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Separation of HDL Particles by Immunoprecipitation

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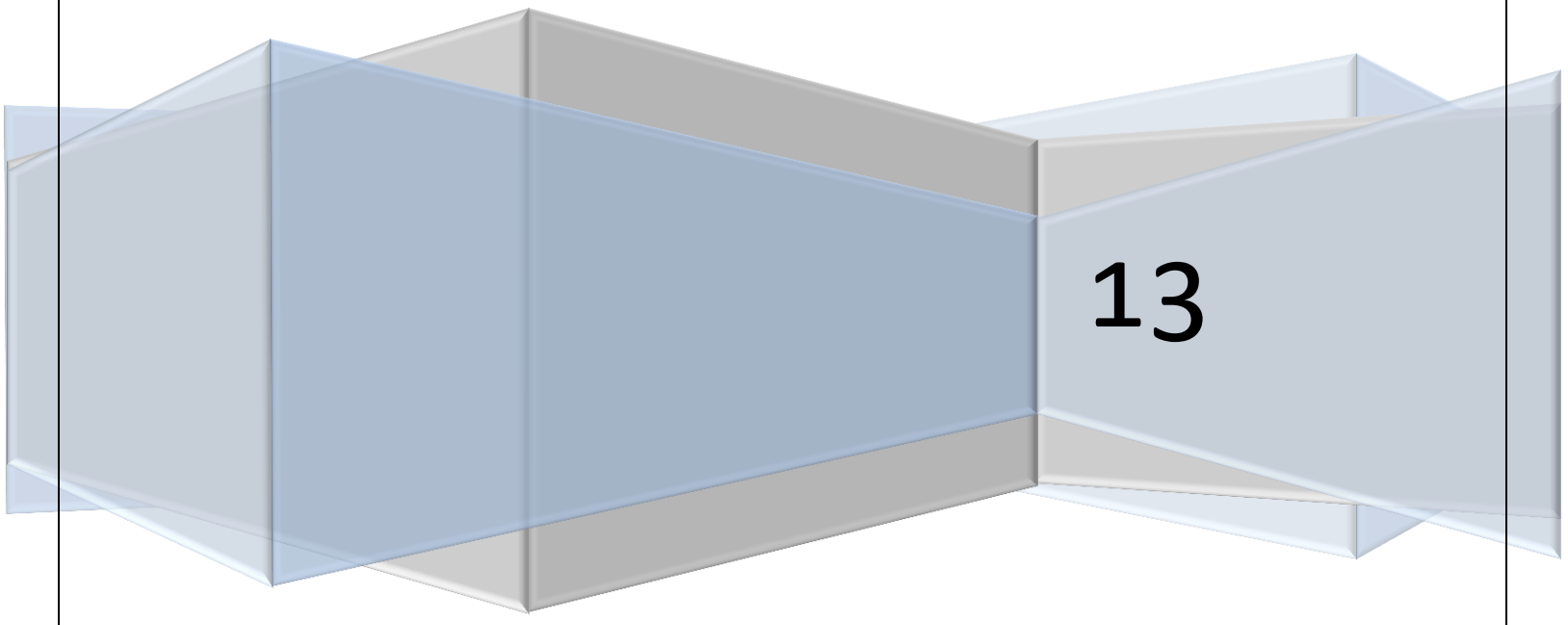

Introduction

We all know that HDL cholesterol concentration is inversely associated with coronary heart disease (CHD) and that the National Cholesterol Education Program (NCEP) recommends HDL cholesterol measurement in all adults as part of the standard lipid profile (1). HDL particles play an important role in reverse cholesterol transport (RCT), the movement of cholesterol from peripheral tissue back to the liver for excretion, and although RCT is presumed to be the primary mechanism for HDL's protective effect, HDL also have anti-inflammatory, antioxidant, and antithrombotic activities, and appear to promote healthy endothelial function (2).

Characterization of high density lipoproteins (HDL) is currently the focus of intense research. The concept of "dysfunctional HDL", HDL particles that are somehow altered and no longer protect against CHD, is relatively new. Clinicians have long noticed the paradox that some individuals with very high HDL cholesterol concentrations develop CHD in the absence of obvious risk factors. Recent data has implicated certain proteins associated with these dysfunctional HDL (3). In addition to HDL function, new classes of pharmaceuticals, including CETP inhibitors, are being developed to raise HDL concentrations. The challenge for these researchers is to isolate HDL particles for measurement of cholesterol and other components that may explain function or better monitor therapeutic efficacy. Traditional approaches such as electrophoresis and ultracentrifugation are labor intensive, technically demanding, and may alter particle composition and structure; chemical precipitation methods may not completely remove apo B-containing lipoproteins or adequately capture all HDL subclasses; and homogeneous methods for HDL cholesterol measurement have proven to be inadequate in patients with certain lipoprotein abnormalities. Immunoprecipitation shares the positive attributes of chemical precipitation methods such as ease of use, but also allows quantitative separation of HDL particles from apo B lipoproteins, without altering lipoprotein particle composition.

