leading cause of mortality in America. At least one in every three American adults has a form of Cardiovascular Disease (CVD). Cardiovascular diseases include heart disease, stroke, high blood pressure, heart failure and several other conditions including arrhythmias, atrial fibrillation, cardiomyopathy and peripheral arterial disease. [2]

CVD has been the leading cause of death in the United States every year since 1900 except during the 1918 flu epidemic. In 2004, the age-adjusted CVD death rate per 100,000 persons was 288.0, compared to 307.7 deaths per 100,000 people in 2003. In 2005, CVD was listed as the underlying cause of death in 869,724 cases, compared to 911,163 deaths in 2003. Cancer was the second-leading cause of death, responsible for 553,888 lives lost. Stroke, when considered separately from other CVD, was the nation's third-leading killer totaling 150,074 deaths, followed by accidents at 112,012 fatalities. CHD, even when considered separately from other cardiovascular diseases, was still by far the nation's single leading cause of death totaling 451,326 fatalities. Although the full statistics for 2008 are not in at this time, from January 1, 2008 to May 20, 2008, 250,735 people have been killed by CVD; 210,937 people died because of cancer; and 57,556 deaths are attributed to stroke. [3] Atherosclerosis, the major cause of CHD, is

the narrowing, inflammation, and endothelial cell dysfunction of the coronary arteries due to fatty build-up of plaque. The death rate from Coronary Heart Disease in America has dropped 30.2% in the last ten years. Cardiovascular disease (CVD) death rates are declining, but CVD is still the number one cause of death in the United States, and according to the most recent data from the American Heart Association's *Heart Disease and Stroke Statistics – 2008 Update*, the risk factor control remains a challenge for many.

In 2001, the National Cholesterol Education Program (NCEP) published a report on the detection, evaluation, and treatment of high cholesterol in adults. [4] One of the major causes of CHD is an elevated level of low-density lipoprotein (LDL) cholesterol. The first step in risk assessment is a measurement of LDL cholesterol as a part of lipoprotein analysis. NCEP suggests that adults, ages 20 and older, should have a fasting lipoprotein profile performed once every five years. [4]

Major risk factors for Coronary Heart Disease are [5]:

- LDL cholesterol higher than 160mg/dL
- Total cholesterol higher than 200mg/dL
- High Density Lipoprotein (HDL) cholesterol lower than 40mg/dL, but above 60mg/dL

In addition to elevated LDL cholesterol, other known risk factors are [5]:

- Cigarette smoking
- Obesity
- Hypertension

- Family history of heart disease
- Age (men \geq 45 years; women \geq 55 years)

Background on Lipoproteins

Lipids are essential for cellular structure, cell signalling, and energy provision in the human body. Highly hydrophobic lipids cannot travel freely in the aqueous plasma. Lipoproteins are the way that intact lipids are transported throughout the body. Lipoproteins are pseudo-micellar in structure comprised of proteins known as apolipoproteins and lipids. [5] The lipid components are cholesterol, cholesterol esters, phospholipids, and triacylglycerols. (See Figure 1) Lipoproteins differ in protein/lipid distribution, and vary from chylomicrons containing 98% lipids, to mature HDL that contains 50 % lipids. The hydrophobic lipid core consists of triacylglycerols and cholesterol esters, whereas the surface of the lipoprotein particle is a combination of apolipoproteins, phospholipids, and cholesterol. Table 1 summarizes the compositional differences between lipoprotein classes. [5]

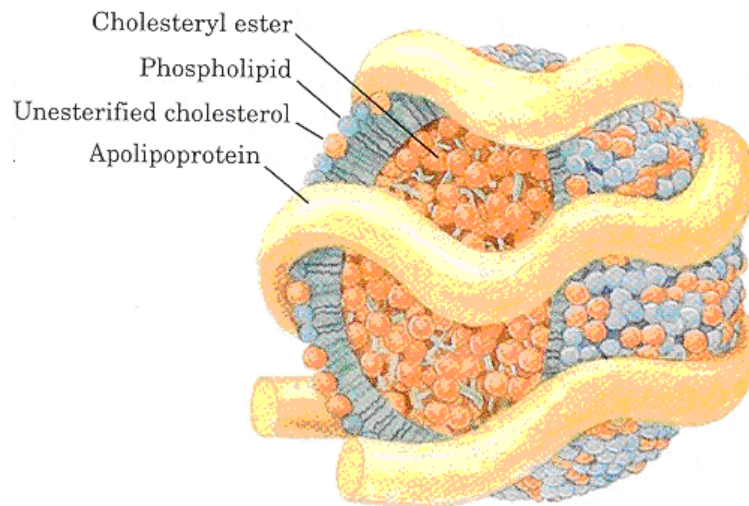


Figure 1: Structure of a lipoprotein. This diagram illustrates the architecture of lipoproteins; a core of water insoluble compounds, triacylglycerols and cholesteryl esters, and an amphiphatic membrane of phospholipids, free cholesterol and apolipoproteins. [6]

Table 1: Composition of Human Serum Lipoproteins

Component	Chylomicrons	VLDL	IDL	LDL	HDL
Phospholipids (Weight %)	6-9	16-20	20-24	24-30	24-30
Free Cholesterol (Weight %)	1-3	4-8	8-9	9-12	2-5
Cholesteryl Esters (Weight %)	3-6	9-13	13-28	28-30	10-20
Triglycerides (Weight %)	82-86	50-60	12-50	7-11	4-5
Proteins (Weight %)	1-2	8-11	11-20	20-22	41-58
Density (kg/L)	0.93-1.006	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.21

The protein moiety of lipoproteins is comprised of various apolipoproteins. (See Table 2) Apolipoproteins are present in each lipoprotein and serve as enzyme activators, ligands for cell receptors, and aid in metabolism of the lipoprotein.

Table 2: Human Apolipoproteins Present in Lipoproteins and Their Molecular Mass

Apolipoprotein	Lipoprotein	Molecular Mass
Apo A-I	HDL, Chylomicrons	28,000
Apo A-II	HDL, Chylomicrons	17,000
Apo A-IV	Chylomicrons, transfers to HDL	46,000
Apo B-100	LDL, VLDL, IDL	550,000
Apo B-48	Chylomicrons	260,000
Apo C-I	VLDL, HDL, Chylomicrons	6,600
Apo C-II	VLDL, HDL, Chylomicrons	8,900
Apo C-III	VLDL, HDL, Chylomicrons	8,750
Apo D	HDL	19,300
Apo E	VLDL, IDL, HDL, Chylomicrons	34,000

The major functions of the apolipoproteins are lipid transport between the liver and the peripheral tissues and metabolic control via the activation or inhibition of enzymes, seen below in Figure 2.

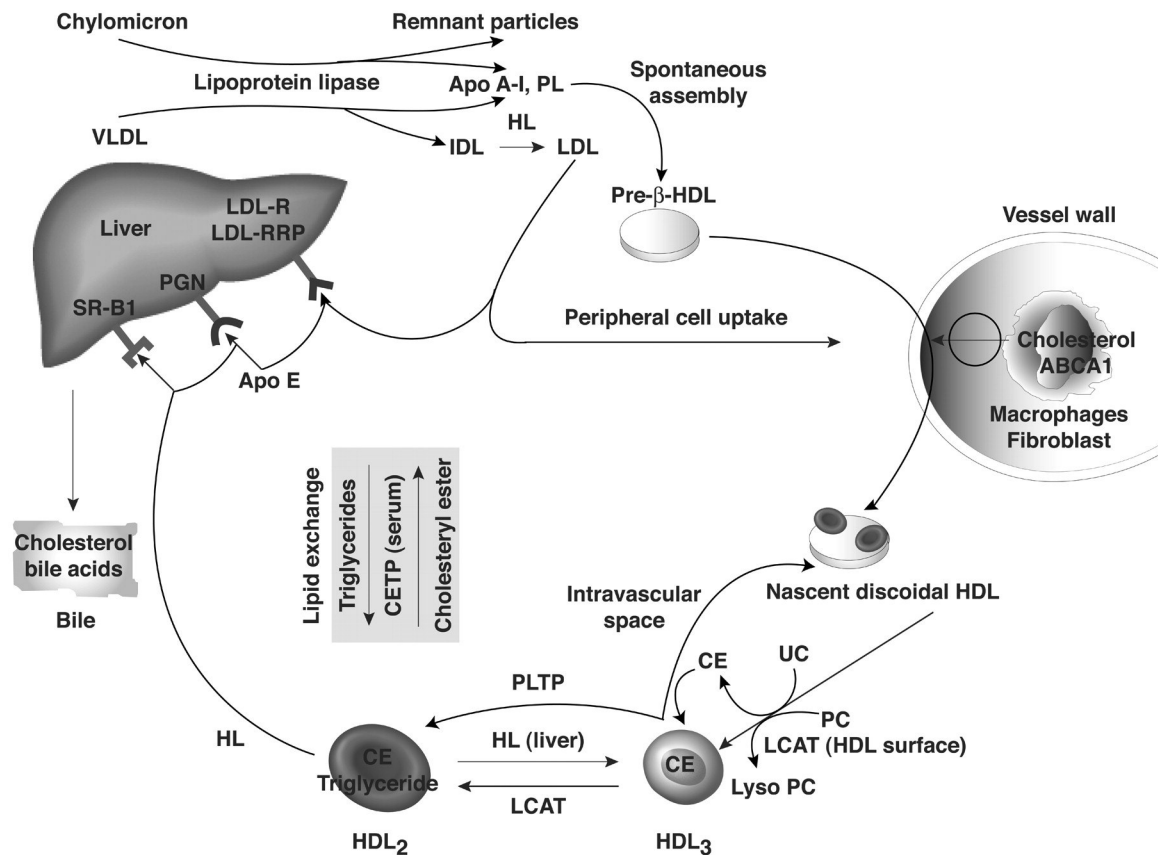


Figure 2: Lipid transport between the liver and peripheral tissues. Pathways involved in the generation and conversion of HDL. ABC1 indicates adenosine triphosphate-binding cassette transporter 1; Apo A-I, apolipoprotein A-I; Apo E, apolipoprotein E; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; LDL-RRP, low-density lipoprotein receptor-related protein; Lyso PC, lysophosphatidylcholine; PC, phosphatidylcholine; PGN, proteoglycans; PL, phospholipids; PLTP, phospholipid transfer protein; SR-B1, scavenger receptor B1; UC, unesterified cholesterol; and VLDL, very-low-density lipoprotein. [7]

Biomarkers in the Blood

Rapid developments in biochemical markers of cardiovascular disease have revolutionized the approach to diagnosis and management of acute coronary syndromes in recent years. [8]

Known Biomarkers in Cardiovascular Disease [9, 10]:

- Creatine Kinase, anything above normal levels of 25 to 200 U/L (units per liter)
- Myoglobin, anything above normal levels of 50 ng/mL
- Lactate Dehydrogenase, anything above a typical range is 105 - 333 IU/L (international units per liter).
- Blood levels of C-reactive protein, anything above 4.9mg/L
- Oxidized LDL

Continuing Search for Risk Markers

Traditional risk factors—smoking, obesity, cholesterol, etc.—can only tell a person so much about the possibility developing heart disease later in life. It is well known, that more than 50 percent of all future vascular events occur in people without overt hyperlipidemia, an elevation of lipids in the bloodstream. Studies have also shown that 20 to 25 percent of all future heart attacks occur in people with only one traditional factor. [8] This is why it is important to detect risk factors early in life to help prevent future risks of heart disease.

Apolipoprotein C-1 Enriched High Density Lipoprotein

Apolipoprotein C-1 (apo C-1), a 6.6-kDa plasma protein, has a basic pI because of its high lysine content (16 mol %) and lack of histidine, tyrosine, cysteine, and carbohydrate. [11] It contains 57 amino acids, and is synthesized in the liver; it's secreted following a co-translational cleavage of 26 amino acid peptide from the N-terminal. [12] Normal apo C-1 concentration in circulation is 6 mg/dL. Apo C-1 is a component of very-low-density (VLDL), intermediate density (IDL), and high-density (HDL) lipoproteins. [13, 14]

Apo C-1 contains alpha-helix regions that contribute to lipid binding. Apo C-1 activates both LCAT and LPL and may inhibit the clearance of triglyceride rich lipoproteins by altering the binding of apolipoprotein E (apo E) to the LPR receptor in the liver.

Problematic apo C-1 displaces apolipoprotein E (apoE) from VLDL and IDL and thereby decreases their clearance from plasma. [11] ApoC-1decreases the binding of B-VLDL to its remnant receptor (LDLR)—LDL receptor related protein—and apoE mediated binding of VLDL and IDL to the LDLR. Apolipoprotein C1 inhibits cholesterol ester transfer protein and phospholipase A2 activity. It also stimulates lecithin cholesterol acyl transferase up to 80% of that of apolipoprotein A-1 (Apo A-1). [5]

A. Apolipoprotein C-1 Enriched HDL and Atherosclerosis

Over the past decade, research has indicated that premature plaque rupture due to death of aortic smooth muscle cells (ASMC) is a major contributor of atherosclerosis. [15, 16]

Treatment of ASMC with apo C-1 or apo C-1-enriched HDL particles induces apoptosis, and therefore may contribute to the complications of atherosclerosis. [17]

Transgenic mice, expressing a wild-type human apo C-1 background or in combination with knockout of apo E or LDLR, were studied to determine the effect of apo C-1 without apo E. This combination created a hyperlipidemia effect, or high levels of lipids in the bloodstream, caused by delayed remnant clearance.[11, 16, 18-20] Research on apo C-1 transgenic mice with an apo E-null background proved positive for apo C-1-enriched HDL and an increase in atherosclerosis. However, it was the apo C-1-enriched HDL, not VLDL, which had an inhibitory effect on the hepatic lipase. [18]

In humans, it has been shown that a significant enrichment of apo C-1 in VLDL remnants can contribute to early signs of atherosclerosis in normolipidemic patients with coronary artery disease. [21, 22]

B. Apolipoprotein C-1 Enriched HDL in SGA Infants

It has been reported that low birth weight is associated with increased risk for CVD in adults. [23] Therefore, Kwiterovich and coworkers studied lipoproteins present at birth in cord blood of low birth weight infants. Results showed that a significant number of infants have increased levels of large HDL particles and also HDL particles enriched in apoC-1.

As seen in Figure 3, patients can be placed in different “groups” based on the gray intensity scale of apo C-1-enriched HDL that appears in their lipid profile. These groups are classified as Groups 0 to 3 based on the intensity of the area between the LDL and HDL in a serum profile.

