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High-density lipoproteins (HDL) are complex particles composed of specific proteins and lipids that facilitate blood and tissue cholesterol homeostasis by transporting excess peripheral cholesterol to the liver. In association with cholesteryl ester transfer protein (CETP) and the enzyme, lecithin: cholesterol acyltransferase (LCAT), HDL contributes to the transport of hydrophobic lipids, including cholesteryl ester and triglycerides through the blood. The studies presented here involve the evaluation of horse serum HDL as a carrier of water insoluble drugs and an improved process to isolate and purify horse serum HDL utilizing hydrophobic affinity chromatography.

Dilauryl fluorescein (DLF) has been chosen as a model compound for the study of horse HDL as a drug carrier. The prepared HDL/DLF particles have similar flotation densities and size properties to native horse serum HDL. The amount of DLF incorporated into HDL is 30μg/mg protein. Various cancer cell lines internalized DLF from horse HDL/ DLF particles successfully. While human plasma contains cholesterol ester transfer protein (CETP), horse plasma does not. Horse plasma/ serum can be supplemented by human plasma to study the role of CETP in drug transport and the stability of the horse HDL/drug complex.

HORSE SERUM HIGH DENSITY LIPOPROTEINS AS DRUG TRANSPORTERS.

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HORSE SERUM HIGH-DENSITY LIPOPROTEINS (HDL) AS DRUG TRANSPORTERS

THESIS

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Introduction

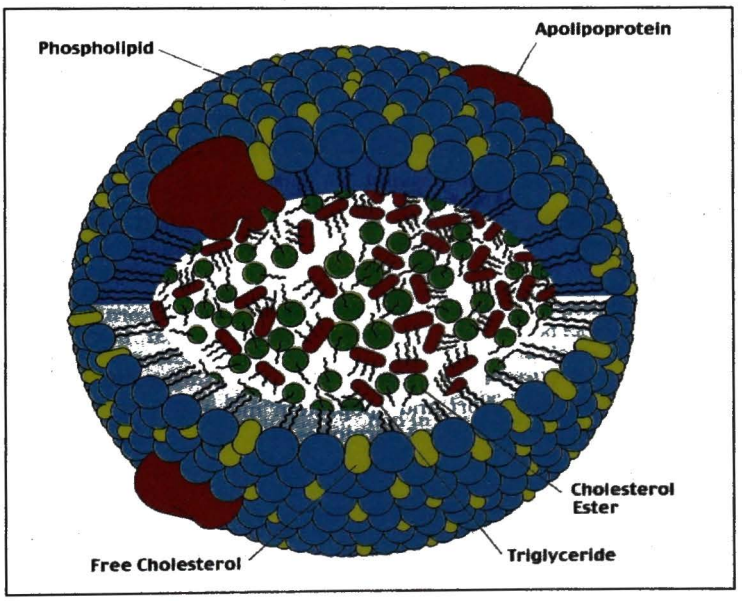
Lipoproteins

Lipoproteins are spherical particles with an outer shell of amphipathic components consisting of free cholesterol, phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin), and apolipoproteins, while their inner core contains cholesteryl esters, triglycerides, or both. Lipoproteins transport water insoluble molecules in the body. Apolipoproteins act as structural lipoprotein components, cofactors for enzymes in lipoprotein metabolism and they facilitate the interaction of lipoprotein particles with target cells receptors in specific tissues (37). There are four basic classes of lipoproteins that can be separated by preparative ultracentrifugation, based on their respective buoyant densities. Lipoproteins also have varying size (diameters). The two larger classes are chylomicrons and very low-density lipoproteins (VLDL) that transport triglycerides. The two smaller lipoproteins low-density lipoprotein (LDL) and high-density lipoproteins (HDL) transport cholesterol esters and a small amount of triglycerides. There is also a fifth, intermediate density lipoprotein (IDL) class that is usually a very minor component of normal/healthy plasma. The major apolipoprotein of HDL is Apo AI. There are at least nine apolipoproteins in human lipoproteins, which are differentiated by their structure, function and contribution to lipoprotein deficiency syndromes and dyslipemia.

High Density Lipoproteins

High-density lipoprotein (HDL) is a key component in cholesterol metabolism and transport, as it carries excess cholesterol from peripheral tissues to the liver via reverse cholesterol transport. HDL is synthesized in the liver and in the small intestine. Some of the HDL precursors (pre-β HDL) are generated from chylomicron or VLDL remnants. These precursor molecules are converted to circulating (mature) HDL particles by the action of the enzyme lecithin: cholesterol acyltransferase (LCAT) to generate larger lipoproteins rich in cholesteryl esters.

<u>Figure 1</u> shows a schematic structure of lipoproteins with an outer shell and an inner core. The outer shell has detergent properties, relatively hydrophobic and the inner core contains highly water insoluble components (triglyceride and cholesterol esters).

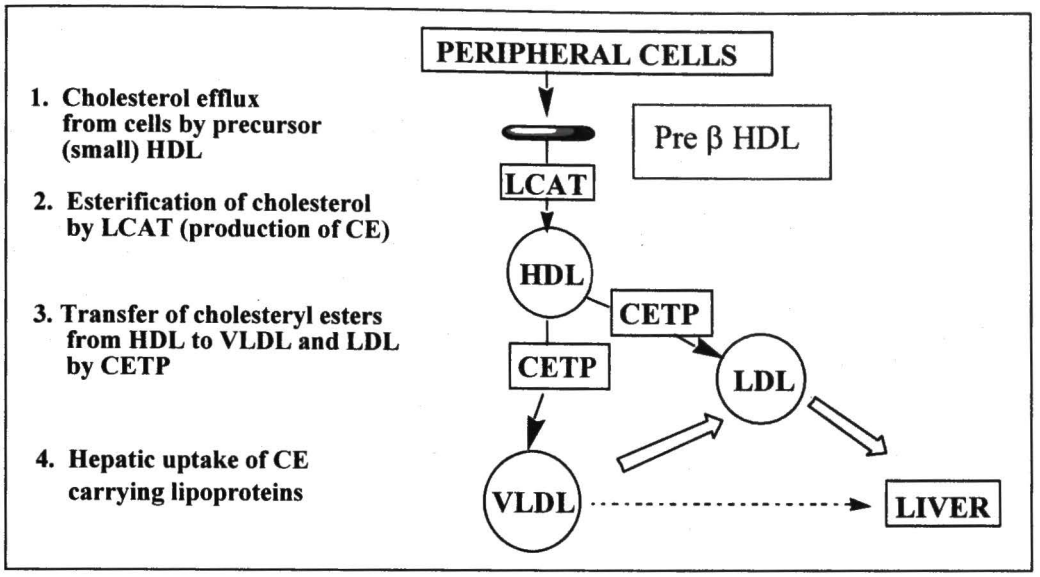


(Weinberg RB. Hosp. Pract. (1987) 22:223-7)

Human plasma HDL contains a number of apoproteins, Apo AI, Apo AII, Apo AIV, Apo CI, Apo CII, Apo CIII, Apo D, Apo E, and Apo F. It also contains a number of enzymes to facilitate its maturation, including phospholipid transfer protein (PLTP), lecithin: cholesterol acyltransferase (LCAT), and cholesteryl ester transfer protein (CETP).

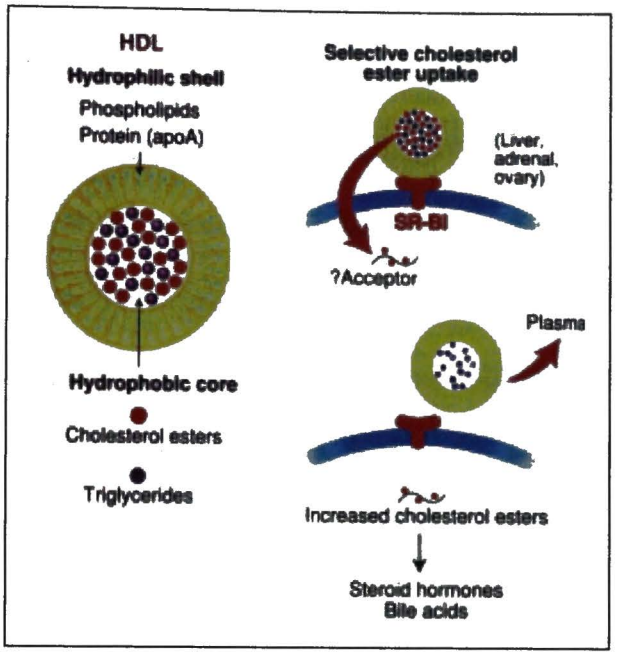
The liver is the only organ that is able to convert cholesterol to bile acids. Consequently, it is the recipient of most of the excess cholesterol in mammals. HDL carries excess cholesterol from peripheral cells to the liver facilitated by the action of LCAT, which converts cholesterol and phosphatidylcholine to cholesterol ester and lysophosphatidylcholine (lyso PC). In this reaction, LCAT transfers the fatty acid from the C-2 position of lecithin to the C-3-OH of cholesterol. The cholesteryl ester produced accumulates in the inner core of the growing (maturing) HDL particle and is subsequently transported to the liver where it can serve as a component of *de novo* hepatic lipoprotein synthesis or as a precursor for bile acids to be incorporated and subsequently excreted in the bile. In serum, the cholesteryl ester in the core of HDL is transferred to Apo B containing lipoproteins, while triglycerides are exchanged for cholesterol ester by CETP making HDL less dense (Figure 2).

Figure 2. Steps involved in human reverse cholesterol transport.