Antibodies directed against apolipoproteins provide the most specific method available for the separation of lipoproteins. This manuscript discusses immunoprecipitation of lipoproteins, and how this straightforward technique provides a simple and effective means of isolating HDL particles for further characterization and analysis.

Chemical Precipitation

Chemical precipitation methods are generally effective in separating HDL particles from apo B-containing lipoproteins. Typically, polyanions such as heparin, dextran sulfate, and sodium

phosphotungstate are used with a divalent cation, such as magnesium or manganese. Polyethylene glycol (PEG) is also sometimes used to precipitate apo B-containing lipoproteins, although it is not a polyanion. Various concentrations of these components have been evaluated over the years to optimize the separation of HDL from other lipoproteins. The dextran sulfate magnesium chloride precipitation method, using dextran sulfate with an approximate molecular weight of 50,000 was optimized to minimize HDL loss and maximize apo B lipoprotein precipitation. It is the most popular precipitation method in the US, and is recognized as an important component of the designated comparison method (DCM) for HDL cholesterol measurement (4).

Triglyceride-rich lipoproteins (TRL) in high concentrations may not completely precipitate, as evidenced by turbidity in the supernatant. According to Warnick and colleagues, chemical precipitation methods may slightly overestimate HDL cholesterol due to incomplete precipitation of VLDL and LDL (5). All methods fail with high triglycerides presumably because the higher densities of the TRLs make sedimentation more difficult with low speed centrifugation. In one study, the percentage of samples requiring additional treatment because of incomplete precipitation were 4%, 7.5%, 10%, 11%, and 12% for PEG (10%), dextran sulfate/magnesium chloride, heparin/manganese, and PEG (7.5%), respectively (6).

An additional drawback to chemical precipitation methods is that lot-to-lot differences in dextran sulfate can affect the specificity of the chemicals for lipoproteins. Warnick et al, in the classic precipitation procedure (5), determined that both dextran sulfate/magnesium and heparin/manganese left small amounts of apo B in the supernate, while precipitating small amounts of HDL (Table 1). Dextran sulfate precipitated slightly more apo B-containing lipoproteins than heparin, but also precipitated slightly more HDL. More recently, we have observed with our lot of dextran sulfate no loss of HDL even at high dextran sulfate concentrations but relatively more apo B lipoproteins remaining in the supernatant.

Given these flaws with chemical precipitation, we sought to develop an alternate method that provides similar ease of use but greater specificity and consistency. Immunoprecipitation of apo B lipoproteins provides the answer.

Immunoprecipitation

Monospecific antibodies directed against apolipoproteins are the most specific method available for the separation of lipoproteins. There are many examples of their use to separate lipoproteins in the

literature. Rastogi et al. used antibodies attached to magnetic beads to separate HDL particles with apo AII (LpAI/AII particles) from HDL without apo AII (LpAI) (7). A once popular method for separating LDL particles was the Direct LDL™ immunoseparation kit from Genzyme Diagnostics (8). This method used antibodies against apo AI and apo E attached to latex beads to remove VLDL and HDL from LDL, which was recovered in the filtrate.

Polyclonal antiserum to apo CI was used by Anderson et al. to precipitate VLDL and IDL for the measurement of LDL-apolipoprotein B (9). A 5:1 ratio of antisera to sample completely removed all VLDL and IDL without altering LDL concentrations. Puchols and colleagues compared ultracentrifugation and chemical precipitation methods to immunoprecipitation for the isolation of HDL and reported that only immunoprecipitation completely separated apo AI and Apo B containing particles (10). Chemical precipitation and ultracentrifugation failed to separate 4% to 20% of HDL particles (Table 2).

Similar results were reported by Heuck and colleagues using anti-apo B antisera to isolate HDL particles (Table 3). No beta or pre-beta lipoproteins were present in the supernatant, with all alpha lipoproteins remaining (11). Antisera developed against apolipoproteins AI, CI, CII, and CIII was also used to successfully isolate LDL particles (11).