Group 0 infants had no inflection above the baseline (no detectable Apo C-1 Enriched HDL); Group 1 had a small bump above the baseline (1 to 5 pixels on scale) (possible Apo C-1-enriched HDL); Group 2 had a peak above baseline in the range of >5 to <50 pixels on scale (probable Apo C-1-enriched HDL); and Group 3 had a peak greater than 50 pixels on scale (elevated Apo C-1-enriched HDL). [17]

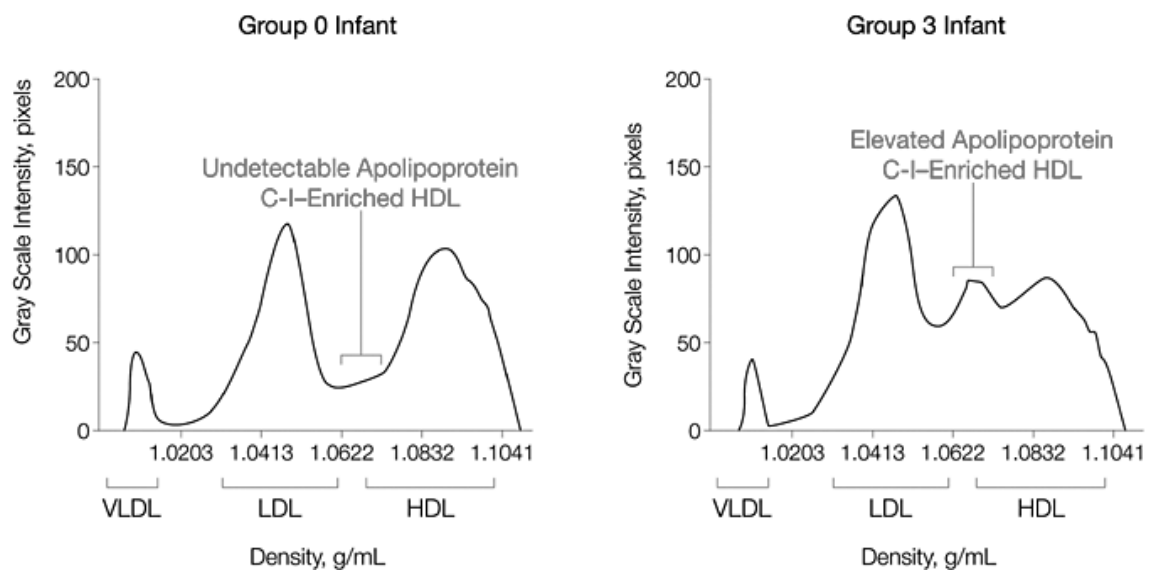


Figure 3: Lipid profiles depicting Apo C-1-enriched HDL groups. [17]

The basis for this thesis is to locate this probable apo C-1-enriched HDL particle with a density range of 1.062 and 1.072 g/mL. The peak for lipoprotein(a) (Lp(a)) is between 1.050 and 1.100g/mL, [24] which could occur at the same density as apo C-1-enriched HDL. Therefore, it is important to future research to obtain Lp(a) values.

Ultracentrifugation as an Analytical Tool for Separation of Lipoproteins

Measurement of VLDL, LDL, and HDL has been a useful research tool in studies of lipoprotein metabolism, pathophysiology, and CVD development. There are several methods, including sequential flotation and rate zone centrifugation and equilibrium density centrifugation, used to separate the different lipids based on density, size, and apolipoprotein composition.

Equilibrium Density Gradient Ultracentrifugation

An equilibrium density gradient ultracentrifugation will be utilized. In density gradient ultracentrifugation, the sample is mixed with a continuous gradient solvent and centrifugation occurs until the lipoproteins form discrete bands according to their density (usually 6 hrs depending on the solvent used). During centrifugation, the solvent generates a gradient, and the proteins migrate to where their density is the same as the gradient material. Isopycnic means “same density”. [25] Isopycnic density gradient ultracentrifugation effectively separates the lipoproteins present in serum into chylomicrons, VLDL, IDL, LDL, and HDL. (Fig 4)

As one moves down the Ultracentrifugation (UC) tube, the density increases and the particle size decreases from chylomicrons to HDL. The lipoprotein fractions also differ in lipid to protein ratios, apolipoprotein constituents, and are constantly changing in size, density, and components.

Advantage of Cs₂CdEDTA Salt as a Density Gradient Forming Solute

Solutions of metal ion complexes of ethylenediaminetetraacetic acid salts (EDTA) were described as a novel approach to produce self—generating density gradients from a homogenous solution during ultracentrifugation. [26-28] The cation influences gradient properties, with the cesium cadmium EDTA (Cs₂CdEDTA) salt, using cesium and cadmium as counterions, forming a less steep density gradient than other EDTA complexes. Within this density profile HDL and protein components of serum shift to a higher position on the tube coordinate scale, i.e. will give a better separation between HDL subclasses. (See Figure 4)

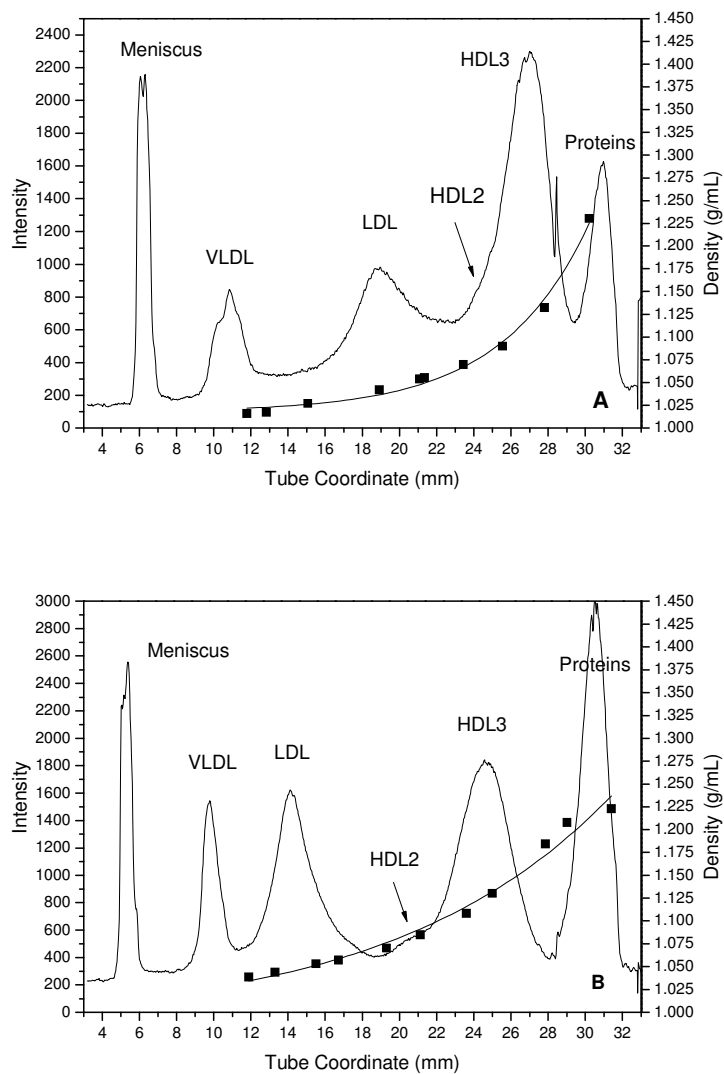


Figure 4: Lipoprotein profiles showing the separation differences between NaBiEDTA (A) and Cs₂CdEDTA (B). Each 6 μ L serum sample were spun at 5 $^{\circ}$ C in 1100 μ L of the respective gradients, 10 μ L of 1mg/mL NBD C₆-ceramide, and 44 μ L distilled water for 6 hours at 120,000rpm.

NBD-C₆ Ceramide Stain for Lipoprotein Imaging

The structure of NBD-C₆ ceramide (6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosine), as shown in Figure 5, is a widely used fluorescent probe for the staining of lipids. In the case of NBD-C₆ ceramide, it has emission at 536nm, absorption at 462nm and EC at 22,000. [29] NBD-labeled lipids are very stable and have suitable fluorescence properties such as: environmentally sensitive, self-quenching at high concentrations, minimal interference with other biological fluorophores. However, in water solutions NBD can suffer from photobleaching, limiting its fluorescence lifetime. [30] Studies done on cellular membranes show that NBD is found at the polar regions of the membrane, whether it is the head or tail of the phospholipid molecule. [30] The hydrophobic tail of the NBD-C₆ ceramide interacts with the surface of the lipoprotein and deep into the lipid core. The quantitative incorporation of the NBD molecule to lipoproteins was studied and showed that when the hydrophobic tail of the NBD molecule is imbedded in the lipoprotein molecule, the molecule fluoresces. [26] If the NBD is not imbedded in a lipoprotein molecule, there is no fluorescence.

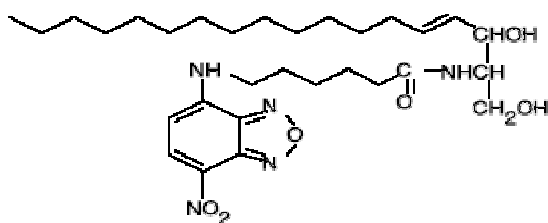


Figure 5: Structure of NBD C₆-ceramide [29]

Capillary Electrophoresis and Lipoproteins

Capillary electrophoresis (CE) has recently emerged as a powerful separation technique for biomolecules. CE has the advantages of obtaining complete separations with high efficiency in a relatively short amount of time. The main advantage of CE is that preparation of samples is quick and easy.

Apolipoproteins have a high affinity for detergents; therefore, adding detergents in the CE background buffer can influence electrophoretic behavior of proteins. Sodium dodecyl sulfate (SDS) is a very common detergent because it has two main effects on apolipoproteins. First, it eliminates protein aggregation by competing for protein-protein interactions. Second, it binds to the lipoproteins to alter their size, shape, and net charge—resulting in a modification of electrophoretic mobilities. [31, 32]

Antibodies and Immunoprecipitation

Antibodies, or Immunoglobulins (I_g), are proteins of molecular weight of approximately 150,000 to 900,000 kD. The basic structural unit of an antibody is a glycoprotein containing two light chains and two heavy chains, which are connected by disulfide bridges, thus forming a capital “Y” shape. Each chain is divided into regions, or domains, consisting of around 110 amino acid residues. The light chain has two domains and the heavy chain has four. The N-terminal domain, the tip of the arms of the “Y” on both the heavy and light chain, is known to be a variable in amino acid sequence composition and is thus called a variable domain; the other domains are called constant for a similar reason. [33-35] (See Figure 6)

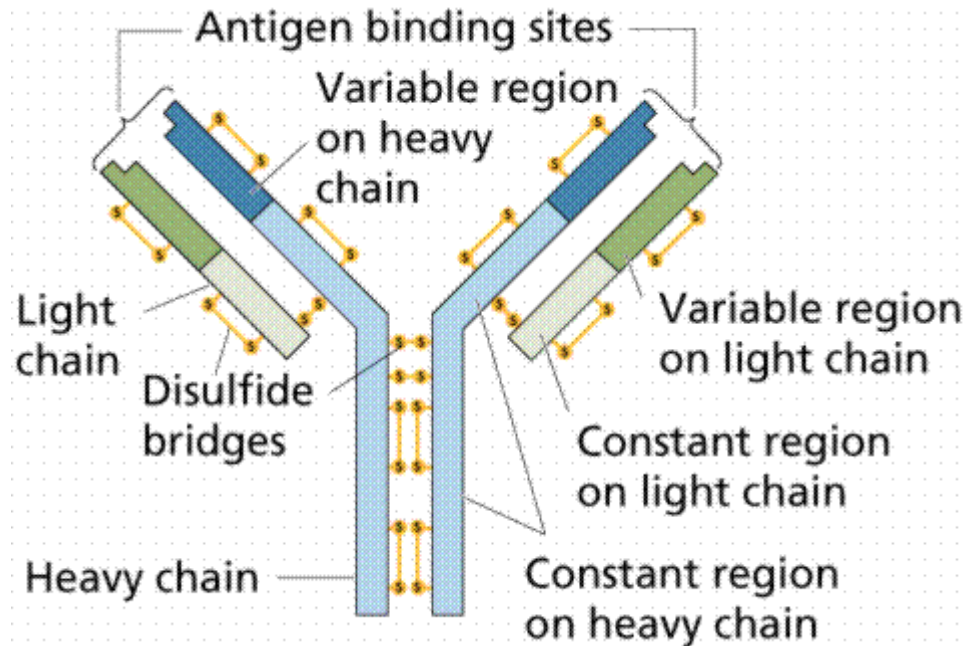


Figure 6: Basic monomer structure of an immunoglobulin molecule. (Adapted from [33])

The “Y” end of the I_g binds to the antigens (F_{ab} portion, so called because it is antigen binding), and the other end is responsible for biological activity (F_c , so called because it is the crystallizable portion of the molecule). [33] The crystallizable portion of the antibody will bind to the Sepharose bead, therefore orienting the antibody such that the F_{ab} portion will bind to the antigen optimally. [36]

Antibodies are capable of recognizing so many antigens because of the variable region of the F_{ab} . Both the variable and constant regions of the antibody are genetically

coded. There are two classes (isotypes) of the light chain called kappa and lambda. Heavy chains have five different isotopes which divide the I_g 's into five different classes, each with different effector functions (in humans IgG1-4, IgA1-2, IgD, IgM, IgE). Each class of heavy chain can combine with either of the light chains, thus giving the different possibilities of binding to different antigens. [33, 37]

Immunoprecipitation is a procedure by which peptides or proteins react with an antibody, and are then captured and are removed from solution. These peptides or proteins are selected by the antibody based upon a physical (molecular weight, color change, isoelectric point, etc.) or chemical (polysaccharide, proteins, etc.) characteristic. [33, 38-41] Contrary to the procedure's name, the precipitate contains only the protein of interest, thus leaving proteins not of interest in the supernatant. (See Figure 6) When this is the case, antigen retrieval plays a key role. [42]

Antigen Retrieval (AR), as developed by Shi et al. in 1991, [43] is a high-temperature heating method to recover the antigens from tissue sections previously masked by formalin fixation. Microwaves, pressure cooker, autoclaving and steam heating are most commonly used. [44] High-temperature heating methods are the most important factor for the retrieval of antigens and yields better results when compared to other methods. The pH of the antigen retrieval solution is another important factor to success in recovering antigens. Some antigens require heating along with a change in pH to achieve the strongest results. [37, 45]

To further advance the results of immunoprecipitation, scientists no longer relying on just a "color change" when studying antibody-antigen reactions. [36, 46]

Agar and agarose gels are quickly becoming common place tools in various areas of emerging biotechnology. Agar, a mixture of galactan derivatives extracted from red seaweeds, has limitations because of its ionic moieties. Isolated from agar, agarose (see figure 7), however, has a greater gel strength, gelling and melting temperatures, and electrical properties that are better suited to various analytical techniques involved in immunoprecipitation. [47] Agarose gel particles (See Figure 7) are sold under trade names such as Sepharose (Pharmacia) and Bio-Gel (Bio-Rad).