The Apo B containing lipoproteins then transport the cholesteryl esters to the peripheral cells or to the liver. The liver and peripheral cells incorporate cholesteryl esters from the apo B containing lipoproteins via receptor-mediated endocytosis. On the other hand the removal of cholesteryl esters from HDL occurs without the uptake of HDL itself but instead via a separate (SR-BI) receptor system (figure 3). Most of the HDL cholesterol is taken up as cholesteryl esters by a scavenger receptor BI (SR-BI) protein. This allows the selective uptake of cholesteryl esters into cells that express the SR-BI receptor. The rest of the HDL is then sent back into the bloodstream where it interacts with remnants from chylomicrons and VLDL. The cholesterol depleted HDL can then take up cholesterol once again from stored extrahepatic tissues.

Figure 3. The selective uptake of cholesterol esters by an SR-BI receptor with the release of HDL.



(Steinburg D. Science, 1996 271:460-1)

HDL from Horse Plasma

In the horse, HDL is the major lipoprotein, Apo AI being the major apolipoprotein component (34) although it also contains Apo E, Apo CII, Apo CIII, and Apo AII (34). The first isolation of horse plasma lipoproteins was by Macheboeuf (1929)(19). It was not until many years later that Robie et al (1975)(21-22) brought attention to horse plasma by describing its lipoprotein profile. Since then horse serum lipoproteins have been investigated by sequential ultracentrifugation, density gradient ultracentrifugation, rate-zonal ultracentrifugation, and agarose gel filtration, to characterize lipids, proteins, size, density limits, and profiles and distribution of lipoproteins in order to define the pathways of horse lipoprotein metabolism (29, 16, 17, 34-36). The molecular weight of

horse plasma HDL is between 86,000-250,000 (17). The composition of horse plasma HDL varies from report to report. The protein is from 40-65%, the phospholipids 19-33%, cholesteryl esters range from 13-20%, the free cholesterol 0.8-5%, and triglyceride from 0.1-8.8% (16, 32, 36). According to several authors (18, 29, 10, 25) there is just a single band of horse plasma HDL, isolated by different types of ultracentrifugation and gel filtration chromatography. There are only negligible amounts of triglycerides in horse HDL. In the HDL range, total cholesterol and phospholipids are higher than in human HDL_2 and HDL_3, and as the HDL fractions increase in hydrated density there is a decrease of lipid content and an increase in protein (16). Apo AI is the major apolipoprotein in horse serum and HDL is the major lipoprotein (16, 34).

CETP and Animals without CETP

There are several steps in the process of reverse cholesterol transport (Figure 2) that involve acquiring of cholesterol from peripheral tissues by HDL, esterification of the cholesterol by lecithin: cholesterol acyltransferase (LCAT), and the transfer of the cholesteryl ester by cholesterol ester transfer protein (CETP) to LDL and VLDL for hepatic removal by the liver (2). Ultimately cholesterol is secreted as free cholesterol or as bile acids in the bile; 10 % of this is excreted from the intestine during each cycle while 90% is returned to the liver via the enterohepatic circulation. This pathway operates in humans, rabbits and a number of other species while animals like rodents and horses lack CETP and thus operate altered pathways of cholesterol transport (36, 8, 6, 30-31). These animals have relatively large HDL particles that are rich in cholesteryl ester and Apo E and are designated HDL_1 or HDL_c (28). The accumulation of cholesteryl ester in

HDL is due to the LCAT reaction, transferring a free fatty acid residue from phosphatidylcholine to cholesterol by LCAT. Subsequently the cholesterol ester is not exchanged with triglyceride rich lipoproteins, because in horse plasma CETP is absent (5, 34). These large HDL particles are recognized by Apo E on the surface (26), **and** removed from circulation by hepatic receptors in the liver (34). Studies show that the rat and pig HDL-CE are directly cleared from HDL (32, 7). Even though these animals lack CETP, there is still a transfer of cholesterol ester from HDL to low density lipoprotein (LDL) (6), but not to VLDL. According to Terpstra (30-31) HDL-CE may be transferred to VLDL remnants and subsequently converted to LDL CE by plasma lipases. These findings suggest that these animals lacking CETP may have an alternate pathway by which HDL-CE is transferred to LDL (6, 30-31). CETP as well as HDL play important roles in reverse cholesterol transport (figure 3)(27) as CETP transfers cholesterol from HDL to low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) prior to removal by the liver (8). Horse serum HDL represents a relatively homogeneous population of particles (18, 29, 17, 10) that is similar to human HDL_3 (35). While there is no apparent heterogeneity in horse plasma HDL, it has been suggested that this is due to the absence of CETP activity (25). Horse HDL does not distribute into subfractions with the characteristics of human HDL_1 or HDL_2 (35). This could be due to limited transfer of free cholesterol and phospholipid from triglyceride lipoproteins during lipolysis (35). The CETP deficiency is thus not due to the presence of an inhibitor from horse plasma but to the lack of expression of CETP protein (35).

Even without CETP, reverse cholesterol transport can take place (6). Horse plasma HDL and VLDL/LDL can serve as substrate/donor for CETP supplemented from humans (35) even though it has no CETP of its own. Horse plasma HDL composition does however depend on LCAT, which is responsible for the large amounts of cholesterol ester in HDL.

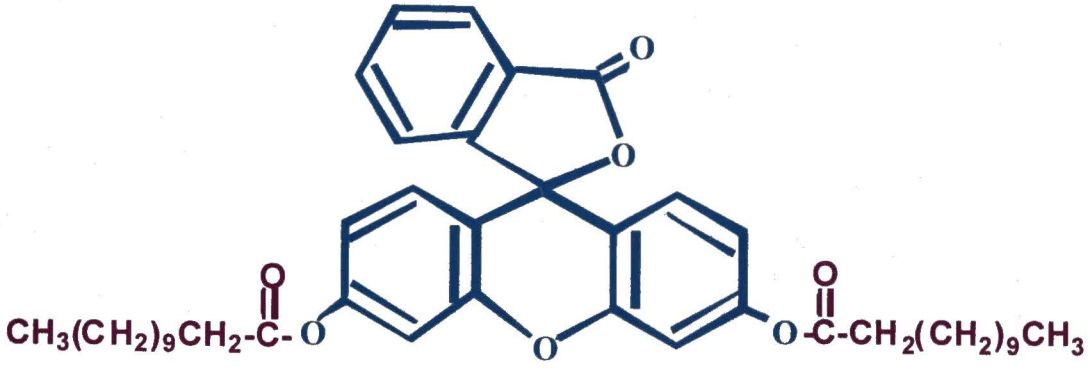
Horse HDL/drug delivery system

Horse plasma HDL will be used in these studies to allow the distinction between facilitated and spontaneous transfer of a hydrophobic test compound from HDL to other lipoproteins because of the absence of CETP. Horse plasma (HP) HDL was chosen for the present study to determine the role of cholesterol ester transfer protein (CETP) and alternate mechanisms in the transfer of drugs from the core region of HDL. Because HP lacks CETP (35-36), the two mechanisms can be studied independently by using the native HP and HP supplemented with partially purified CETP from human plasma.

Fluorescent Labeling

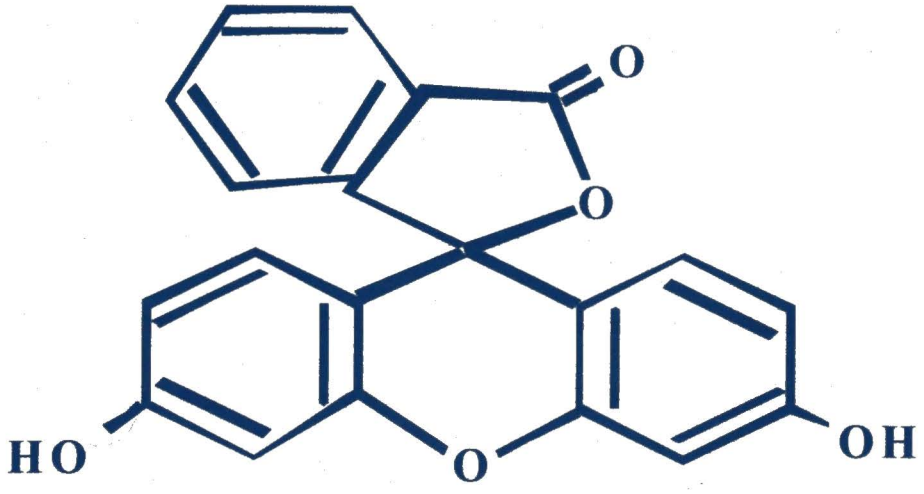
The highly hydrophobic compound dilauryl fluorescein (DLF; Figure 4) has been successfully incorporated into horse plasma HDL as a surrogate (model) for hydrophobic drugs.

Figure 4. **Structure of dilauryl fluorescein.**



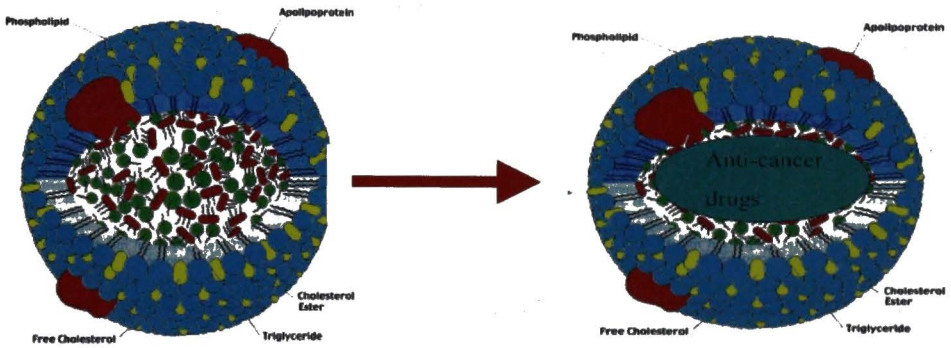
HDL takes up this compound as a core component because it is hydrophobic. DLF is suitable as fluorogenic substrate for esterases/lipases. Its molecular formula is $C_{44}H_{56}O_7$ molecular weight is 696.91(Sigma-Aldrich). After hydrolytic removal of the fatty acid chains, DLF changes to fluorescein (figure 5) and is fluorescent (wavelength 490nm excitation and 515nm emission).