Development of a Robust Immunoprecipitation Reagent

IP reagent was prepared from delipidated and stabilized goat anti-apo B antisera. Dose-response studies indicated that equal volumes of sample and reagent completely precipitated all apo-B containing lipoproteins to 300 mg/dL with no effect on HDL (Figure 2). Incubation time (1 – 60 min), centrifugation speed (8,000-14,000 rpm) and centrifugation time (5 – 15 min) had little effect on results (Eppendorf microcentrifuge). For subsequent experiments 200 or 250 µL of reagent was added to an equal volume of sample and vortexed for 10 seconds, incubated for 10 minutes at RT, and centrifuged at 12,000 rpm for 10 minutes. Precision was assessed by immunoprecipitation of 10 replicates of a serum pool. HDL-C results for 25 serum samples with apo B concentrations from 45-138 mg/dL were determined by IP and dextran sulfate/$MgCl_2$ precipitation. Specificity was determined by measuring apos AI and B in 25 sera before and after immunoprecipitation.

Total imprecision was 2.2% and 5.0% for HDL-C and HDL-apo AI, respectively (Table 4) after immunoprecipitation. HDL-C by IP gave excellent agreement to dextran sulfate/$MgCl_2$ precipitation

(Figure 3). The mean recovery of apo AI and B in 25 serum samples after IP was 98.3% and 1.0%, respectively; all apo B results were < LOD of the assay.

Conclusion

Apolipoprotein recognition by antibodies can be used as a highly specific tool for identifying and characterizing lipoprotein subclasses. One can easily see the advantages of immunoprecipitation to isolate HDL for measurement of cholesterol or a number of other components. Once immunoprecipitation is completed, total cholesterol or other analyte measured in the supernatant is highly specific for HDL. This is an important advantage over homogeneous assays for HDL-C, as total cholesterol measurement is inexpensive, better standardized and more accurate.

References

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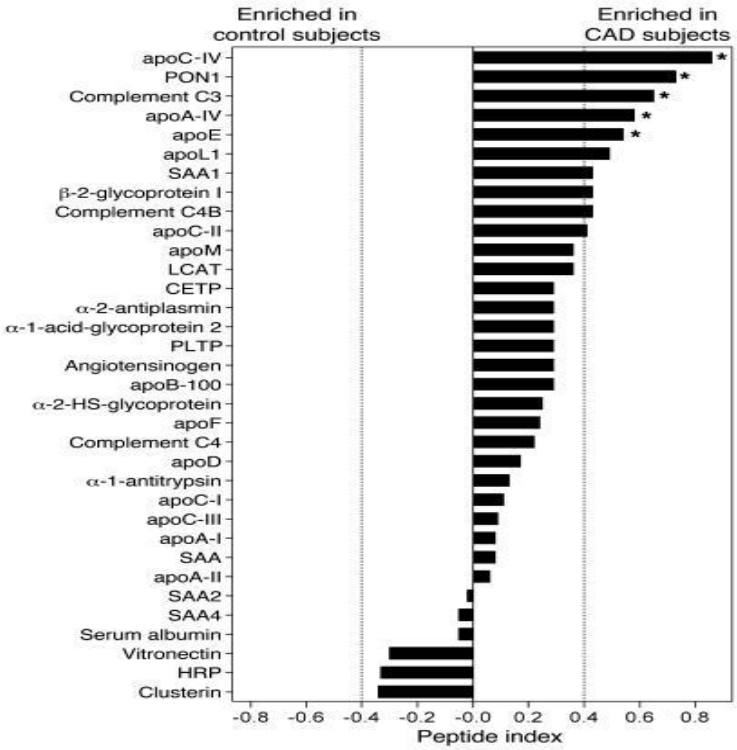
Figure 1: Relative Abundance of Proteins Isolated from HDL_3.



From: Vaisar et al, J Clin Invest 2007; 117:746-756.