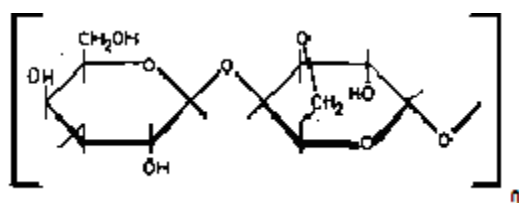


Figure 7: Agarbiose: basic repeating unit of agarose, (1, 3)-β-D-galactopyranosyl [1, 4]-3, 6 anhydro-α-L-galactopyranose. Adapted from [47]

An antibody is bound physically or chemically to the agarose particle interacts specifically with antigens of interest, and thus remove these particles from complex solutions. (See Figure 8)

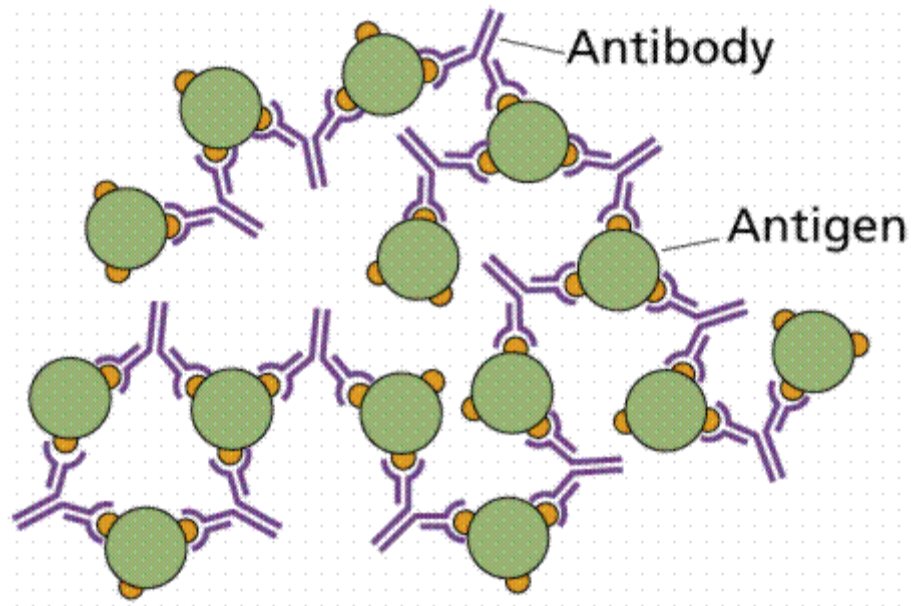


Figure 8: Binding of antigens to antibodies. [33]

Immunoprecipitation is a technique that can be formatted to fit one's needs. CNBr-activated Sepharose is a highly cross-linked agarose mixture that is reacted with cyanogen bromide (CNBr). This coupling makes the gel more rigid and allows for large proteins and other molecules to be directly coupled to the medium. Multi-point attachment of proteins allows for good chemical stability, high yield in products, and reproducibility of the gel. Mean particle size is 90 μm , with the size ranging from 45 to 165 μm . The Sepharose beads are 4% agarose and are spherical in structure. [48]

Application of Antibodies with Lipoprotein Research

Immunoprecipitation can be used for many different purposes. Among these are: (1) determination of molecular weight and isoelectric points of precipitated proteins by SDS-PAGE [49], (2) verification that an antigen of interest is synthesized by a specific tissue [44], (3) characterization of the type of carbohydrate present on glycoproteins [46], (4) identification of individual's drug use through forensics [36], (5) determination of heroin metabolites in urine by capillary electrophoresis [35] , and (6) study of lipoprotein remnants in human serum [50]. Although studies have been done with antibodies and lipoproteins, most of these studies isolated lipoproteins utilizing more than one antibody and more than one analytical technique—SDS-PAGE, CE, immunoaffinity chromatography, mass spectrometry, ultracentrifugation, and ELISA. [35, 37, 45, 46, 50-52]

For this research, immunoprecipitation will be utilized with one specific antibody, Anti-Apolipoprotein C1, in order to isolate apolipoprotein C-1 in serum obtained from patients with or without CVD. This research will show that patients with CVD have an apoC-1-enriched HDL, as detected in the recovered lipoprotein profile. However, non-CVD patients will not have any apoC-1 recovered after antigen retrieval, and will have no recovered HDL profile.

CHAPTER II

EXPERIMENTAL

This chapter focuses on the description of the experimental settings for all the procedures developed or applied for the research reported in this thesis. Detailed protocols for all experimental sections can be found in Chapter IV of this thesis.

Blood Draw and Serum Collection

A. Materials

Serum samples were obtained by venipuncture into a 7mL, vacuum tube with a sterile interior containing STT gel and clot activator (Beckton Dickinson Vacutiner Systems, Franklin Lakes, NJ).

B. Methods

Blood from normolipidemic subjects was drawn into a Vacutainer-brand series collection tubes following a 12-h fast. Serum was separated from red blood cells by centrifugation at 3200rpm for 20 minutes at 4°C. The supernatant (serum) was aspirated from the red blood cells, aliquotted into 250 μ L increments, and used immediately or stored at -86°C until used.

Coupling Antibody to CNBr-activated Sepharose

A. Materials

Apolipoprotein C-1 Antibody solution was purchased through Academy Biomedical Sciences in Houston, TX. The CNBr-Sepharose beads were purchased from Amersham

Biosciences. The following solutions were prepared and used during the binding of antibody to the Sepharose beads and the binding of serum to the antibody-Sepharose gel: (1) Coupling buffer—0.1M NaHCO₃ and 0.5M NaCl, pH 9.0, (2) 1mM HCl, (3) Blocking buffer—1M Tris-Base, pH 9.0 in coupling buffer, (4) low pH wash buffer—0.1M HCl, 0.5M NaCl, and (5) 0.05M Tris-HCl.

B. Methods

An optical density reading at 280nm (OD₂₈₀) of the antibody solution was taken to determine the protein concentration in the antibody solution. 62.5μL coupling buffer (0.1M NaHCO₃ and 0.5M NaCl, pH 9.0) was then added to 125μL antibody solution obtained. [17] The Sepharose beads were mixed with 1mM HCl and allowed to swell for approximately 15 minutes at room temperature. Supernatant was then removed, and the Sepharose beads were washed with 1mM HCl solution for 1 minute, with this process repeated twice.

The next two steps were done with *no pausing* in between. Swollen agarose beads were washed with 5mL coupling buffer to prepare for the addition of the antibody-coupling buffer solution, and after the addition, the tube was rotated gently at room temperature for 4 hours. After the beads settled, the supernatant was removed, and the OD₂₈₀ was taken. If the OD₂₈₀ is at least 10-fold lower than before coupling, one can continue with the procedure. If OD₂₈₀ is not at least 10-fold lower than in step 1, coupling did not proceed as expected. The primary cause for failure was not transferring gel to antibody solution quickly enough.

The coupled medium was washed twice with 1mL coupling buffer, prior to addition of 1mL blocking buffer (1M Tris-Base, pH 9.0 in coupling buffer) and incubation for 2 hours at room temperature. The supernatant was then removed, and the gel was washed 4x, alternating washes between low pH wash buffer (0.1M HCl, 0.5M NaCl) and high pH wash buffer (coupling buffer). The first and fourth washes were saved, and the OD₂₈₀ of each was checked to determine that all antibody in solution was removed, thus showing an OD₂₈₀ reading below ~0.01.

Coupling Serum to Sepharose-Antibody Gel

The AB-Sepharose gel, that was freshly made and washed with coupling buffer, was mixed with 50µL serum and 600µL 0.05M Tris-HCl in a 1.5mL Eppendorf tube. The tube was gently rotated for 4 hours at 1400rpm at room temperature. After rotation, the Sepharose gel was allowed to settle and the depleted serum, approximately 600µL, was removed. Using a 3,000 molecular weight cut-off (MWCO) filter the supernatant, containing everything that did not bind to the AB, was concentrated for UC separation. (See Figure 9)

Recovering Serum from the Sepharose-Antibody Gel

After removing the depleted serum from the AB-Sepharose gel, the beads were washed twice with coupling buffer to ensure removal of residual, bound serum in the Eppendorf tube. Afterwards, 600µL of 1mM HCl was added to the beads. The tube was gently rotated at 1400rpm for 5 hours at 37°C. After rotation, the Sepharose gel was allowed to settle and the recovered lipoproteins, approximately 600µL, was removed.

The Sepharose-AB gel was washed with coupling buffer twice and then stored in 20% ethanol to prevent microbial growth at 4°C.

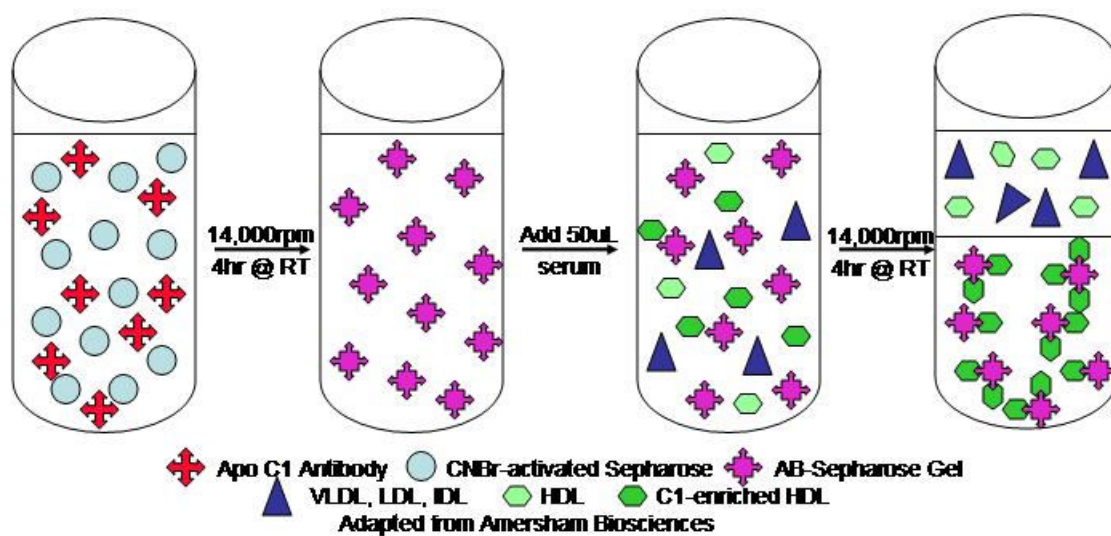


Figure 9: Schematic showing the steps involved for immunoprecipitation.

Separation of Lipoprotein Particles by Single Spin Ultracentrifugation

A. Materials

A 0.175M and 0.300M solution of Cs₂CdEDTA was used to form the density gradient used for the separation of lipoprotein classes. The various EDTA complexes were synthesized from H₄EDTA, the appropriate alkali carbonate, and the heavy metal carbonate. The reagents were combined stoichiometrically in 100mL of DI H₂O, followed by a two hour reflux, yielding a clear solution. Sodium carbonate or cesium carbonate was then added to the clear solution to bring the final pH range to 6-7. The final solution volume was reconstituted to 100 mL to account for evaporation during reflux to give stoichiometric solutions with a final concentration of 0.200 M. [28] A 10% (w/v) solution of sodium-bismuth-EDTA (NaBiY) in water was also used as a density gradient. NaBiY is commercially available (E086, TCI America, Portland, OR, USA).

In either density gradient, serum samples were stained with NBD C₆-ceramide as a 1 mg/mL or 2 mg/mL solution in dimethyl sulfoxide (DMSO, 99.9%, MX1458-6 Burdick & Jackson, Muskegon, MI, USA). NBD C₆-ceramide (NBD) was purchased from Molecular Probes (Eugene, OR).

Each sample was pipetted into an ultracentrifugation tube (1.5 mL, thick-walled, polycarbonate, Beckman-Coulter, Palo Alto, CA). Ultracentrifugation was carried out in a Beckman Optima TLX tabletop ultracentrifuge equipped with a 30° fixed angle TLA 129.2 rotor.

B. Methods

Lipoproteins were separated based on their hydrated densities as discussed previously. [26, 28] Six microliters of Apo C-1 enriched HDL serum samples were individually mixed with 10 μL of 1 mg/mL NBD, 1100 μL of 0.175 M Cs_2CdEDTA or 10% NaBiEDTA, and 44 μL of distilled water. The samples were allowed to stand for 30 minutes at 5°C so the NBD C_6 -ceramide could be incorporated into the lipoproteins. A 1000 μL volume of each sample was transferred to an ultracentrifuge tube. Both density gradients were formed by ultracentrifugation for 6 hours, 120,000 rpm and 5°C. [26, 27]

Layering Ultracentrifugation Tubes

After the UC spin, each tube was layered with 200 μL distilled water to further separate the buoyant TRL from the dense TRL found in the meniscus.

Digital Analysis

A fluorescence imaging system was developed in order to improve sensitivity and versatility to the lipoprotein fingerprinting method as previously reported. The light source used was a Fiber-Lite MH -100 Illuminator (MH100A, Edmund Industrial Optics). The camera used was a digital color microscope camera (S99808, Optronics, Goleta, CA). The camera and the light source were placed orthogonally from each other on an optical bench. A tube holder, suspended by a post/post holder, was placed 8 cm away in order to collimate the excitation beam. A gain of 1.0000 and an exposure time of 15.8 ms were chosen using the accompanying Micro Fire camera software. A blue-violet excitation filter (BG-12, Schott, Edmund Industrial Optics) with a bandwidth at 407 nm and a yellow emission filter (OG-515, OEM, Edmund Industrial Optics) with a

bandwidth centered at 570nm were chosen to match the excitation and emission of NBD C₆-ceramide.

Lipoprotein Fraction Collection after Ultracentrifugation

A. Materials

By using a high-speed thin blade (0.254 mm wide) scroll saw (Model 1672, 16 inches, 2-speed, Dremel, Racine, WI) and liquid nitrogen, lipoprotein fractions could be collected and saved for further analysis.

B. Methods

After ultracentrifugation and imaging, the ultracentrifugation tubes containing Apo C-1 Enriched HDL serum were slowly frozen in liquid nitrogen, without disturbing the gradient. [53] By cutting the tube at the appropriate positions with a high-speed thin blade scroll saw, lipoprotein fractions were collected. Cut positions were determined from the profile obtained from the digital imaging software.

C₁₈ Solid Phase Extraction, Desalting, and Delipidation

A. Materials

The proteins in VLDL, LDL, and HDL were delipidated and purified using a tC₁₈ Light cartridge (Sep-Pack, 36805, Waters, Milford, MA, USA). Also used were trifluoroacetic acid (TFA, T-6508, Sigma, St. Louis, MO, USA), and acetonitrile (ACN, 99.8%, AX0145-1, EM Science, Gibbstown, NJ, USA). Solutions of 0.1% TFA in distilled water and 0.1% TFA in acetonitrile were prepared and used for this experiment.

B. Methods

Apo C-1 enriched HDL fractions were prepared for capillary electrophoresis (CE) and kept intact by removing the density gradient medium from the fraction by ultrafiltration in a 100,000 MWCO filter (Millipore). The samples were filtered with CE background buffer seven times for 3 minutes at 6,000 rpm.

Apo C-1 enriched HDL fractions were delipidated using a tC₁₈ Light Cartridge. The tC₁₈ cartridge was conditioned with 5 mL 0.1% TFA in ACN slowly, allowing no air to enter the cartridge, and then with 5 mL 0.1% TFA in water, allowing no air to enter the cartridge.

The sample, mixed with 0.1% TFA and water, was slowly loaded onto the cartridge. The cartridge was rinsed with 0.1% TFA in water to remove any salts from the cartridge. The proteins are then eluted with four 50 µL 0.1% TFA in ACN aliquots with 1 mL air pushed through the cartridge. The 2nd through 4th aliquots were collected and combined. The combined aliquots were immediately Speed-Vacuumed to dryness and then reconstituted to 50µL with the CE buffer of 12.5mM Sodium Borate, 3.5mM 70% SDS, and 20% ACN.

Apolipoprotein C-1 HDL Characterization by Capillary Electrophoresis

A. Materials

All capillary electrophoresis experiments were performed using a P/ACE MDQ instrument (Beckman Coulter, Fullerton, CA).

B. Methods

Table 3 shows a capillary electrophoresis time program used for the separation of lipoproteins in the Apo C-1 enriched HDL fractions and for characterization of the Apo C-1 standard used in perfecting the immunoprecipitation protocol. Protein samples were kept at a temperature of 20°C during testing.