Figure 5. **Structure of fluorescein.**



Dilauryl fluorescein is used to monitor the loading and transfer of core components to other lipoproteins. The HDL particle loaded with DLF is expected to have similar physical characteristics to the native HDL. This research project has been developed to study the incorporation and transport of dilauryl fluorescein (DLF) by using horse serum high-density lipoproteins (HDL) as a drug transporter (figure 6).

Figure 6. Incorporation of hydrophobic compounds (including drugs) into lipoproteins.



DLF a derivative of fluorescein has been used as a surrogate for hydrophobic drugs to characterize the DLF/HDL complexes and the movement of DLF to other lipoproteins.

Research Plan

Objective1.

Preparation and characterization of Horse HDL/ DLF complex.

The first part of this research project focuses on using dilauryl fluorescein (DLF) as a model compound for delivery by horse serum HDL to cancer cells. DLF is used as a model compound for this project because it is a highly hydrophobic compound and thus a potential candidate for the encapsulation into the hydrophobic core of horse plasma HDL particles. DLF is actually not fluorescent but it is a derivative of the fluorescent

compound fluorescein. After hydrolytic removal of the fatty acid chains, DLF changes into fluorescein. It was used to monitor the loading and uptake of core components from horse HDL by various cancer cells.

Objective 2

The role of CETP in the transfer of Drug (DLF) from the core of HDL to other lipoproteins.

The second part of this research consists of the distribution of core components from the horse HDL/DLF complex to the low-density lipoproteins. Horse HDL /DLF complex labeled with ^{3}H cholesterol oleate was incubated with and without human plasma, to determine the dependence of horse HDL core component movement on CETP. Horse serum can be supplemented with human plasma containing CETP and transfer its CE to low-density lipoproteins.

Objective 3

An alternate isolation procedure for horse HDL for better yield and bulk production.

The third part of this project was the isolation and purification of horse plasma HDL. Horse plasma was isolated by DDA-Agarose chromatography and purified by affinity purification using anti-horse albumin. HDL composition was measured using protein and enzymatic colorimetric assay kits. Horse HDL was characterized by molecular sieve chromatography and electrophoresis followed by immunoblot. The advantage of this procedure over the other HDL isolation procedures is its capacity for bulk production and better yield.

Materials and Methods

Preparation of Dodecylamine (DDA) Agarose/ Sepharose.

The DDA-agarose affinity resin was prepared as follows. Settled sepharose 4B (500ml) was suspended in 1 L of 2M Na_2CO_3 and the suspension was stirred vigorously while 50 ml of CNBr solution (1g/ml) in acetonitrile was added (14). The activation reaction was allowed to continue for 1 minute (20) and the suspension was rapidly filtered and washed with 0.3M $NaHCO_3$, pH 10.2 on a sintered glass funnel. Next 30 g of DDA, dissolved in 800 ml of ethanol, was added to the moist activated sepharose beads and suspension was gently stirred overnight at 4°C. Finally, the freshly prepared DDA-agarose was washed exhaustively with ethanol and deionized water to remove the unreacted DDA.

Priming of the DDA column.

The DDA-agarose was equilibrated with 0.3 M NaCl, 5 mM PO_4, pH 7.4 and large amounts of the supernatant prepared from horse plasma by polyethylene glycol precipitation (33) was applied to saturate the column with plasma lipoproteins (mainly HDL). The supernatant was prepared by adding polyethylene glycol to horse plasma to achieve a 6% (w/v) concentration for removing low-density and very low-density lipoproteins.

Preparation of horse plasma HDL by DDA affinity chromatography.

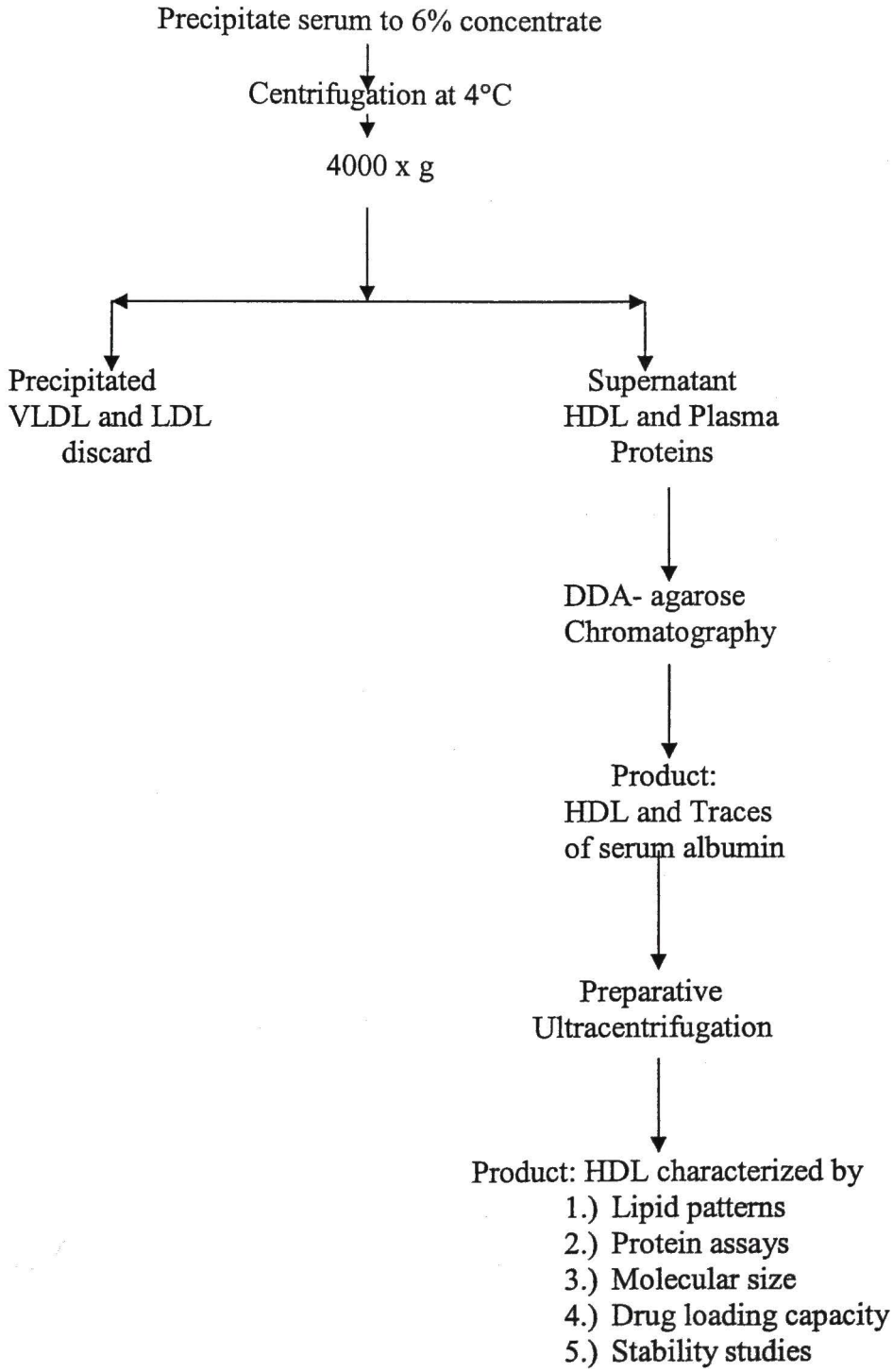
The horse serum was treated by adding polyethylene glycol to make a 6% (w/v) concentration in order to remove the lower density lipoproteins. The suspension was stirred overnight in the cold room and the precipitate serum was removed by centrifugation (3000Xg) at 4°C. The supernatant containing HDL and most of the other plasma proteins was collected and 100ml was applied to the DDA column that had previously been equilibrated with a high ionic strength buffer (0.3 M NaCl, 5 mM PO_4, pH 7.4). The conductivity (measured by Markson Model 500) of the supernatant was adjusted to ~30-35 mho and the pH to 7.4 before loading on the column. The column was then washed with high ionic strength buffer (0.3 M NaCl, 5 mM PO_4, pH 7.4) to remove unbound contaminants (plasma proteins). The HDL was subsequently eluted by lowering the ionic strength (deionized water, pH 7.4). LCAT and serum albumin are co-eluted with HDL during this process (13). Protein was read at an absorbance of A_{280} (Spectronic Genesis 5) and cholesterol was monitored in the column fractions subsequent to elution from the DDA column.

Preparative Ultracentrifugation.

The isolated horse HDL was prepared for ultracentrifugation by using 0.3g KBr/ ml of HDL (for total of 1.2g for 3ml). It was then layered with three density solutions 1 ml of 1.22 g/ml, 4 ml of 1.066 g/ml and 3ml of 1.009 g/ml (from highest to lowest on top). The plasma was then placed in SW40 ultracentrifuge tubes and ultracentrifuged in a SW40 swing-bucket rotor for 24 hours at 4°C and 40,000 rev/min in an Optima ™ LE

80K Beckman ultracentrifuge. Then the separated lipoproteins were collected in 1ml fractions, a total of 12 fractions. The total cholesterol in all 12 fractions was analyzed, and the fractions with the highest cholesterol were pooled and dialyzed overnight. The preparation of isolated HDL followed by ultracentrifugation is shown in figure 7.

Figure 7. Preparation of HDL isolation procedure.



Preparation of HDL drug complexes/ Determination of drug carrying capacity of horse serum HDL.

Horse Serum HDL was prepared by DDA-agarose chromatography followed by preparative ultracentrifugation and used in loading experiments to find the maximum loading capacity of the particle. The horse HDL was labeled with ^{3}H cholesterol oleate (Amersham Pharmacia Biotech). The ^{3}H cholesterol oleate (90μci (500,000 counts)) was dried down in a glass tube under low nitrogen until it was completely dry. Then one added 20ml of the isolated horse HDL to the glass tube and vortexed for 2 minutes; it was then incubated for an hour at 37°C. Dilauryl fluorescein (Sigma) stock solution (200mg/ml) was made in acetone. DLF was injected into horse HDL solution (5ml) with constant stirring. The horse serum HDL/ DLF complexes were incubated for 4 hours at 37°C covered in foil. The unincorporated DLF was removed by dialysis overnight against 137mM NaCl, 2.7 mM KCl, 4.3mM Na_2HPO_4 and 1.2mM KH_4PO_4 and H_2O at pH 7.4 (Phosphate Buffer Saline-PBS) in a porous tube (molecular weight 8000), from BioDesign Dialysis Tubing™ and centrifuged for 20 minutes, 6400 rpm, to remove denatured material. The amount of DLF in horse HDL/DLF complexes was estimated by fluorescence measurements (FL 600 microplate fluorescence reader). The preparations were incubated with 200μl of 1M NaOH overnight prior to the fluorescence measurement to release the fatty acyl chains to make the complex fluorescent. The fluorescence was measured at an excitation of 490/25nm (slit width) and an emission wavelength of 515/35nm. The horse HDL/DLF complexes with increasing amounts of DLF were prepared by the above-mentioned procedure.

Cell Culture studies.

The established cancer lines were obtained from ATCC and grown according to procedures provided by the ATCC. Briefly, cells were cultured in RPMI 1640 media with 1% penicillin and streptomycin mixture and 10% fetal bovine serum (obtained from Gibco/Life Technologies). Fetal bovine serum was obtained from Sigma. HGL5, non-malignant ovarian granulosa cells, a gift of Dr. William H. Rainey of Southwestern Medical Center, Dallas TX, were grown in DMEM F-12 media (Gibco/Life Technologies) with 1% ITS (BD Bio Science) and 10% FBS. Cells were grown in 75cm^2 flasks (Corning Inc.) and split, using 0.25% trypsin (Gibco) to detach the cells from bottom of the flasks, once 80-90% confluency was reached. Then their respective media was added to the flask to inhibit trypsin digestion. The cells were spun at 4000rpm, 4°C for 7 minutes, 30 seconds (CT 422 centrifuge by Juan). The supernatant was removed and the cells were resuspended in the complete media. An aliquot of the homogeneous suspension of the cells was taken to count the number of cells. The cells were counted on a hemacytometer (Fisher Scientific Catalog No. 0267110) and the required number was plated in multi-well plates (Costar) with the respective complete media.

Cellular Uptake Study.

The cells were plated in a 48 well plate (Costar) in complete medium. After 24 hours the medium was removed from the wells and washed with PBS. The cells were incubated with different concentrations of horse HDL/DLF preparations containing serum free media for 2 hours at 37°C in the presence of 5% CO_2. A control with no horse HDL/DLF was maintained and the cells were counted. Then the horse HDL/DLF was removed

from the wells and washed with PBS. Then 200µl of 1M NaOH was added to each well (except the control wells) and the plate was then incubated another 4 hours at 37°C. The fluorescence was measured at an excitation wavelength of 490/25nm (the slit width) and an emission wavelength of 515/35nm to see the uptake of DLF from horse HDL/DLF preparations. The uptake was calculated per number of cells presented.

Stability of HDL/DLF Complexes.

Time dependent incubations of HDL /DLF were done with and without CETP. HDL was isolated from horse serum by DDA procedures followed by preparative ultracentrifugation. Horse HDL was loaded with DLF (.0299mg/ml) and then labeled with ^{3}H-labeled cholesterol oleate to monitor the movement of cholesterol ester during incubation. The horse HDL/DLF complexes were incubated with human plasma (which contains CETP) and horse plasma (which does not contain CETP) at various times (0 and 24 hours) at 37°C. All the samples were subjected to preparative ultracentrifugation after the incubation. The isolated fractions from each sample were used for analysis. The distribution of the DLF among lipoprotein fractions was determined by fluorescence measurements. The distribution of ^{3}H-labeled cholesterol esters among lipoprotein fractions was also determined by radioactivity measurements.

Purification of HDL by Immunoaffinity chromatography.

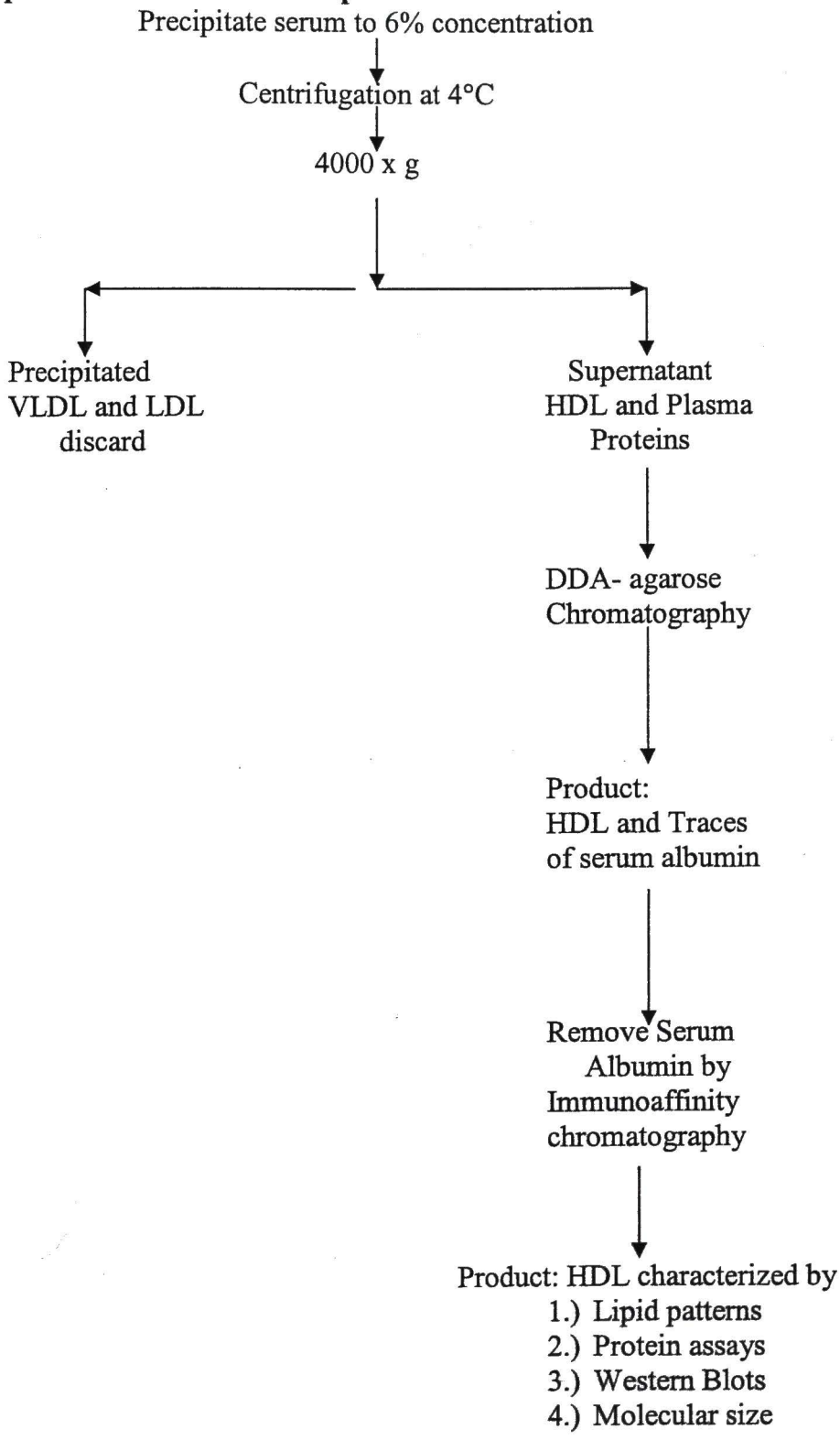
Horse albumin (Sigma) antibody was generated in goat and the anti serum was collected Immunoglobulin G (IgG) was precipitated with ammonium sulfate overnight at 4°C and coupled to CNBr activated agarose to make the horse anti albumin column.

First, equilibrated the horse anti albumin column with a low buffer containing 0.025M Tris-HCl, 0.15 NaCl, 0.05% NaN_3, and 0.05% EDTA, pH 7.5. Then applied 20 ml of HDL (540mg/dl) (isolated by DDA-chromatography) to the column. Horse serum HDL was removed with low ionic strength buffer. The column was eluted with a 3M NaSCN buffer to remove the albumin bound to the column. The fractions were analyzed by measuring the absorbance at 280 (A_{280}), total cholesterol, and conductivity. The isolation of HDL followed by immunoaffinity chromatography scheme is on figure 8.