Step	Time	Function	Duration
1		Buffer Rinse	2.00 min
2		Inject EOF	5.00 s
3		Inject Sample	5.00 s
4		Inject Buffer	2.00 s
5	0.00	Separate-Voltage 20 kV	30.00 min
6	0.00	Auto Zero	
7	30.00	Stop Data	
8	30.00	Rinse 0.1N NaOH	1.00 min
9	31.00	Rinse dH ₂ O	1.00 min
10	32.00	End	

CHAPTER III

RESULTS AND DISCUSSION

Influence of Cs₂CdEDTA vs. NaBiEDTA on Lipoprotein Profiles

In the Cs₂CdEDTA solute system (seen in Figure 10B and 10C), the slope of the density gradient was less steep than the NaBiEDTA system (as seen in Figure 10A), causing the HDL and protein regions of the lipoprotein profile to shift to a higher position on the tube coordinate scale, thus further separating the LDL and HDL regions. There was essentially no difference in the LDL region of the lipoprotein profiles between these two solute systems, but the intensity of the VLDL peak in the Cs₂CdEDTA system was elevated. In each case due to the inherently higher molecular weight of the counterion, it can be seen that Cs⁺ solutions yield a steeper density profile than those containing the Na⁺.

It was also noticed that when a serum sample is spun in a solute containing Cs⁺, the corresponding density of the lipoprotein subclasses were higher than those spun in a solute containing the Na⁺ ion. At a pH of 7, lipoproteins are macromolecular anions, so the Na⁺ and Cs⁺ ions will form an ionic atmosphere around them and increase their density relative to their natural state. Since Cs⁺ is heavier than the Na⁺, the density of the lipoprotein can be greatly altered by substituting the counterion of the EDTA complex. These findings suggest that the ionic atmosphere configuration is retained under the

influence of the forces operating during ultracentrifugation as the lipoproteins migrate to their isopycnic positions.

Because there were concerns with the region between the LDL and HDL, the Cs_2CdEDTA density gradient was selected for further study. Using this particular density gradient increased recovery of the lipoprotein fractions because the LDL and HDL fractions are more widely separated allowing more room to cut between the lipoprotein classes.

Now that it has been established to use Cs_2CdEDTA density gradient, one must decide on what concentration. In Figure 10, the Cs_2CdEDTA is at 0.175M. (See Figure 10B) When the molarity is increased to 0.300M (Figure 10C), the solution becomes denser and further separates the HDL. This higher molarity is useful when the HDL subclasses want to be separated. One disadvantage to this particular density gradient is that the VLDL, IDL, and LDL are all sandwiched together and are inseparable for further analysis.

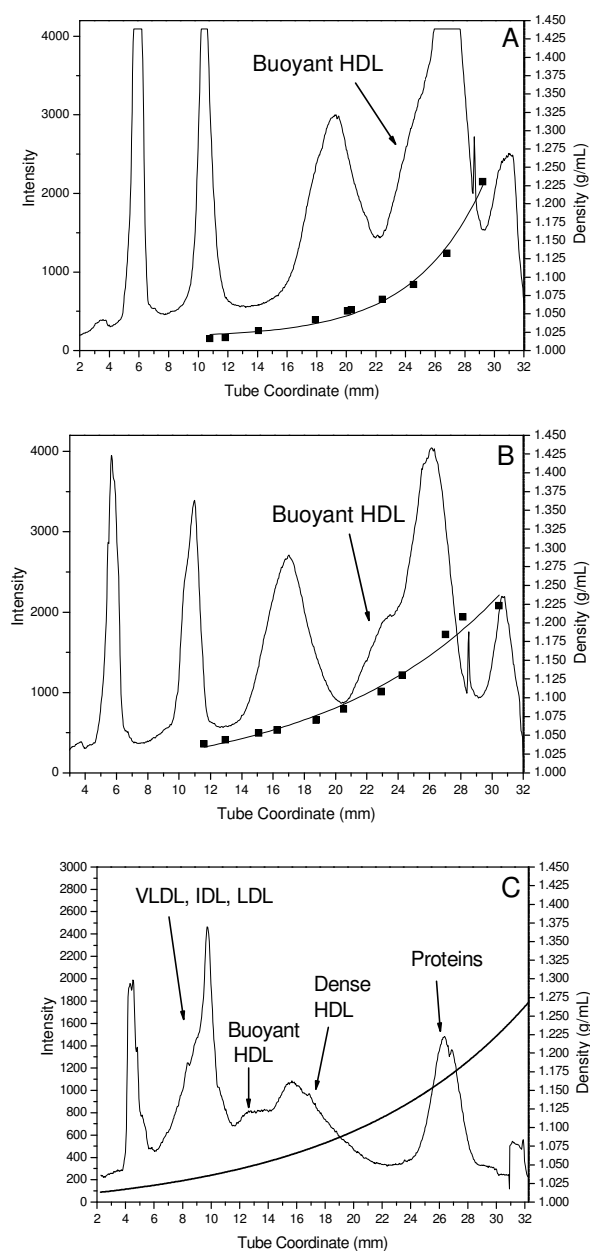


Figure 10: Ultracentrifugation profiles showing differences in densities and separations of LDL and HDL between 0.200M NaBiEDTA, 0.175M Cs₂CdEDTA and 0.300M Cs₂CdEDTA. Profiles show 0.200M NaBiEDTA (A), 0.175M Cs₂CdEDTA (B), and 0.300M Cs₂CdEDTA (C). Each 50 μ L serum sample were spun at 120,000rpm at 5 $^{\circ}$ C in 1100 μ L of the respective gradients, 10 μ L of 1mg/mL NBD C₆-ceramide, and 44 μ L distilled water for 6 hours.

Coupling Apolipoprotein C-1 Standard to Sepharose-Apolipoprotein C-1 Antibody

After attaching the Apolipoprotein C-1 antibody to the CNBr-Sepharose beads as shown in the previous chapter, one had to make sure that the Sepharose-Antibody beads did in fact remove the Apo C-1 in solution. Therefore, 50 μ L of Apolipoprotein C-1 Standard (Human Apo C-1, 31P-101, 0.1mg, Academy Biomedical Company, Houston, Texas) [54] was added to 600 μ L 0.05M Tris-HCl in a 1.5mL Eppendorf tube. The Apo C-1 standard was collected from fresh human plasma that has been purified by a series of ultracentrifugations in which the VLDL was isolated from human plasma. Purity was verified by SDS-PAGE. [54] An optical density reading at 280nm (OD_{280}) of 300 μ L of the Apo C-1 Standard-Tris-HCl solution was taken to determine the starting protein concentration being added to the system.

Apolipoprotein C-1 STD + NaN₃ Tris-HCL $OD_{280} = 0.187$

The tube containing the Apo C-1 Standard and Tris-HCL solution was gently rotated for 4 hours at 1400rpm at room temperature. After rotation, the Sepharose gel was allowed to settle and the depleted serum, approximately 600 μ L, was removed. An optical density reading at 280nm was taken of the supernatant to determine if the Apo C-1 Standard was successfully attached to the Antibody-Sepharose beads and removed from the solution.

Depleted Apolipoprotein C-1 STD + NaN₃ Tris-HCL $OD_{280} = 0.005$

After the supernatant was removed, the gel was washed twice with coupling buffer to ensure that all residual traces of Apo C-1 Standard and NaN_3 Tris-HCl were removed. Afterwards, 600 μL of 1mM HCl was added to the beads. The tube was gently rotated at 1400rpm for 5 hours at 37°C. After rotation, the Sepharose gel was allowed to settle and the recovered Apo C-1 Standard, approximately 600 μL , was removed and an optical density reading was taken.

Recovered Apolipoprotein C-1 STD + HCL $\text{OD}_{280} = 0.183$

Although the absorbances of each solution show the Apo C-1 standard being removed and recovered, each sample was analyzed by Capillary Electrophoresis as a second method to prove the theory. After all supernatants were collected, each was Speed Vacuumed to dryness and reconstituted using 50 μL of the CE buffer consisting of 12.5mM NaBorate, 3.5mM SDS (70%), and 20% ACN. Each electropherogram is shown below in Figures 11-13.

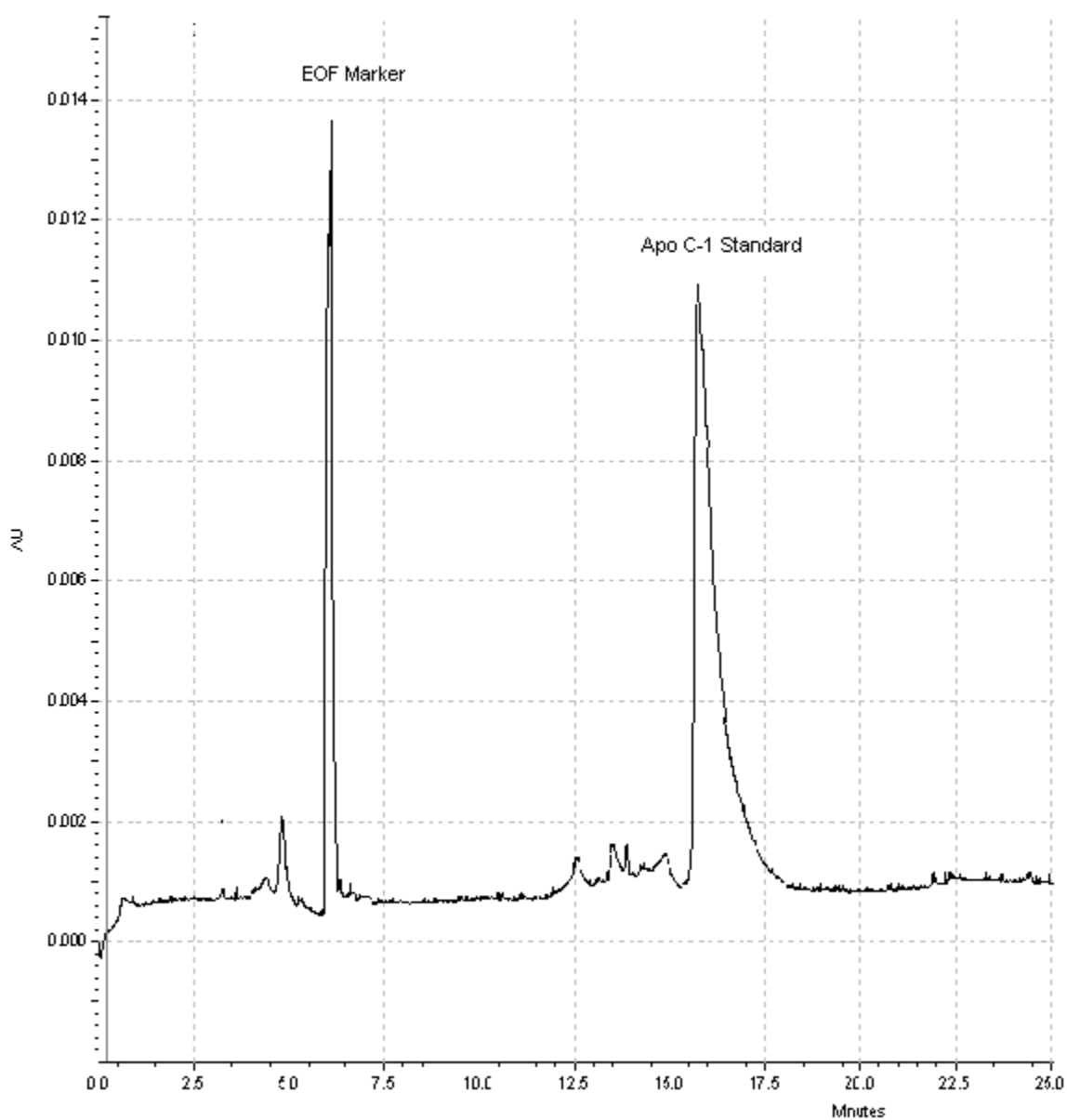


Figure 11: Electropherogram of apolipoprotein C-1 standard. Background electrolyte consisted of 12.5mM Sodium Borate, 3.5mM SDS, and 20% (v/v) acetonitrile, pH 9.25.

EOF marker consists of 0.05% DMSO with a 5sec 0.5psi injection. Under these conditions this Apo C-1 Standard has an effective mobility of $-26.3 \times 10^{-5} \text{ cm}^2/\text{Vs}$.

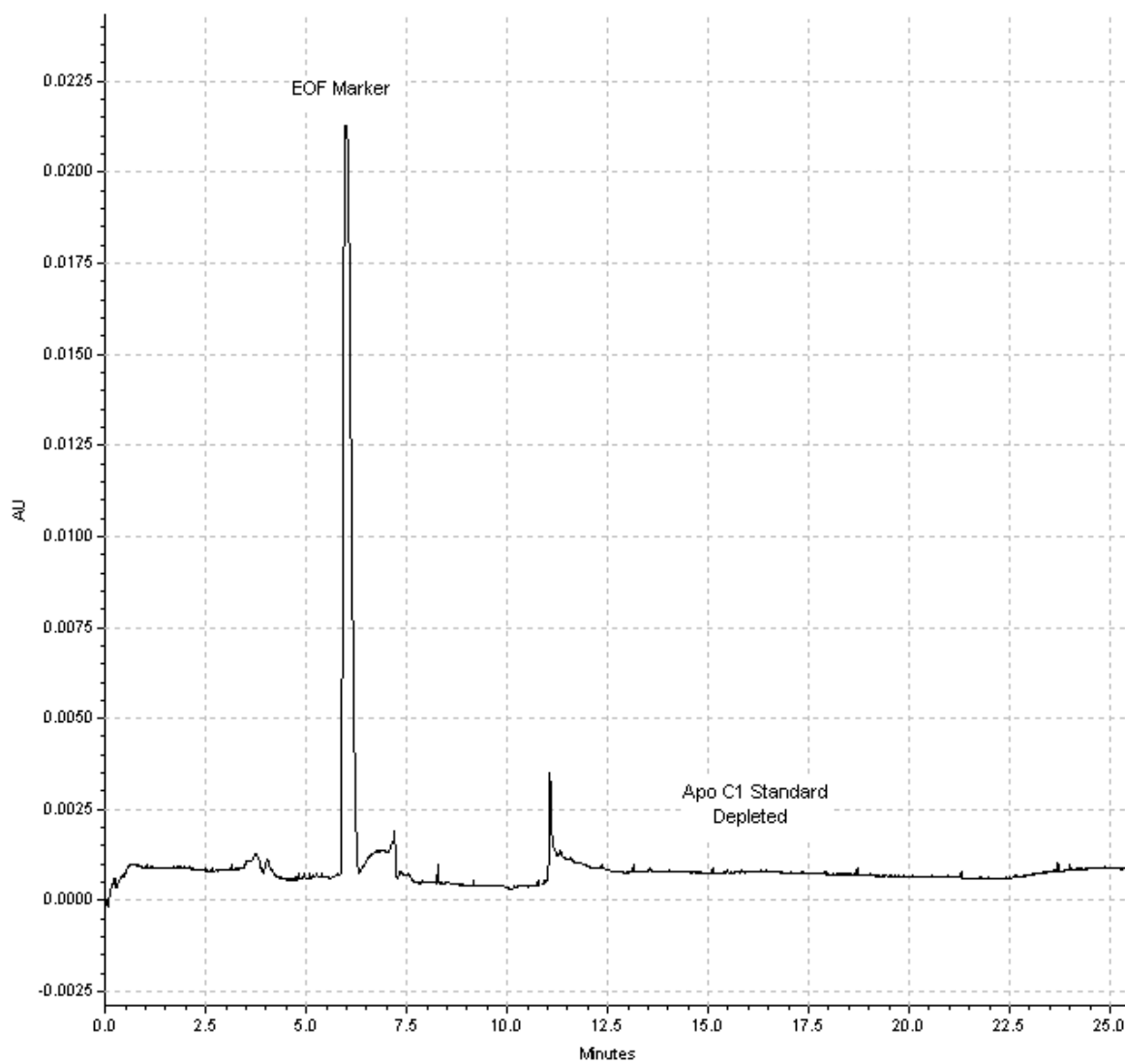


Figure 12: Electropherogram of depleted apolipoprotein C-1 standard. Background electrolyte consisted of 12.5mM Sodium Borate, 3.5mM SDS, and 20% (v/v) acetonitrile, pH 9.25. EOF marker consists of 0.05% DMSO with a 5sec 0.5psi injection. The only peak detectable is that of the EOF.