Ultracentrifugation Rudel (23) Procedure.

The solvent density was raised to *d*1.225g/ml by adding solid KBr (0.3517g of KBr/ml of plasma). Plasma (*d*1.225g/ml; 10ml) was then placed in SW40 ultracentrifuge tubes and overlayed with *d*1.225g/ml solution, which was prepared by the addition of solid KBr to the buffered *d*1.006g/ml solution (24). Tubes were ultracentrifuged in a SW40 swing-bucket rotor for 48 hours at 4°C and 40,000 rpm in an Optima ™ LE-80K Beckman ultracentrifuge. The top 1.5ml containing the lipoprotein concentrate was removed from each tube. The lipoprotein concentrate was then applied to an agarose chromatography column. The column was prepared using Bio-Gel A-5m (Bio-Rad Laboratories, Inc. U.S.A.) at room temperature.

Figure 8. Preparation of HDL isolation procedure.



Characterization of Horse HDL.

Content of protein was estimated using Bio-Rad protein assay reaction mixture. The enzymatic colorimetric kits from Wako Chemicals analyzed phospholipid and free cholesterol, total cholesterol was analyzed by enzymatic colorimetric kits from Thermo DMA. The concentration of cholesterol ester was obtained as the difference between total and free cholesterol values.

Molecular weight determination.

The HDL was further characterized by molecular sieve chromatography using Biogel.A-5m (Bio-Rad) (6 x 87cm column). Column was standardized with standards (700Kda – 67Kda) apoferritin, alcohol dehydrogenase, serum albumin, and oval albumin (Bio-Rad chemicals). The samples were prepared with 0.2% sucrose, added to the column, and eluded with PBS. The fractions were collected in one ml fractions (BioFrac^{TM} Fraction Collector/ BioLogic LP- BioRad).

Characterization of LCAT by Western Blot analysis.

Samples were run on a 10% gradient polyacylamide gel (Bio-Rad) in the presence of sodium dodecyl sulfate (SDS-PAGE). Samples for each lane were 30µl and 10µl of 4X loading dye. Samples were boiled for 5 minutes and centrifuged at 1000g before loading. The gel was then run at 130V for 90 minutes and then subsequently electroblotted to a nitrocellulose membrane (Bio-Rad) at 30V for 1 hour. The membrane was blocked for 1 hour at 4°C in 3% nonfat dry milk (Carnation), in PBS. The human rLCAT antibody (Betagene, polyclonal goat) was diluted 1:5000 into the blocking solution. The membrane was incubated with the antibody solution overnight at room temperature.

After rinsing with PBS containing Tween 20 (Acros Organics) (1X PBST), a mouse anti-goat antibody conjugated to alkaline phosphatase (Sigma, diluted 1:5000) was incubated with the blot for 1 hour at room temperature. The blot was rinsed three times in PBS Tween 20 and visualized with nitro blue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) (KPL).

Results

HDL drug complex/ Determination of drug carrying capacity of horse serum HDL.

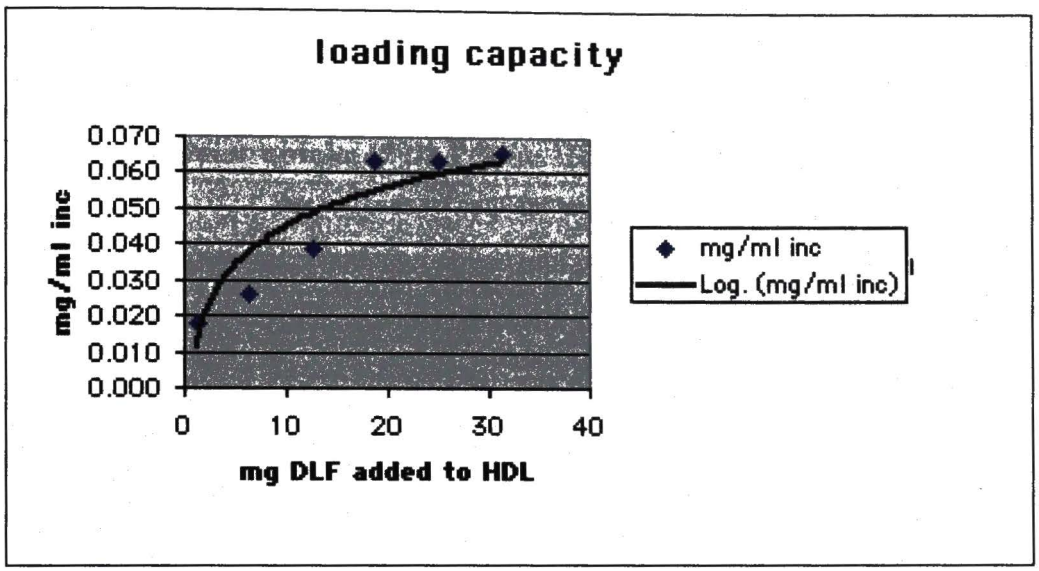
Horse HDL complexes, containing dilauryl fluorescein (HDL/DLF) were prepared by using HDL isolated by DDA-agarose chromatography followed by preparative ultracentrifugation. DLF stock was prepared in acetone (200 mg/ml). Different amounts of DLF varying from 1.25 mg – 31.2mg was added to HDL (135 mg of protein) with constant stirring and incubated for 4 hours at 37°C. After dialysis overnight the HDL/DLF complexes were spun and the supernatant collected. The fluorescence was read after 200µl of 1M NaOH treatment. The amount of DLF incorporated was computed based on standard curves established for pure DLF (Table 1). The various amounts used for loading and the amount of DLF incorporated was given in figure 9. The molecular weight of these complexes were about the same, around 148,000-152,000, the last three preps were pooled together and had an incorporation of .0299mg/ml. These were established by gel filtration chromatography as described in the methods.

Table 1. Amount of DLF incorporated from Horse Serum HDL/DLF complexes

HDL/DLF complexes	Amount of DLF in HDL/DLF
Prep 1	.018mg/ml
Prep 2	.0262
Prep 3	.0388
Prep 4	.0631
Prep 5	.0631
Prep 6	.0653

Incorporation studies were done in triplicate.

Figure 9. Loading of horse serum HDL with increasing amounts of DLF. As the amount of DLF is increased it appears to plateau off.



Cellular Uptake Study

It has been reported that the uptake of HDL core components by the cells were facilitated by SR-B1 receptors (15). Earlier studies indicate the proliferation of cancer cells is dependent on the uptake of HDL or HDL components (4,11) cancer cells take up cholesterol from HDL by an SR-BI receptor as a nutrition source for metabolism. Cancer cells have more of these receptors than normal/surrounding cells in the body. The HGL5, non-malignant ovarian granulosa cells are used as a positive control for SR-B1 expression because this transfected cell line expresses a high level of SR-BI receptors. The various cancer cells lines selected for these studies are DU145, prostate carcinoma; OV1063, ovarian carcinoma; and MCF7, breast carcinoma. The ovarian HGL5 cell line was used as the control in these studies to compare the ability of the cancer cells to incorporate DLF as core components from HDL. The data suggest that all the cell lines were able to

incorporate DLF from HDL in a concentration dependent manner. These data (Fig 10-12) along with our previous studies (15) suggest that cancer cell lines (DU145, OV1063, and MCF7) express SR-B1 receptor that allows the selective uptake of DLF from HDL/DLF complexes.

Figure 10. Cellular uptake of DLF from HDL/DLF complexes

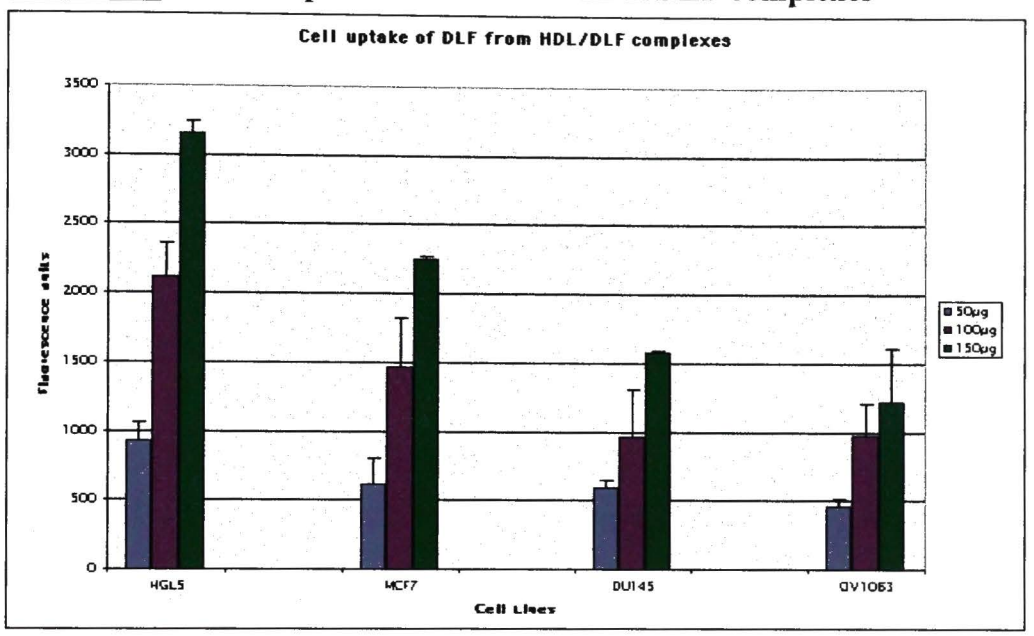


Figure 11. Cellular uptake of DLF from HDL/DLF complexes

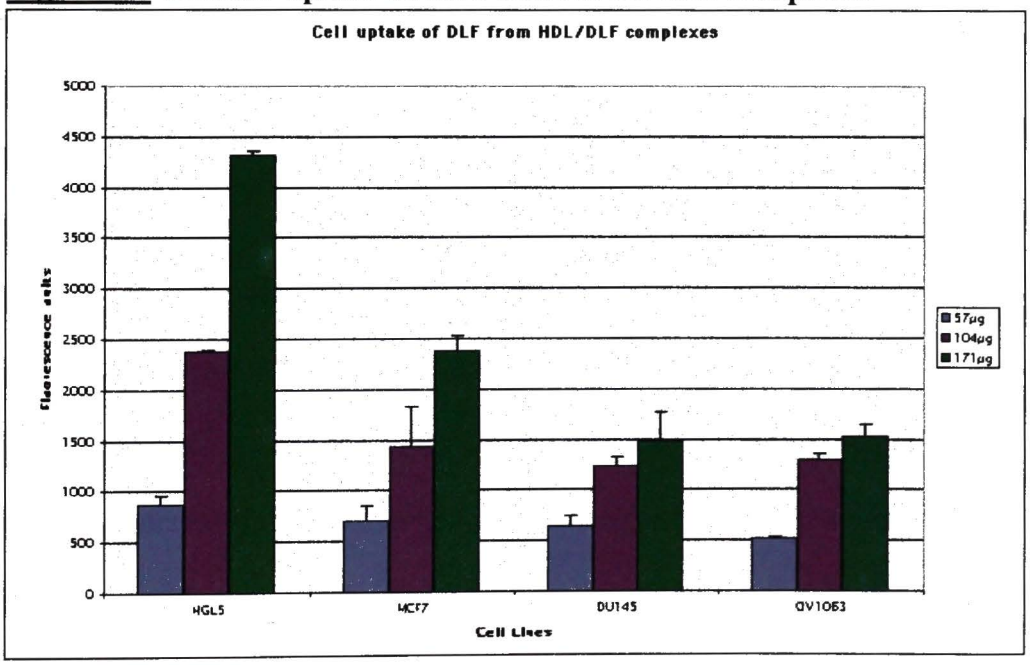
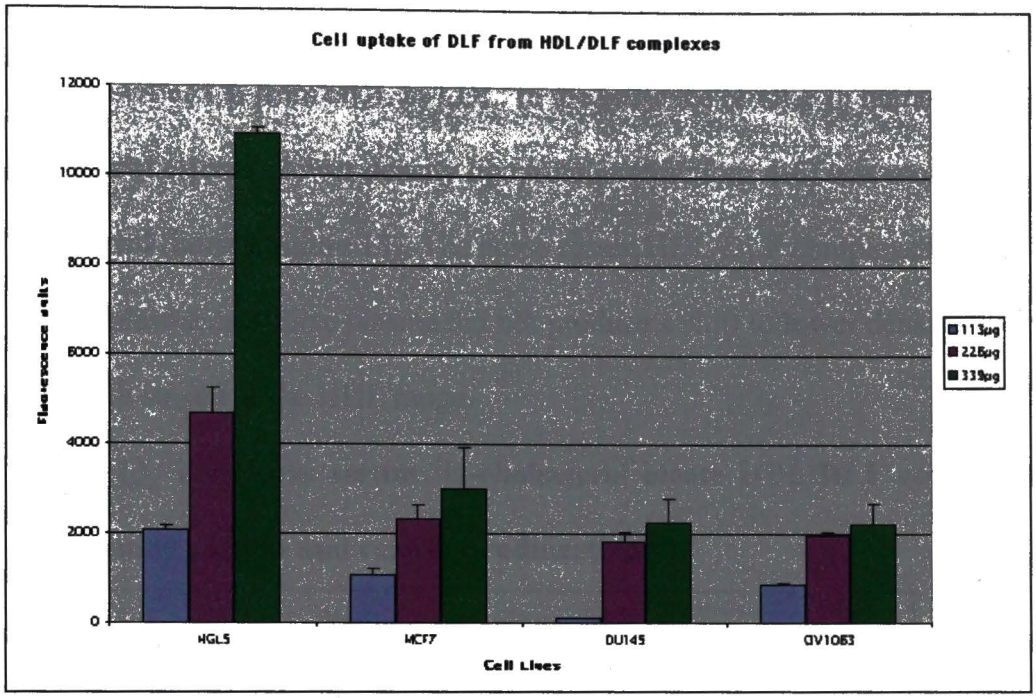


Figure 12. Cellular uptake of DLF from HDL/DLF complexes



Figures 10-12 show that the cancer cells were able to efficiently incorporate DLF from horse HDL/DLF complex in a concentration dependent manner.

Stability of the HDL/DLF complexes

This study was to evaluate the transfer of HDL components to other lipoproteins, by examining the dependence of DLF and cholesterol ester movement on CETP. The horse plasma HDL/DLF complex labeled with ^{3}H cholesterol oleate was incubated with human plasma and horse plasma to establish the dependence of the transfer of DLF/ cholesterol ester components to other lipoproteins on CETP. Horse plasma HDL labeled with ^{3}H-cholesterol oleate was used as the control. Figure 13 showed that the cholesterol esters were transferred from HDL to lower density lipoproteins when incubated with human plasma. Incubation with horse plasma did not produce a shift to the left (lower density

lipoproteins), signifying that there was no movement of cholesterol ester to the extent seen with the human plasma (Figure 15). This data suggest the involvement of CETP in the transfer of CE from HDL to LDL. In the presence of DLF in human, the shift of cholesterol esters to lower density lipoproteins (fractions 4-6) was more pronounced than the control (horse)(Figure 13). The horse serum HDL/DLF complexes incubated with human plasma and horse plasma did not produce a shift to lower density lipoproteins, they both stayed in the HDL range.

Figure 13. The horse serum ^{3}H-cholesterol oleate HDL/DLF complexes incubated with human plasma and read for radioactivity.

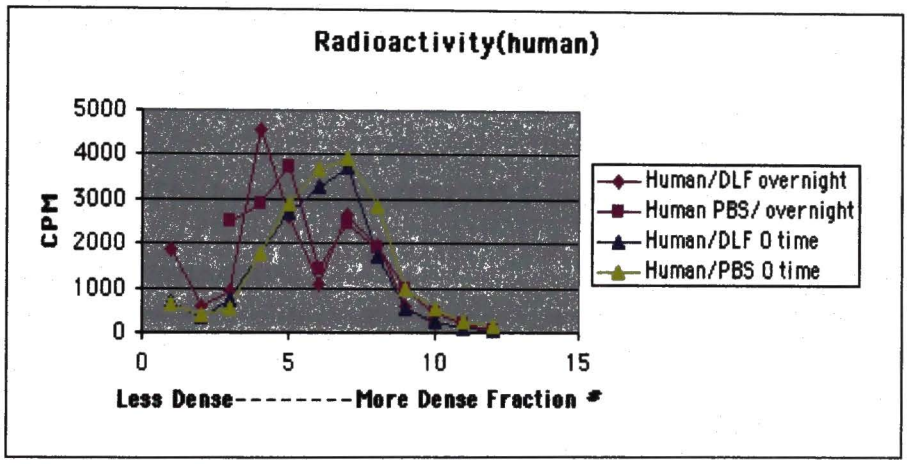


Figure 14. The horse serum HDL/DLF complexes incubated with human plasma and read for fluorescence.

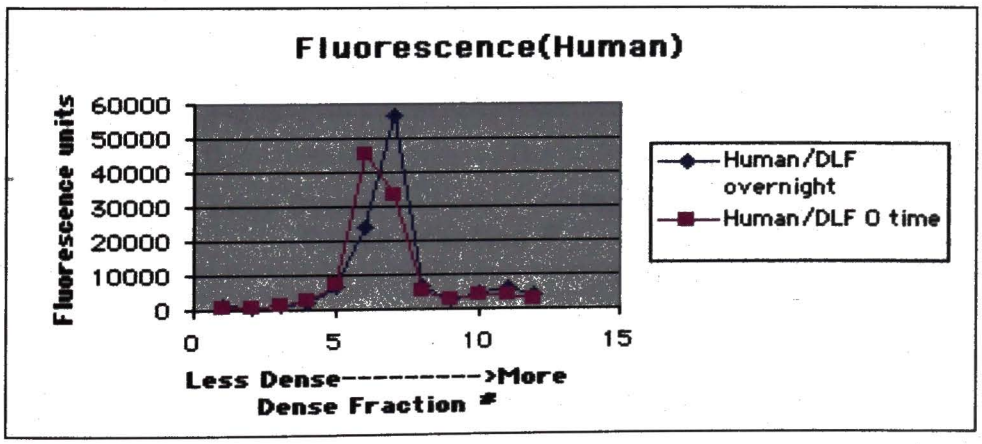


Figure 15. The horse serum ^{3}H-cholesterol oleate HDL/DLF complexes incubated with horse plasma and read for radioactivity.