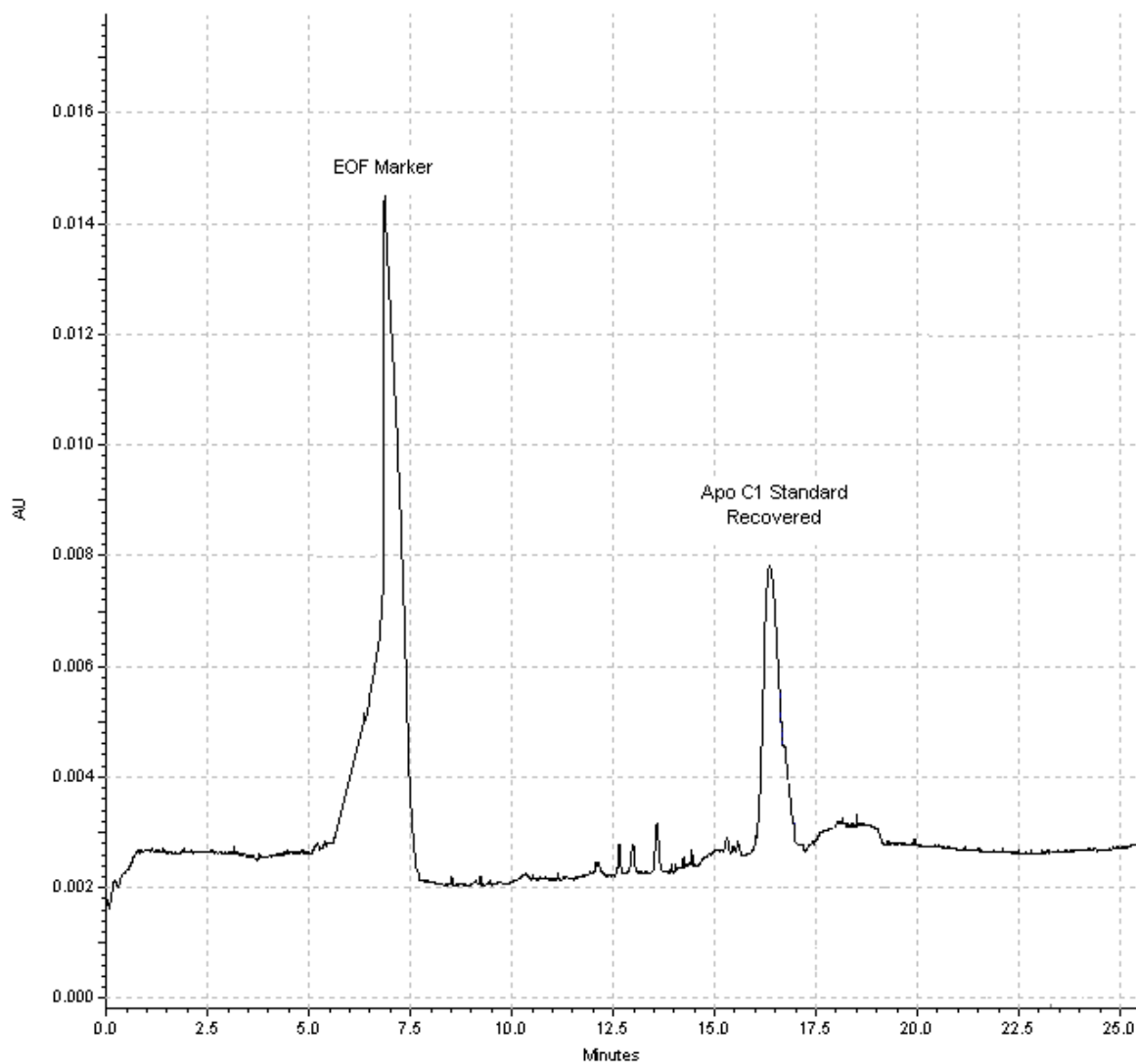


Figure 13: Electropherogram of recovered apolipoprotein C-1 standard. Background electrolyte consisted of 12.5mM Sodium Borate, 3.5mM SDS, and 20% (v/v) acetonitrile, pH 9.25. EOF marker consists of 0.05% DMSO with a 5sec 0.5psi injection. Under these conditions this Recovered Apo C-1 Standard has an effective mobility of $-26.1 \times 10^{-5} \text{ cm}^2/\text{Vs}$.

Influence of Serum Volume on Antibody-Sepharose Lipoprotein Profiles

Serum volume can affect the lipoprotein intensity and the separation differences. Too small a volume and the intensity of the peaks are weakened; too concentrated a serum volume and the intensity and separation of peaks increases. An experiment was designed to test this theory using 6 μ L, 50 μ L, and 60 μ L of serum in 0.175M Cs₂CdEDTA. The results are shown in Figure 14. All three experiments used the same reagents: 1100 μ L 0.175M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, and X μ L distilled water. NBD was added in the same volume and concentration so that any changes in intensities were due to the difference in serum volumes. The amount of distilled water (X μ L) is dependent on the volume of serum that is added to the UC tubes. The distilled water and serum total must be equal to a volume of 80 μ L. Therefore, for a 6 μ L serum sample, the volume of distilled water must be equal to 74 μ L.

After a six-hour ultracentrifugation spin at 120,000rpm at 5°C, a huge increase in intensities was noticeable for all lipoproteins between the 6 μ L and the 50 μ L runs, but not so much between the 50 μ L and 60 μ L runs. In fact, there was a huge decrease in the dense HDL intensity in the 50 μ L and 60 μ L runs. Both the buoyant and dense HDL fractions were higher in intensities in the 50 μ L spins; therefore, all immunoprecipitation will be run with 50 μ L serum.

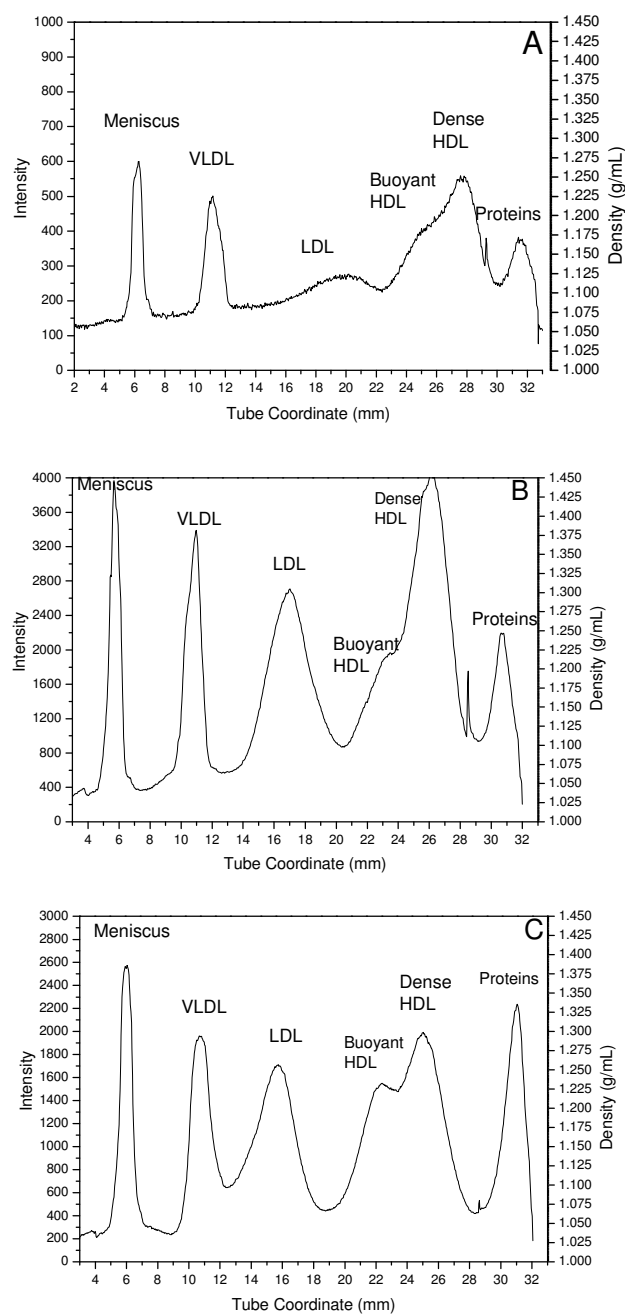


Figure 14: Ultracentrifugation profiles showing volume differences using 6µL (A), 50µL (B), and 60µL (C) of human serum. Each sample was spun at 120,000rpm for 6 hours at 5°C in 1100µL of 0.175M Cs₂CdEDTA, 10µL of 1mg/mL NBD C₆-ceramide, and 74µL (A), 30µL (B), and 20µL (C) distilled water.

Influence of 1mM HCl on Lipoprotein Profiles

A major step in the immunoprecipitation experiment is the recovery of the captured lipoproteins. As stated in Chapter I, antigen retrieval will be done with a technique utilizing a change in pH. [44] Because the immunoprecipitation reactions were run in neutral and basic solutions, a change to an acidic pH opened the “Y” and released the antigen from the antibody.

Using the acid as the antigen retrieval solution, one should be certain that only the antigens will be removed from the precipitate. Therefore, 100mL of 1mM HCl was added to 0.05g pre-swollen and washed Sepharose beads without the apo C-1-antibody. The beads were pre-swollen and washed so that no absorption of acid would be seen; i.e. volume of acid in will equal volume of solution out. Data was collected by recording the absorbance at 280nm. Table 4 below shows the results of the experiment.

Table 4: Effect of 1mM HCl on Sepharose Beads without Antibody	
	ABS at 280nm
1mM HCl (Blank)	0.000
1mM HCl after Sepharose addition	0.077

As seen in Table 4, the addition of 1mM HCl to the Sepharose beads brought a little amount of impurities off the beads. If the acid had little effect on the beads, what

effect will the acid have on lipoproteins? To test this, 50 μ L serum was added to 80 μ L distilled water or 80 μ L 1mM HCl in addition to 1100 μ L 0.175M Cs₂CdEDTA and 10 μ L 1mg/mL NBD. Each tube was mixed for 2 minutes and allowed to incubate for 30 minutes before the UC spin. (See Figure 15A and 15B) To determine if the acid affected any lipoprotein subclasses (bTRL, dTRL, LDL-1, LDL-2, LDL-3, LDL-4, LDL-5, HDL-2b, HDL-2a, HDL-3a, HDL-3b, and HDL-3c) [5], we decided to compare the coefficient of variation (CV) for all lipoprotein subclasses. This was done by taking the standard deviation divided by the average and multiplying by 100. A high CV means a high variation in lipoprotein subclasses, thus showing a change in lipoprotein structure due to acid digestion. (See Table 5)

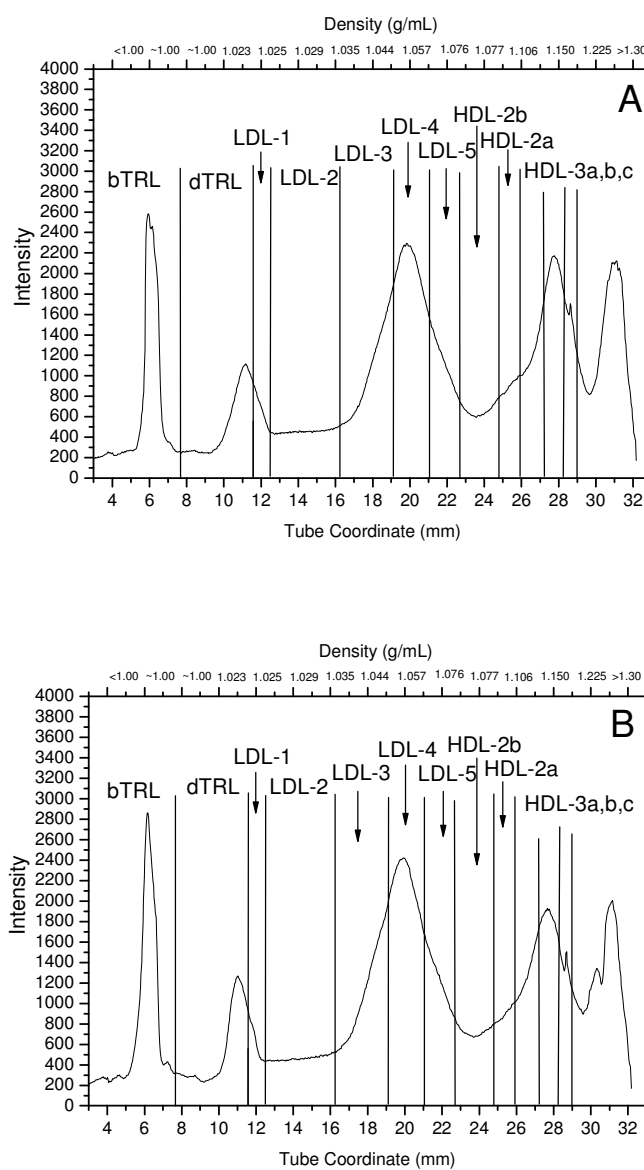


Figure 15: Ultracentrifugation profiles depicting effects of HCl on serum. UC profiles show (A) serum + 80 μ L dH₂O and (B) serum + 80 μ L 1mM HCl with 1100 μ L 0.175M NaBiEDTA and 10 μ L 1mg/mL NBD C₆-ceramide. The UC spin was performed at 120,000 rpm at 5°C for 6 hours.

Table 5: Relative Intensities, Averages, Standard Deviation, and the Coefficient Variation for Figure 15 [5]

	Serum + dH ₂ O	Serum + HCl	Average	Std Dev	CV
bTRL	2999.867	3399.673	3199.77	282.7055	8.835183
dTRL	1939.28	2059.502	1999.391	85.00979	4.251784
LDL-1	668.317	557.118	612.7175	78.62957	12.83292
LDL-2	1723.393	1765.312	1744.3525	29.64121	1.699267
LDL-3	2869.306	3076.634	2972.97	146.603	4.931198
LDL-4	4092.985	4220.742	4156.8635	90.33784	2.173221
LDL-5	1807.865	2068.403	1938.134	184.2282	9.505441
HDL-2b	1364.946	1587.101	1476.0235	157.0873	10.6426
HDL-2a	976.871	989.161	983.016	8.690342	0.884049
HDL-3a	1691.838	1644.919	1668.3785	33.17674	1.988562
HDL-3b	2161.459	2007.024	2084.2415	109.202	5.239414
HDL-3c	1000.647	876.401	938.524	87.85519	9.360995

Influence of 37°C on Recovered Lipoprotein Profiles

Along with a change in pH, heat also has an effect on the recovery of lipoproteins from antibodies. Compared to room temperature retrieval, will adding heat have any influence on increasing the intensities of the recovered lipoprotein profiles? To test this theory, 600 μ L 1mM HCl was added to a freshly reacted Sepharose-AB-Lipoprotein sample and heated to 37°C for 5 hours while mixing on an orbital shaker at 1400rpm. The Sepharose-AB beads were allowed to settle and the supernatant was collected and analyzed by ultracentrifugation in 0.175M Cs₂CdEDTA for 6 hours at 5°C at a speed of 120,000rpm. (See Figure 16A and 16B)

As seen in the data provided, heating the samples, along with changing the pH of antigen retrieval solution, increases the chances of releasing any lipoprotein that was captured during the binding process. Because it was shown that a change in pH and a change in heat hardly affected the lipoproteins, all clinical samples will undergo this combined antigen retrieval method.