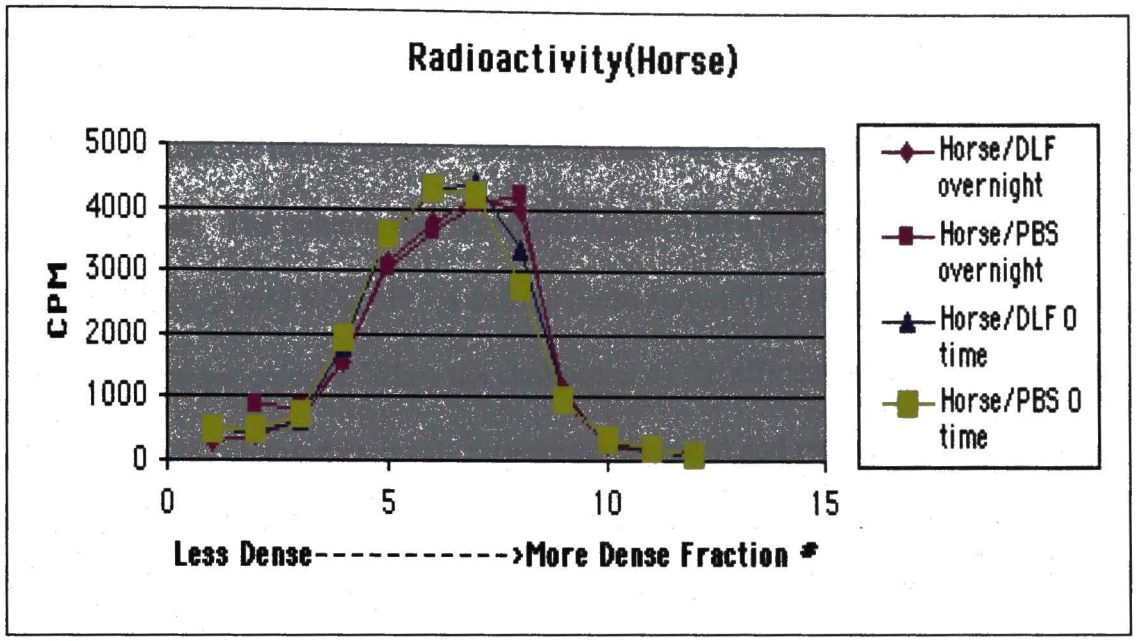
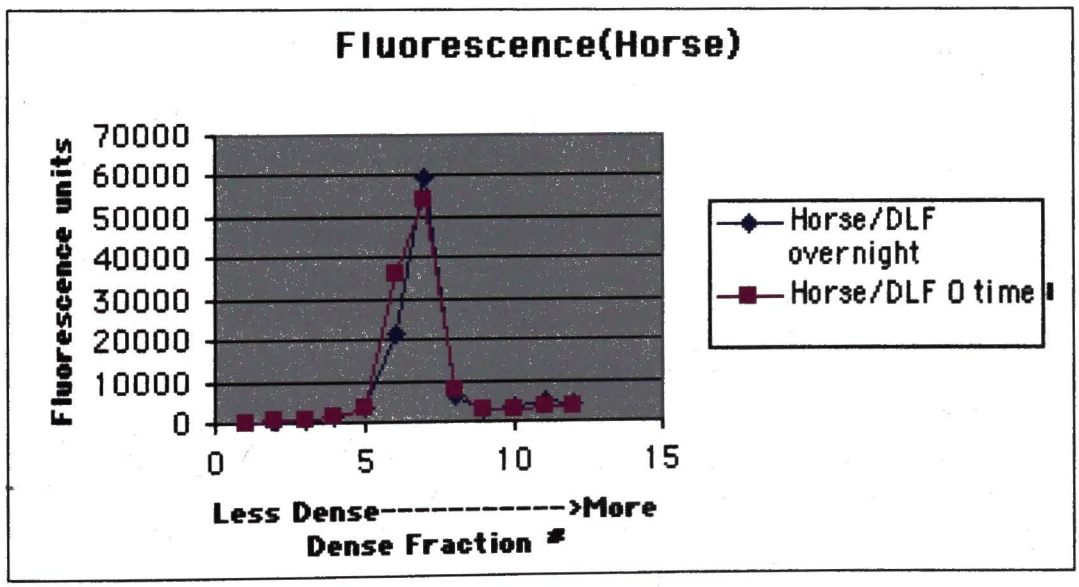


Figure 16. The horse serum HDL/DLF complexes incubated with horse plasma and read for fluorescence.

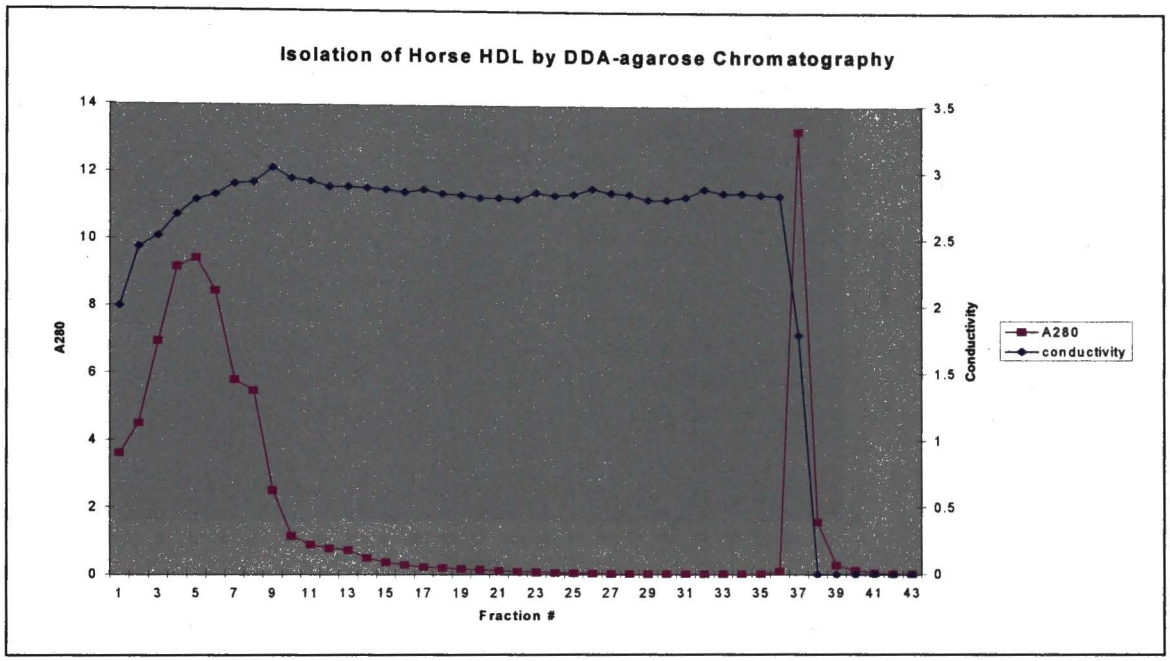


Isolation of Horse Serum HDL by DDA-Agarose Affinity Chromatography

A. DDA chromatography

Horse serum HDL was treated with 50% solution of polyethylene glycol (PEG in water) to a 6% concentration in the plasma. The precipitate was removed by centrifugation 4000 g for 45 minutes. The column was first equilibrated with a high ionic strength buffer with 0.3 M NaCl, 5 mM PO_4, pH 7.4 and the horse serum/PEG supernatant was applied to the DDA-agarose column. When the cholesterol of the effluent matched the cholesterol that goes into the column then it was considered saturated and ready for the affinity chromatography isolation of HDL. The column was subsequently washed with 0.3 M NaCl, 5 mM PO_4, pH 7.4 and the horse serum HDL was eluted from the column by the application of deionized water. The chromatographic pattern of this procedure is shown in Figure 17. In addition to HDL, it appears that HDL some LCAT was also isolated following the deionized water wash.

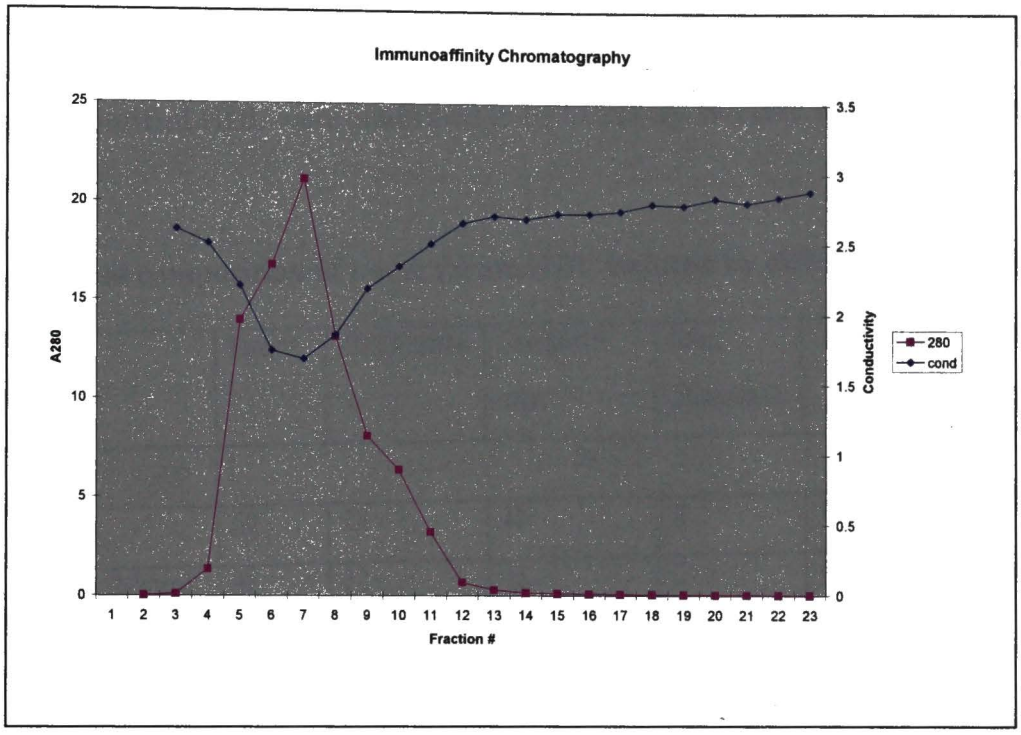
Figure 17. Affinity chromatography of horse serum HDL in a DDA-agarose column. The first peak (pink squares) shows the elution of plasma proteins with a high ionic conductivity buffer wash (blue triangles). The second 280 peak (pink squares), which is horse HDL, is released by a low ionic buffer wash (deionized water) elution.