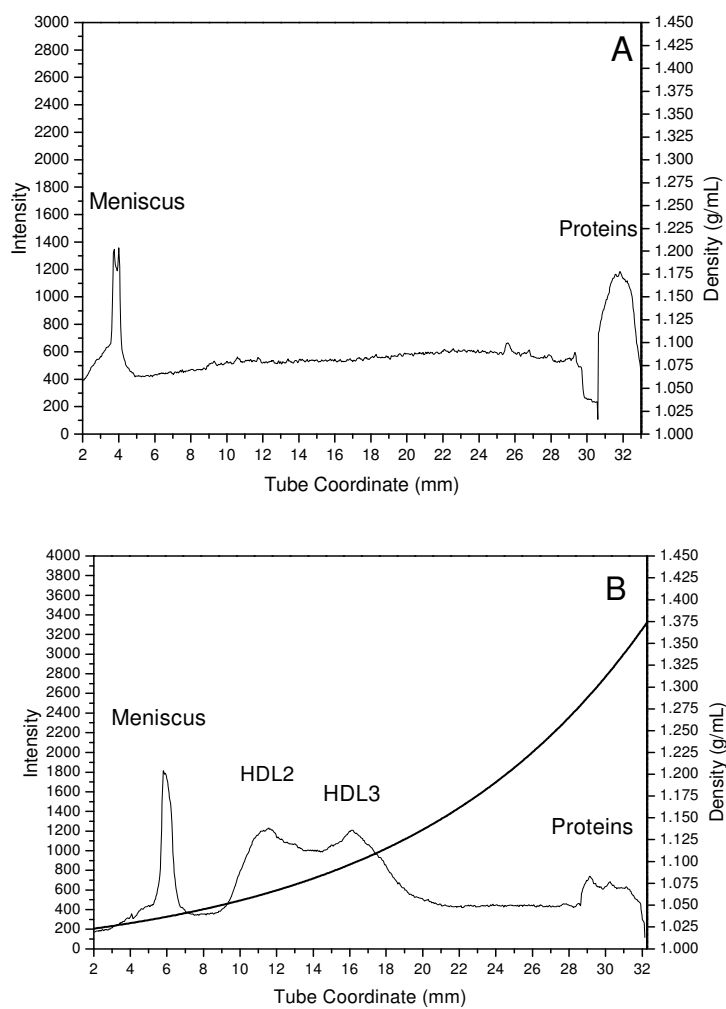


Figure 16: Ultracentrifugation profiles of recovered lipoproteins using antigen retrieval methods such as changes in pH and temperature. UC profiles show 1mM HCl for (A) 1 hour at Room Temperature vs. (B) 5 hours at 37°C. Profiles contained 50 μ L serum, 1100 μ L 0.175M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide and 600 μ L 1mM HCl. Each spin was performed at 5°C for 6 hours at 120,000rpm.

Reproducibility of CNBr-activated Sepharose Gel

In order to increase proficiency with clinical trials, a test to determine whether the AB-Sepharose gel prepared could withstand more than one full experiment—addition of serum for 4 hours and then the recovery process—needed to be run. To test this, an experiment was run using the same Sepharose-AB mixture and a patient's serum twice through. See Figure 17.

As one can see, the AB-Sepharose gel took to both experiments well. The meniscus and VLDL peaks vary because of the human error that was introduced into the sample when the UC tubes were layered with 200 μL distilled H_2O . Because both LDL and HDL profiles are identical, this informs us that we can run multiple samples with the same AB-Sepharose gel without having to make fresh gel for each trial. This will help to save time, money, and resources.

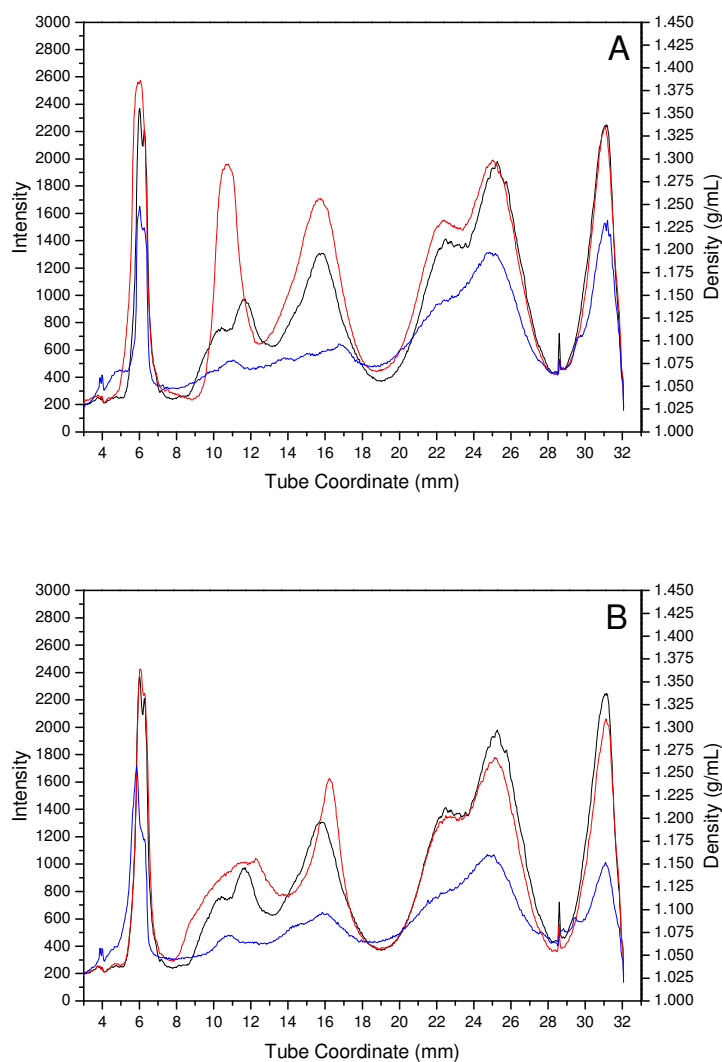


Figure 17: Ultracentrifugation profiles showing reproducibility of CNBr-activated sepharose gel. First (A) and Second (B) uses of Anti-Apolipoprotein C1-Sepharose gel.

Profiles each show a baseline (black), depleted lipoproteins (blue), and recovered lipoproteins (red). Each UC spin was performed with 1100 μ L 10%NaBiEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant at 120,000rpm at 5°C for 6 hours.

CHAPTER IV

APPLICATION: APOLIPOPROTEIN C-1 CHARACTERIZATION IN CASE STUDIES

The purpose of this chapter is to demonstrate the applicability of the methods developed during the course of this research. The protocols used in these samples were the same for all of them and were as detailed in Chapter II, the Experimental Section.

Initial scientific interest in the study of apolipoprotein C1 enriched HDL started with a collaboration with Peter O. Kwiterovich, Jr. and Johns Hopkins involving small gestational age (SGA) infants. [17] After this research was deemed successful in the study of CVD in infants, it was asked as to whether or not the same apolipoprotein C1-enriched HDL can be found in adults. The main goal of this project was to develop a rapid, easy-to-use method for the characterization of Apo C-1 distribution by density. The methods developed in this research were applied to a pilot clinical study involving patients from Scott & White Clinic in Temple, TX. Criteria for each patient included: (1) normal or high HDL, (2) normal LDL, and (3) classification as either CVD or non-CVD as determined by a team of doctors at Scott & White Clinic in Temple, TX. Patients were classified as either “Control” or “CVD” based on a previous angiogram done at Scott & White Clinic.

Thirty patients were selected based on the above criteria, and their serum underwent immunoprecipitation reactions using the apolipoprotein C1-Antibody.

Baseline serum, depleted serum, and recovered serum samples were separated using the ultracentrifugation spin with fluorescent NBD C₆-ceramide stain. Density profiles were constructed for each sample. Figure 18 through Figure 35 show the density profiles for the baseline, depleted serum, and the recovered lipoproteins for six of the thirty samples. HDL₂ and HDL₃ subfractions are indicated in the profiles.

Apo C-1 Characterization CVD Subject 1,

High HDL₂ and HDL₃ Levels in Lipoprotein Density Profile

Sample 1 was collected from a Cardiovascular patient who is a 75 year old Caucasian female with a family history of CVD. Her cardiac risk factors are fairly minimal, but has had elevated LDL levels of 133 mg/dL. HDL levels for this patient, at the time of the blood draw, were 84 mg/dL.

The lipoprotein density profile of this subject shows a bump in the density region between LDL and HDL—labeled HDL₂. (See Figure 18) Classified as a cardiovascular patient, it is expected that if the HDL₂ and HDL₃ regions are Apo C-1 enriched, these two peaks will attach to the Apo C-1 Antibody on the Sepharose beads and disappear from the depleted serum lipoprotein profile. As seen in Figure 19, what is shown in the supernatant after reaction with the Apo C-1 Antibody is LDL, VLDL and chylomicrons, and serum proteins.

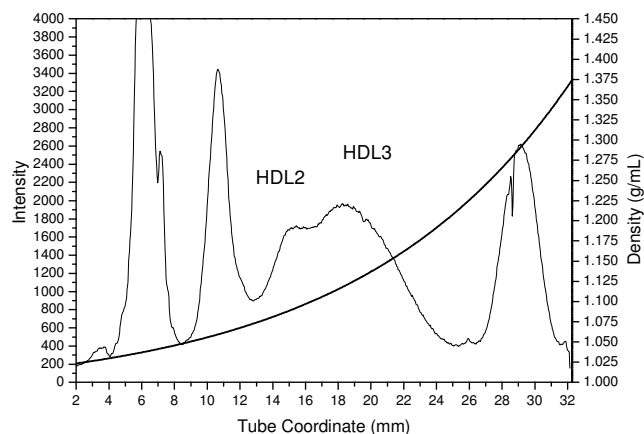


Figure 18: Baseline ultracentrifugation profile for CVD patient 1. Baseline profile shows serum before reaction with the Apo C-1 Antibody-Sepharose beads. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L serum, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours.

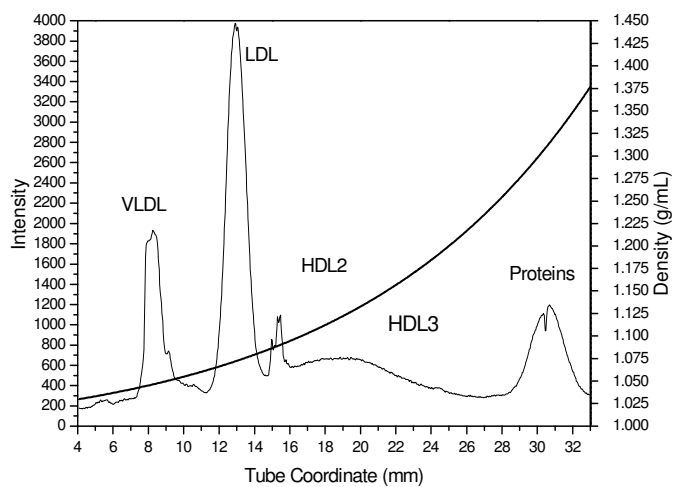


Figure 19: Depleted serum ultracentrifugation profile for CVD patient 1. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L supernatant, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours. The supernatant was collected after reaction with Apo C-1 Antibody-Sepharose beads for 4 hours.

Figure 20 shows the Apo C-1 enriched VLDL, HDL₂ and HDL₃ that is recovered following a 5 hour spin with 1mM HCl wash at 37°C. The lipoproteins that were recovered contained enough Apo C-1 that could attach to the antibodies and be pulled out of solution. By changing pH and temperature, these lipoproteins were released from the antibody and analyzed by ultracentrifugation, and show a perfect case of apo C-1-enriched HDL in human serum. The shift in HDL₂ and HDL₃ in the recovered profile could be due to apo C-1 being pulled off HDL particles. Further analysis and testing, by CE or MALDI-MS, would need to be done to determine if this is indeed what happened.

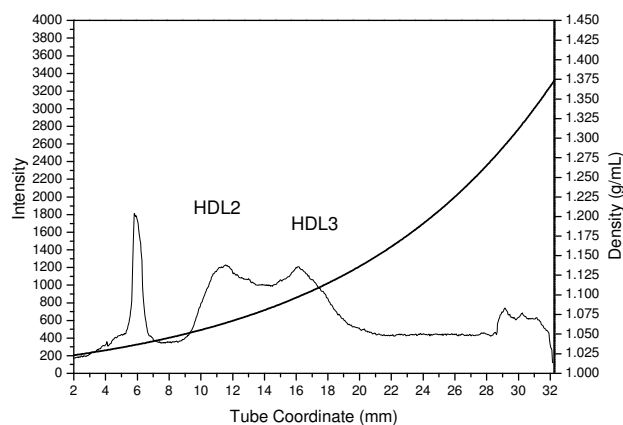


Figure 20: Recovered serum ultracentrifugation profile for CVD patient 1. The recovered lipoprotein profile shows HDL₂ and HDL₃ recovered by antigen retrieval based on change in pH and addition of heat. Each UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant, 40 μ L distilled H₂O at 120,000rpm at 5°C for 6 hours.

Apo C-1 Characterization CVD Subject 2,

High HDL and Lp(a) Levels in Lipoprotein Density Profile

Sample 2 was collected from a cardiovascular Patient 2 that is a 72 year old, Caucasian male with high HDL (>160 mg/dL) and elevated Lp(a) levels—thus resulting in a elevated bump between the LDL and HDL in the baseline profile (Figure 21). This “bump” between the LDL and HDL can be determined to be either Lp(a) or Apo C-1-enriched HDL by using the Apo C-1 Immunoprecipitation procedures discussed in this thesis.

Figure 22 shows the lipoprotein profile for the apolipoprotein C-1 depleted serum of CVD Patient 2. Here, the apolipoprotein C-1 concentrations are noticed in the depleted volume of HDL₂ and HDL₃, but not in the VLDL or serum proteins.

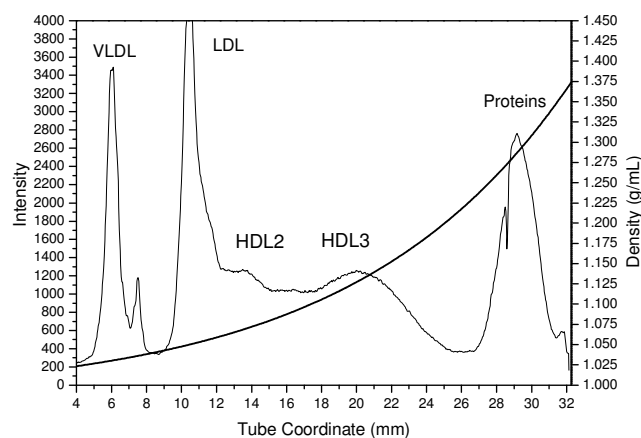


Figure 21: Baseline ultracentrifugation profile for CVD patient 2. Baseline profile shows serum before reaction with the Apo C-1 Antibody-Sepharose beads. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L serum, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours.

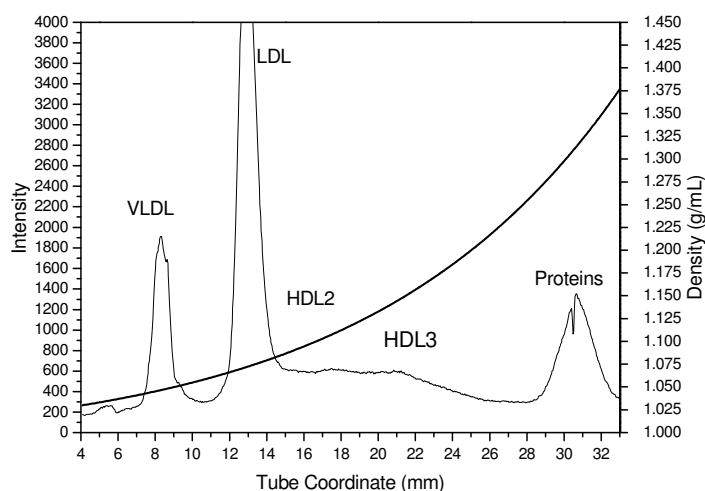


Figure 22: Depleted serum ultracentrifugation profile for CVD patient 2. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L supernatant, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours. The supernatant was collected after reaction with Apo C-1 Antibody-Sepharose beads for 4 hours.

As seen in Figure 23, when the solution's pH and temperature was changed, HDL₂, HDL₃, VLDL, and serum proteins are recovered. Recalling from Chapter I, Apo C-1 can be found in Chylomicrons, VLDL, HDL, and serum proteins. This particular patient helps to show that Apo C-1, which is being pulled from multiple proteins in human serum, can be easily accessible to the Apo C-1 antibodies and/or is enriched on one or more particular lipoproteins. Although this patient has high levels of Lp(a) in his serum, it should be noted that there is also apo C-1-enriched HDL, as seen in the recovered lipoprotein profile, which may attributing to his heart conditions.

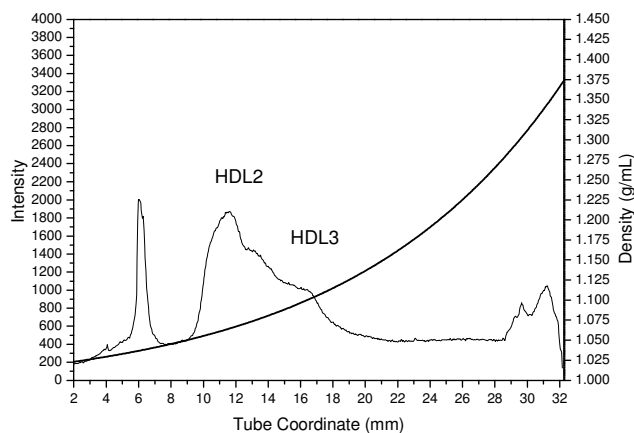


Figure 23: Recovered serum ultracentrifugation profile for CVD patient 2. The recovered lipoprotein profile shows HDL₂ and HDL₃ recovered by antigen retrieval based on change in pH and addition of heat. Each UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant, 40 μ L distilled H₂O at 120,000rpm at 5°C for 6 hours.

Apo C-1 Characterization CVD Subject 3,

High Levels of HDL in Lipoprotein Density Profile with a History of CVD

Sample 3 was collected from a cardiovascular male subject in his fifties with high LDL levels (>160 mg/dL). Although Patient 3 has high levels of HDL, see Figure 24, these levels have not been enough to prevent significant cardiovascular disease. This warning flag makes him a perfect candidate for the Apolipoprotein C-1 Immunoprecipitation protocol.

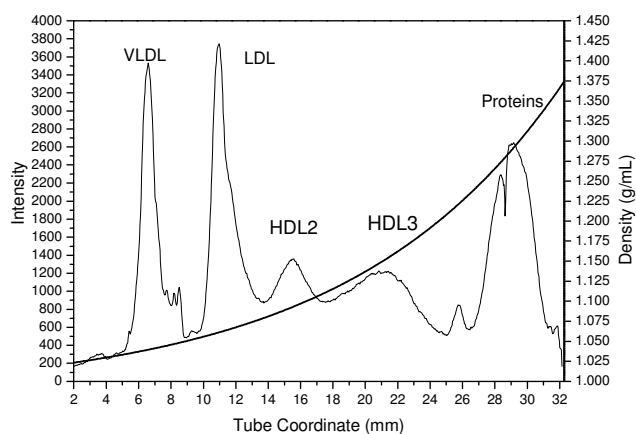


Figure 24: Baseline ultracentrifugation profile for CVD patient 3. Baseline profile shows serum before reaction with the Apo C-1 Antibody-Sepharose beads. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L serum, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours.

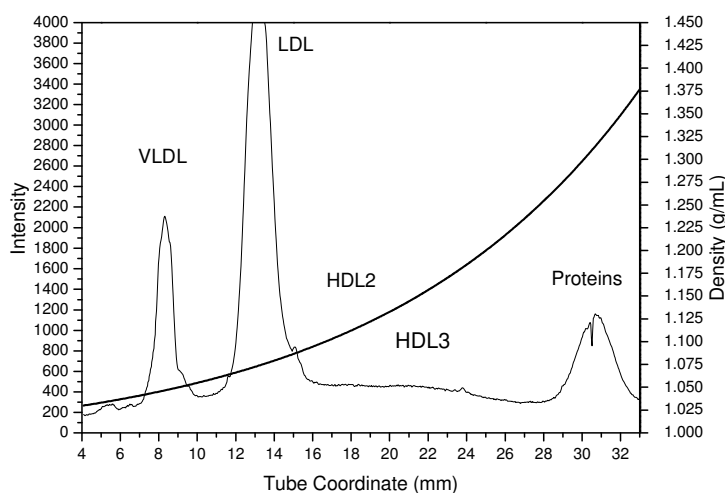


Figure 25: Depleted serum ultracentrifugation profile for CVD patient 3. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L supernatant, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours. The supernatant was collected after reaction with Apo C-1 Antibody-Sepharose beads for 4 hours.

As seen in Figure 26, by changing the solution's pH and temperature, HDL₂ and HDL₃ is recovered in tact and with the same density peaks and separation as seen above in his baseline profile, Figure 24. The shift in HDL₂ and HDL₃ in the recovered profile could be due to apo C-1 being pulled off HDL particles. Further analysis and testing, by CE or MALDI-MS, would need to be done to determine if this is indeed what happened.

The recovery of the HDL₂ and HDL₃ helps to conclude this patient is a CVD subject due to the apo C-1-enriched HDL being recovered versus the depleted-serum profiles showing no apo C-1 being recovered in the HDL regions.

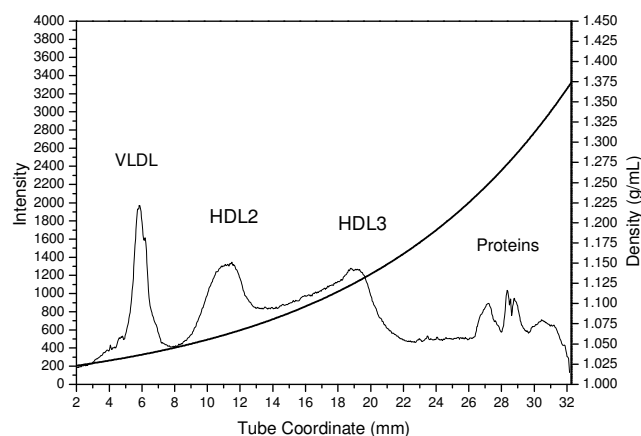


Figure 26: Recovered serum ultracentrifugation profile for CVD patient 3. The recovered lipoprotein profile shows HDL₂ and HDL₃ recovered by antigen retrieval based on change in pH and addition of heat. Each UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant, 40 μ L distilled H₂O at 120,000rpm at 5°C for 6 hours.

Apo C-1 Characterization Control Subject 1,**High Levels of HDL₃ in Lipoprotein Density Profile with a History of CVD**

Sample 4 was collected from a “control patient” who is a 58 year old Caucasian female with low HDL levels (<35 mg/dL) and a family history of CVD. Her LDL levels were between 130-160 mg/dL. As seen in her baseline profile, Figure 27, there is no “bump” between the LDL and HDL indicating no Lp(a) or HDL₂. It is her family history and recent angioplasty by Scott & White in Temple, TX, that has placed her as a control in this study.

Figure 28 shows the lipoprotein profile for the apolipoprotein C-1 depleted serum of sample 4. Here, the VLDL, LDL, HDL, and serum proteins all remain intact, showing no apo C-1-enriched particles that could be attached to the apo C-1-AB-Sepharose beads. As seen in Figure 29, when we change the solution’s pH and temperature, we recover no lipoproteins. This particular patient helps to showcase that with a family history of CVD; there might be no other risk factors for CVD.

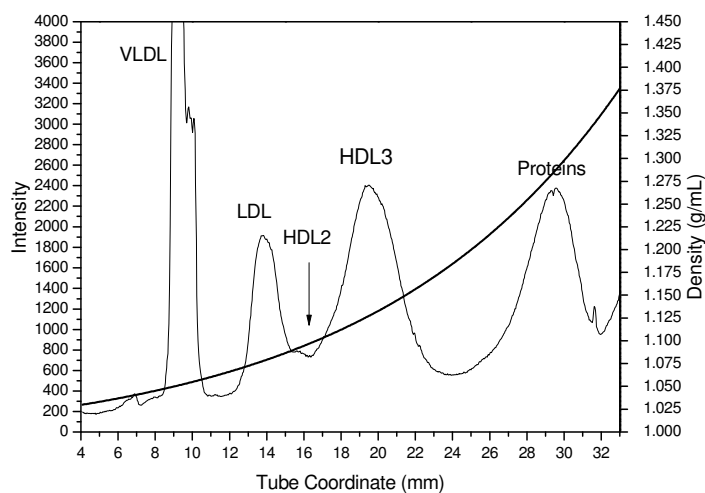


Figure 27: Baseline ultracentrifugation profile for control patient 1. Baseline profile shows serum before reaction with the Apo C-1 Antibody-Sepharose beads. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L serum, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours.

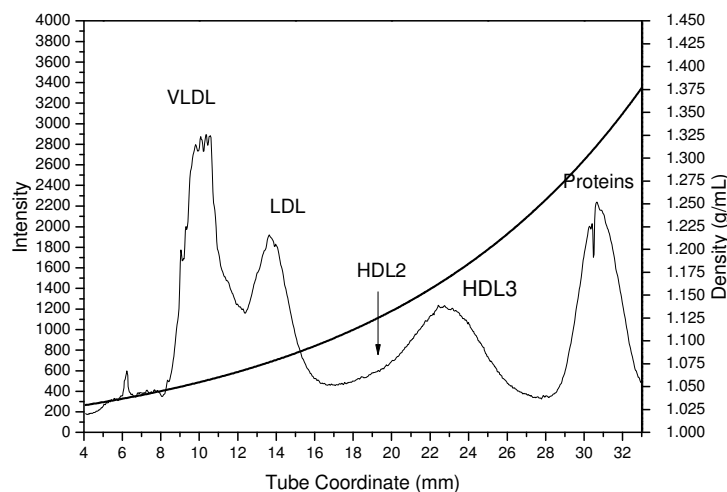


Figure 28: Depleted serum ultracentrifugation profile for control patient 1. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L supernatant, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours. The supernatant was collected after reaction with Apo C-1 Antibody-Sepharose beads for 4 hours.

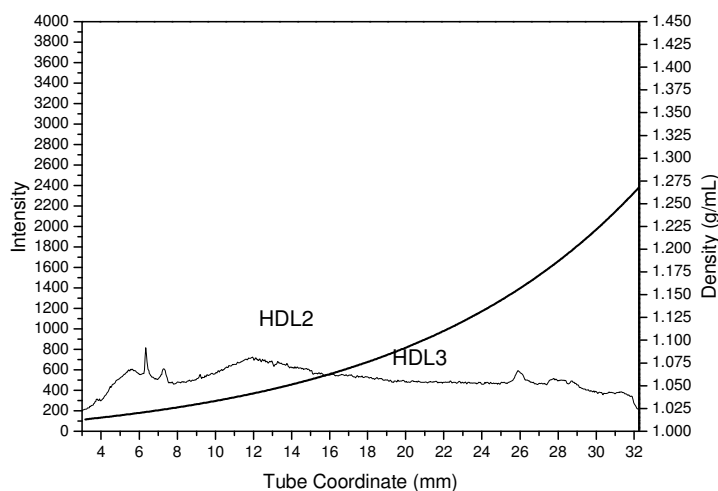


Figure 29: Recovered serum ultracentrifugation profile for control patient 1. The recovered lipoprotein profile shows HDL₂ and HDL₃ recovered by antigen retrieval based on change in pH and addition of heat. Each UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant, 40 μ L distilled H₂O at 120,000rpm at 5°C for 6 hours.

Apo C-1 Characterization Control Subject 2,

Low Levels of HDL in Lipoprotein Density Profile with a History of CVD

Sample 5 was collected from a “control patient” who is a 47 year old female with a family history of CVD. This Caucasian female is a smoker with high LDL levels greater than 160 mg/dL. In previous years, her HDL has fallen below 35 mg/dL. As seen in her baseline profile, Figure 30, HDL₂ connects LDL and HDL₃. Her risk factors—family history, smoking, high LDL levels, and low HDL levels—have placed her in this study.

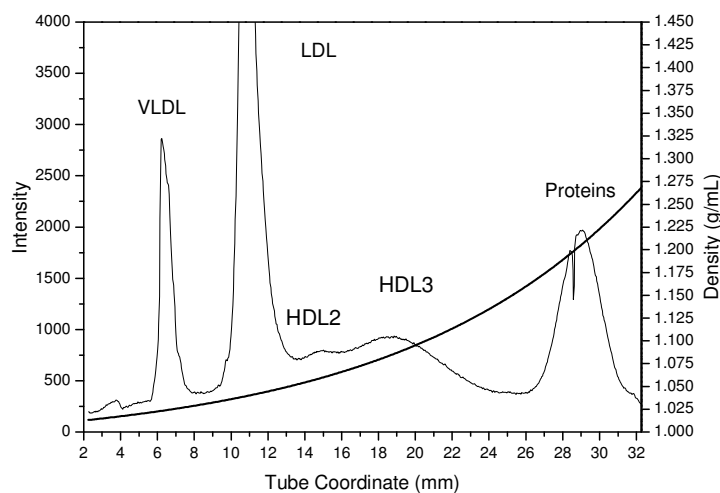


Figure 30: Baseline ultracentrifugation profile for control patient 2. Baseline profile shows serum before reaction with the Apo C-1 Antibody-Sepharose beads. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L serum, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours.

When studying the apo C-1-depleted lipoprotein profile, the VLDL, LDL, HDL, and serum proteins all remain intact, although intensity and placement of the LDL has changed; there are no apo C-1-enriched particles that could attach to the apo C-1-AB-Sepharose beads. (See Figure 31) As seen in Figure 32, when we change the solution's pH and temperature, we recover no lipoproteins. This particular patient helps to showcase that although there is a family history of CVD and high levels of "bad" LDL, apo C-1-enriched HDL might not be to blame for this patient's heart problems.

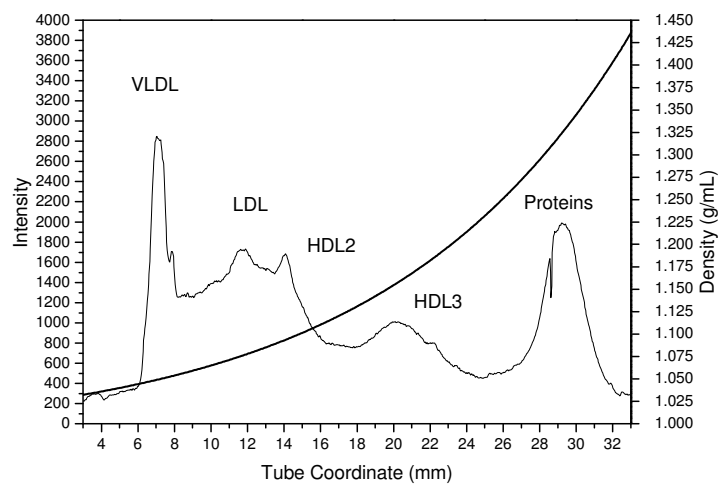


Figure 31: Depleted serum ultracentrifugation profile for control patient 2. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L supernatant, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours. The supernatant was collected after reaction with Apo C-1 Antibody-Sepharose beads for 4 hours.