B. Immunoaffinity chromatography

The small amounts of serum albumin were removed from the HDL preparation by immunoaffinity chromatography. The column was first equilibrated using a buffer containing 0.025M Tris-HCl, 0.015M NaCl, 0.05% NaN_3, and 0.05% EDTA, pH 7.5. The HDL was added to the column and eluted with the same buffer. The fractions were analyzed by measuring the absorbance at 280 nm (A_{280}), total cholesterol, and conductivity. The pattern for purification is shown in Figure 18. **A buffer removed the HDL**, the serum albumin was released subsequently with 3M NaSCN.

Figure 18. Immunoaffinity chromatography of horse serum anti-albumin column. This shows the elution of HDL with a low ionic strength buffer wash. The blue diamonds are the conductivity measurements, and the pink squares the A280.



Characterization of Horse serum HDL

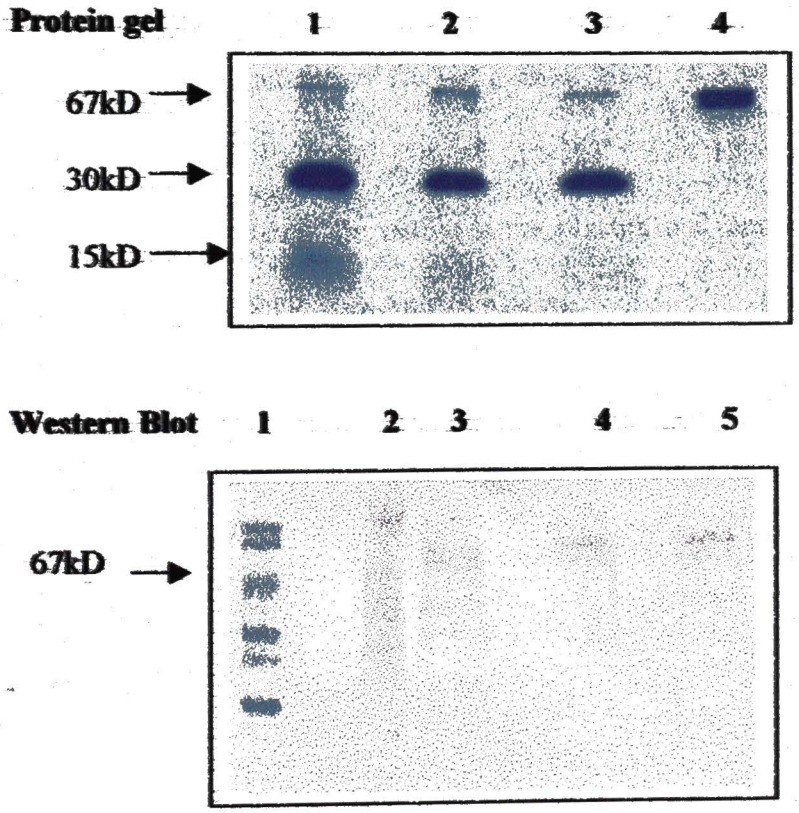
Comparison of the two HDL samples, one isolated by the DDA method vs. the conventional ultracentrifuge/agarose chromatography procedure (23) showed that the HDL isolated by DDA-agarose chromatography was quite similar to the latter preparation but had a lower triglyceride, but higher phospholipid content than the control (Table 2). The molecular weights of the HDL preparations isolated by the two procedures are shown in Table 2. The molecular weight of the HDL isolated by DDA-agarose chromatography very similar to that of the HDL purified by the immunoaffinity chromatography. However, the molecular weight of HDL isolated by the method of

Rudel et al (1974) was larger than that of both the HDL isolated by the DDA-agarose and immunoaffinity chromatography (Table 2). The DDA-agarose chromatography yielded a 60% recovery of HDL cholesterol while the method of Rudel et al. (1974) only gave an 8% recovery. The bands in the albumin range on the SDS gels in the DDA isolated HDL and affinity purified HDL were confirmed to be LCAT by Western Blot analysis (Figure 19).

Table 2. Lipid composition of horse serum HDL isolated by different procedures.

	Protein	Phospholipid	Cholesterol ester	Free Cholesterol	Triglyceride	Molecular Weight
	%					
HDL from DDA	50	28	20	2	0	234k
HDL from DDA followed by affinity chromatography	43	32	18	7	0	234k
HDL from DDA followed by preparative ultracentrifugation	55	30	11	2	2	281k
HDL prepared by Rudel procedure	54	21	9	5	11	269k

Figure 19. **Protein stain of DDA isolated horse HDL (upper pattern). 12% SDS PAGE gel lane 1 DDA isolated HDL, lane 2 DDA isolated HDL followed by immunoaffinity chromatography to remove serum albumin, lane 3 sample from lane 2 run through immunoaffinity chromatography twice, lane 4 serum albumin. Western Blot (lower pattern) of horse HDL blotted with anti-LCAT antibody. Lane 1 & 2 molecular weight standards, Lane 3 DDA isolated HDL, Lane 4 horse HDL from DDA purified by immunoaffinity chromatography, Lane 5 horse HDL from DDA purified by immunoaffinity chromatography run through twice. It shows that the bands in the albumin range on the SDS-PAGE gels are indeed LCAT and not serum albumin.**



Discussion

Recent studies in our laboratory (15) suggested that rHDL is a potential drug delivery vehicle for hydrophobic drug compounds. Current research suggest that the changes in lipoprotein binding of drug compounds have major impact on the efficacy of the therapy, especially because of the fact that these drugs are often administered into patients with abnormal lipid metabolism. Hence it is very important to evaluate the stability of HDL/ drug complex and the role of cholesterol ester transfer protein (CETP) and alternate mechanisms in the transfer of drugs from the core region of HDL.

Horse plasma HDL was used in these studies to allow the distinction between facilitated and spontaneous transfer of the drug from HDL to other lipoproteins because of the absence of CETP in horse plasma (35-36). The two mechanisms can be studied independently by using the native horse plasma and horse plasma supplemented with partially purified CETP from human plasma.

Preparation and characterization of horse HDL/DLF complexes.

We were able to successfully incorporate DLF into horse serum HDL. These studies helped to determine the capacity of the HDL for the encapsulation of the hydrophobic compound DLF. We did establish that horse serum HDL/DLF complex could be taken up by several types of cancer cells in a concentration dependent manner. It has been reported that the uptake of HDL core components by the cells were facilitated by SR-B1 receptors (15). Earlier studies indicate the proliferation of cancer cells is dependent on the uptake of HDL or HDL components (4,11). The HGL5, non-malignant ovarian granulosa cells are

used as a positive control for SR-B1 expression because this transfected cell line expresses a high level of SR-BI receptors. The various cancer cells lines selected for these studies were DU 145, prostate carcinoma, OV 1063, ovarian carcinoma, MCF7, breast carcinoma. The ovarian HGL5 cell line was used as the control in these studies to compare the ability of the cancer cells to incorporate DLF as core components from HDL. The data suggest that all the cell lines were able to incorporate DLF from HDL in a concentration dependent manner. These data (Fig 10-12) along with our previous studies (15) suggest that cancer cell lines (DU 145, OV 1063, and MCF7) express SR-B1 receptor that allows the selective uptake of DLF from HDL/DLF complexes.

Facilitation of water insoluble drugs between lipoproteins by CETP

The horse plasma HDL/DLF complex labeled with ^{3}H cholesterol oleate was incubated with human plasma and horse plasma to establish the dependence of the transfer of DLF/ CE components to other lipoproteins on CETP. Horse plasma HDL labeled with ^{3}H-cholesterol oleate is used as the control for CETP activity. Figure 13 showed that the cholesterol esters were transferred from HDL to lower density lipoproteins when incubated with human plasma. Incubation with horse plasma however did not produce a shift (Figure 15). These data suggest the involvement of CETP in the transfer of CE from HDL to LDL. In the presence of DLF, the shift of cholesterol esters to lower density lipoproteins was more pronounced than the control. In figures14 and 16, the DLF did not move into the lower density range. The difference in transfer of CE may be attributed to the ability of CETP to transfer the drug and lipid separately. It can also be attributed to

the ability of lipoproteins to accumulate more CE than DLF. These data very well correlate with the findings of Kwong et al (12).

In the last part of this research horse serum HDL was isolated by DDA-agarose affinity and purified by immunoaffinity chromatography. We compared the purity, recovery and yield of these horse HDL preparations. The DDA chromatography followed by affinity purification using anti horse albumin did have a better yield with more pure HDL than the conventional Rudel method. It has been our experience that the HDL particles are generally of the size and flotation properties of an authentic HDL species. We have found that the quantitative contributions of the lipid, and protein components to the total mass of the HDL were very similar to the reported values. (16-17, 34-36). The studies presented here involve an improved process to isolate, and purify horse serum HDL utilizing hydrophobic affinity chromatography. The above system of isolation for horse HDL has been developed to provide a basis for studies involving the drug carrying capacity and stability of horse HDL/drug complexes.

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