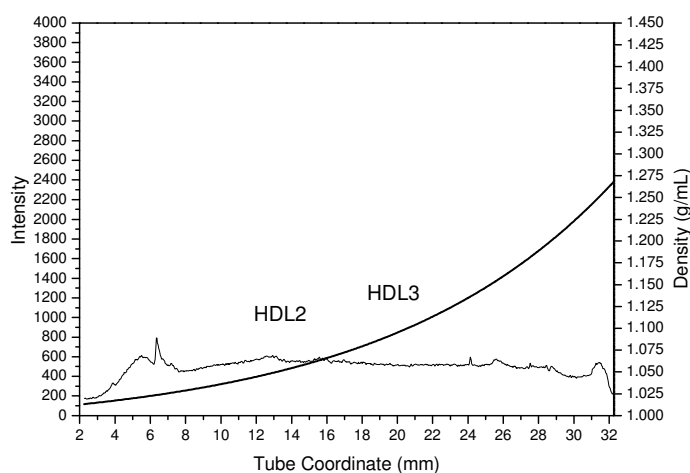


Figure 32: Recovered serum ultracentrifugation profile for control patient 2. The recovered lipoprotein profile shows HDL₂ and HDL₃ recovered by antigen retrieval based on change in pH and addition of heat. Each UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant, 40 μ L distilled H₂O at 120,000rpm at 5°C for 6 hours.

Apo C-1 Characterization Control Subject 3,

Low Levels of HDL₂ and HDL₃ in Lipoprotein Density Profile

Sample 6 was collected from a “control patient” who is a 42 year old Caucasian, male with no family history of CVD. He has high levels of LDL and shows normal HDL levels in his baseline profile, Figure 33. It is his “bump” between the LDL and HDL and his non-apparent risk factors that has placed him in this study.

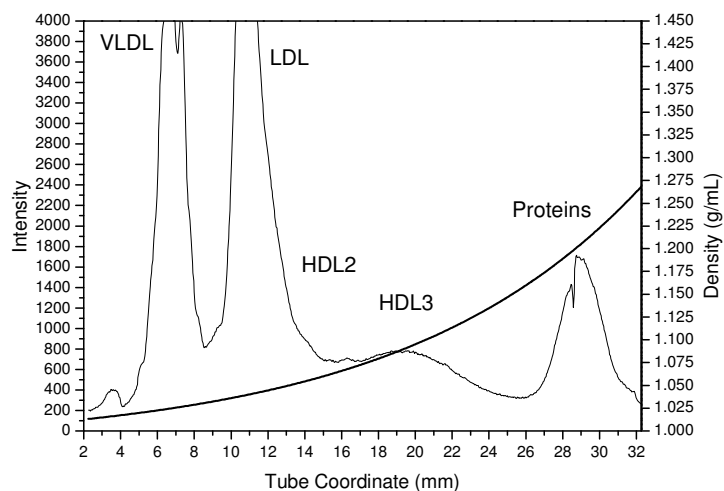


Figure 33: Baseline ultracentrifugation profile for control patient 3. Baseline profile shows serum before reaction with the Apo C-1 Antibody-Sepharose beads. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L serum, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours.

When studying the apo C-1-depleted lipoprotein profile, the VLDL, LDL, HDL, and serum proteins all remain intact, although the VLDL intensity has decreased slightly and tube placement has changed; there are no HDL apo C-1-enriched particles that could attach to the apo C-1-AB-Sepharose beads. (See Figure 34) This shift in placement could indicate an apo C-1 particle being removed from the VLDL, as proven in Figure 35 when solution's pH and temperature was changed, some VLDL was recovered. Sample 6 helps to showcase that although there is a no family history of CVD and low levels of HDL, apo C-1-enriched HDL might not be the cause for this patient's heart condition.

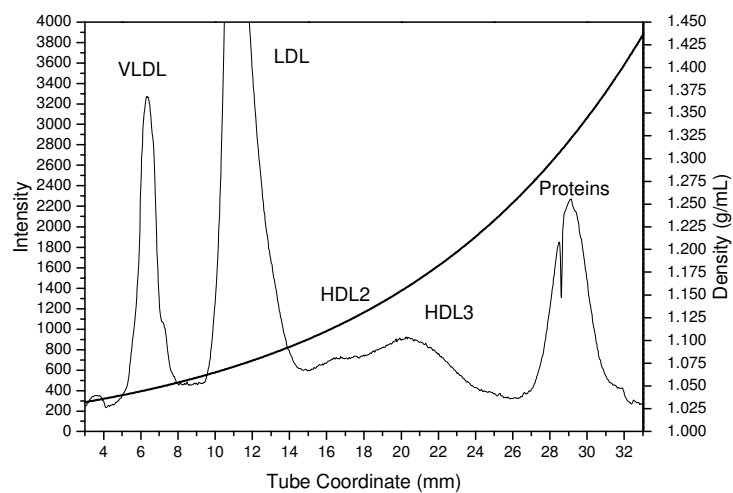


Figure 34: Depleted serum ultracentrifugation profile for control patient 3. The UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1 mg/mL NBD C₆-ceramide, 50 μ L supernatant, and 40 μ L distilled water at 120,000rpm at 5°C for 6 hours. The supernatant was collected after reaction with Apo C-1 Antibody-Sepharose beads for 4 hours.

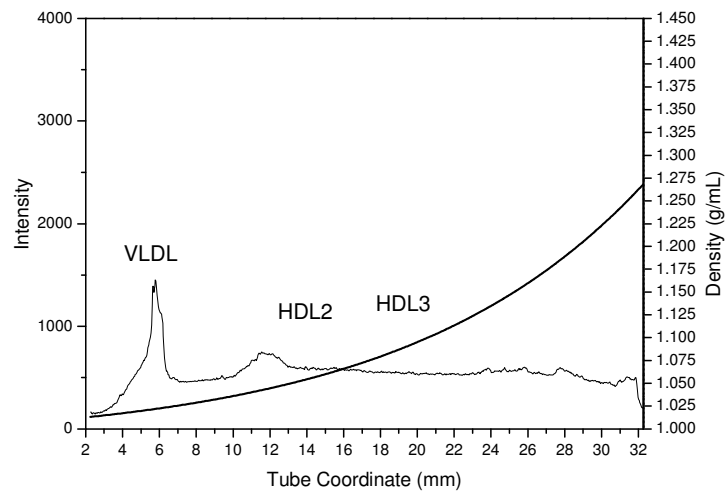


Figure 35: Recovered serum ultracentrifugation profile for control patient 3. The recovered lipoprotein profile shows HDL₂ and HDL₃ recovered by antigen retrieval based on change in pH and addition of heat. Each UC profile contains 1100 μ L 0.3M Cs₂CdEDTA, 10 μ L 1mg/mL NBD C₆-ceramide, 50 μ L serum/supernatant, 40 μ L distilled H₂O at 120,000rpm at 5°C for 6 hours.

CHAPTER V

SUMMARY AND CONCLUSIONS

The study reported here demonstrates the analytical power of linking ultracentrifugation with immunoaffinity separations for the characterization of serum lipoproteins. The primary objective to this research was to develop a rapid method for the separation, purification, and characterization of Apo C-1-enriched HDL from human serum. The objective was met by linking Apo C-1 Antibody with CNBr-activated Sepharose beads and using a density gradient ultracentrifugation method for lipoprotein separation.

Apolipoprotein C-1-enriched HDL was selectively removed from human serum by using a 50 μ L sample of serum that is diluted in TRIS HCl buffer (pH 7.5) and incubated with CNBr-activated Sepharose (Amersham) containing antibodies to apo C-1 (Academy Bio-medical Company). The apo C-1-depleted serum is removed by centrifugation and all apo C-1-containing lipoproteins are released from the Sepharose beads at pH 2. The apo C-1-depleted sample and the apo C-1-containing sample were ultracentrifuged to obtain a lipoprotein density profile in the absence and presence of apo C-1. Density Lipoprotein Profiling (DLP) gives relevant information of lipoproteins such as density and subclass characterization, and is a novel approach to purify apo C-1-enriched HDL. An additional advantage of this approach is that lipoprotein-a (Lp(a)), which is often an interfering component in the HDL density region, is eliminated.

Apolipoprotein C-1 was quantitatively removed from serum and recovered efficiently. All apo C-1 recovered by the methods described in this thesis retained its inherent hydrated density. Apo C-1 was purified by ultracentrifugation, and by utilizing an apo C-1 standard, characterization was achieved by capillary electrophoresis.

The main objective of the research, to show feasibility that these methods could be used in a clinical setting, was achieved. In conclusion, this methodology will yield a precise and quantitative profile of the distribution of apo C-1 for all lipoprotein particles including HDL.

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APPENDIX A

Coupling Antibody to CNBr-Activated Sepharose

This protocol is to be followed to obtain an Antibody-Sepharose gel to be coupled with human serum.

Materials

1mL Eppendorf tubes

Coupling Buffer – 0.1M NaHCO₃

Antibody solution

CNBr-Activated Sepharose

1mM HCl

Blocking Buffer - 1M Tris-Base, pH 9.0 in coupling buffer

M-60 Orbital Shaker

Procedure

1. Using 300 μ L, carefully add antibody solution to a micro well plate and measure absorbance at 280nm to determine protein concentration.
2. In a 1.5mL Eppendorf tube, add 62.5 μ L coupling buffer (0.1M NaHCO₃ and 0.5M NaCl, pH 9.0) to 125 μ L antibody solution.
3. In a second 1.5mL Eppendorf tube, swell the Sepharose beads in 600 μ L 1mM HCl for approx. 15 min at room temperature.

4. Remove supernatant and wash with 600 μ L 1mM HCl solution for 1 min. Vortex tube for 1 minute at 1400rpm, allow gel to settle, and remove supernatant.

Repeat.

**The next two steps should be done with no pausing in between steps 5 and 6.

5. Wash gel with 1mL coupling buffer. Vortex tube for 1 min, allow gel to settle and remove supernatant.
6. Using a clean pipette tip, transfer the antibody solution to the tube containing Sepharose beads. Vortex the samples briefly to homogenize the solution.
7. Rotate the tube gently using the M-60 Orbital shaker set to 13,000rpm at room temperature for 4 hours.
8. Spin down beads using a table-top centrifuge for 1min at 2000rpm. Remove supernatant and take OD₂₈₀. If OD₂₈₀ is at least 10-fold lower than in step 1, continue with procedure. If OD₂₈₀ is not at least 10-fold lower than in step 1, coupling did not proceed as expected. (Mainly caused by not transferring gel to antibody solution quickly enough.)
9. Wash Sepharose beads with 1mL coupling buffer. Vortex tube for 1 min, allow gel to settle and remove supernatant. Repeat twice.
10. Add 1mL blocking buffer (1M Tris-Base, pH 9.0 in coupling buffer). Vortex tube for 1 minute and allow to stand for 2 hours at room temperature.
11. Remove the supernatant and wash the gel 4 times, alternating between low pH wash buffer (0.1M HCl, 0.5M NaCl) and high pH wash buffer (coupling buffer).

(2x low pH and 2x high pH) The first and fourth washes were saved and the OD₂₈₀ of each was checked. (The OD₂₈₀ readings should be below ~0.01)

12. After removal of the supernatant, the gel is washed with Coupling buffer and is ready to be coupled with serum.

Coupling Serum to AB-Sepharose Beads

This protocol is to be followed to obtain a lipoprotein-depleted serum sample and a lipoprotein-recovered serum sample.

Materials

1.5mL Eppendorf tubes

Coupling Buffer - 0.1M NaHCO₃, 0.5M NaCl, pH 8.3

1mM HCl

Procedure

1. Obtain AB-Sepharose gel that was freshly made.
2. Mix 50 μ L serum, 10 μ L 1mg/mL NBD, and 600 μ L 0.05M Tris-HCl in a 1.5mL Eppendorf tube.
3. Wash gel with 1mL coupling buffer (0.1M NaHCO₃, 0.5M NaCl, pH 8.3).
Vortex for 1 min, allow the gel to settle and remove supernatant.
4. Using a clean pipette tip, transfer the serum mixture into the tube containing the AB-Sepharose beads.
5. Gently rotate the tube for 4 hours using a M-60 Orbital shaker set to 14,000rpm at room temperature.

6. Allow gel to settle for approx. 15 min. Remove supernatant (~600 μ L). Wash gel with coupling buffer prior to recovery.
7. To recover lipoproteins bound to the Sepharose-Antibody gel, add 600 μ L 1mM HCl to the Eppendorf tube. Gently rotate the tube at 37°C at 1400rpm for 5 hours.
8. Allow gel to settle for approx. 15 minutes. Remove the supernatant (~600 μ L). Wash the gel in coupling buffer and store in 20% ethanol to prevent microbial growth.

Concentration of Depleted and Recovered-Lipoprotein Samples

This protocol is used to concentrate lipoprotein samples prior to ultracentrifugation.

Materials

3,000 molecular weight cut-off filter

Table top centrifuge

1.5mL Eppendorf tubes

Density gradient

Procedure

1. Transfer 300 μ L of the depleted serum solution to a 3,000 molecular weight cut-off filter.
2. Centrifuge for 3 minutes at 6,000 rpm. Discard filtrate (bottom chamber).
3. Add 400 μ L of density gradient. Repeat until initial sample volume has been replaced 2.5 times.

4. Concentrate sample by centrifuging for 7 minutes at 6,000 rpm.
5. Recover depleted-serum sample by inverting the top chamber of the filter into a clean Eppendorf tube and centrifuging for 8 minutes at 10,000 rpm.
6. 50 μ L of the concentrated supernatant, 40 μ L dH₂O, 10 μ L 1mg/mL NBD and 1100 μ L 0.175M Cs₂CdY were mixed and allowed to stain for 30 minutes.
7. Aliquot 1000 μ L solution to an ultracentrifuge tube. Follow UC protocol established for the density gradient used. For 0.3M Cs₂CdEDTA, tubes were spun at 120,000rpm at 5°C for 6 hours.
8. After the ultracentrifugation spin, tubes were removed, layered with 200 μ L of dH₂O, and digitally imaged.

Imaging of UC tubes for Apo C-1-Enriched HDL

1. Use the Optronics Microfire Camera (System II) using the BG12 filter for NBD excitation and OG515 for emission.
2. Increase the exposure time from 15.8 ms to 50 ms. This will increase the Lp(a) peak fluorescent intensity. Keep the exposure time at 15.8 if sample is to be compared with a lipoprotein density profile.
3. All other settings are to be kept the same. Target intensity of 30%, Gain of 1.000, MONO and 12 Bit Snap adjustment.
4. Take the picture, save the picture, and obtain a lipoprotein profile using Origin 7.0

VITA

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During her graduate studies, she developed methods for the analysis, characterization and removal of apolipoprotein C-1 in human serum, including separation by immunoaffinity, purification by ultracentrifugation and characterization by capillary electrophoresis. She defended her research in June 2008, and received her Master of Science degree in December 2008.

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