Figure 2: Immunoprecipitation of 250 µL serum sample with Various Volumes of Precipitation Reagent

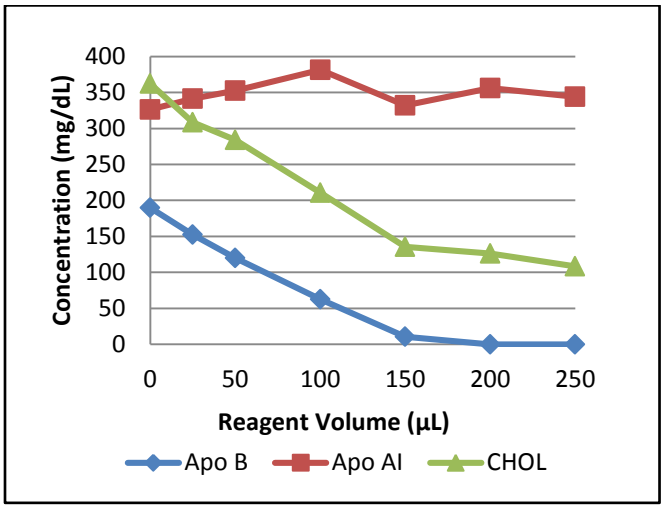


Figure 3: Comparison of HDL Cholesterol Measurement after Immunoprecipitation with Dextran Sulfate/Magnesium Chloride Precipitation

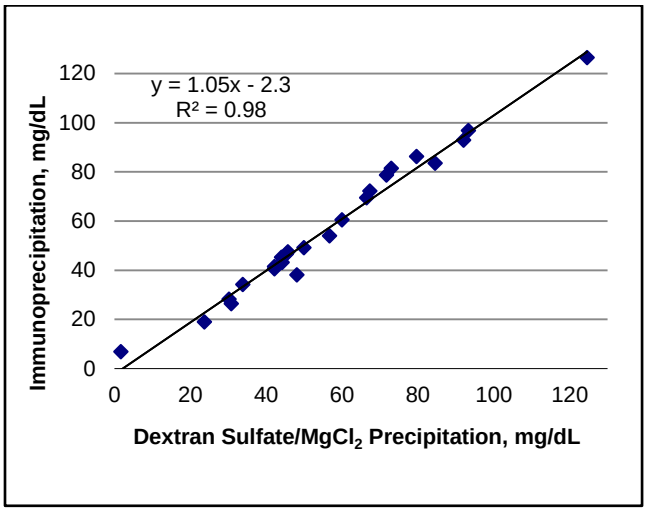


Table 1: Incomplete Precipitation of Apo B-Containing Lipoproteins and Co-Precipitation of HDL with Chemical Precipitation Methods

				Supernatant CHOL		Supernatant Apo B- CHOL		Precipitate Apo AI	
Specimen	**n**	**Plasma CHOL**	**Plasma TRIG**	**HM**	**DSM**	**HM**	**DSM**	**HM**	**DSM**
Overall	48	250 ± 56	180 ± 177	49.4	47.6	1.0	0.1	3.1	4.7
Hypercholesterolemic	9	310 ± 27	147 ± 52	46.1	44.7	1.3	0.1	3.8	4.8
Hypertriglyceridemic	9	232 ± 29	340 ± 111	32.4	32.3	0.3	0	3.6	4.6
Hypercholesterolemic and hypertriglyceridemic	4	351 ± 40	569 ± 360	44.6	40.0	3.2	0	4.4	8.9
Adapted from Warnick et al, Clin Chem 1982; 28:1379-88. Concentrations are mg/dL.									

Table 2: Concentrations of Apolipoproteins and Cholesterol in Plasma and in Five Different Preparations of HDL

	Apo AI	Apo AII	Apo B	Apo CIII	Apo E	CHOL
Serum	153	78	102	8.3	8.5	161
HDL (d>1.063)	117	55	1.7	3.4	3.3	42.1
Heparin/manganese	141	70	0.1	4.1	2.9	50.2
Phosphotungstate/magnesium	137	70	0.5	4.6	2.0	46.7
Anti-apoB immunoprecipitation	148	76	0	5.2	4.9	56.4
Adapted from Puchols et al, Clin Chem 1987; 33:1597-1602. Concentrations are mg/dL						

Table 3: Cholesterol Concentrations in Supernates after Immunoprecipitation of Apo B-Containing Lipoproteins

	Serum Sample, µL Precipitated with 200 µL antisera			
Serum Cholesterol	**40**	**30**	**20**	**10**
452	81.9	71.4	71.4	70.3
278	56.0	40.5	38.6	38.2
166	40.2	25.1	24.7	25.1
Adapted from Heuck et al, Clin Chem 1985; 31:252-256. Concentrations are mg/dL.				

Table 4: Total Variability (Includes Variability Due to Immunoprecipitation and Measurement Error)

Sample	CHOL	APOA1	APOB
1	24.1	70.9	0
2	23.4	71.6	0
3	23.9	68.4	0
4	22.6	68.0	0
5	23.2	72.4	0
6	24.2	78.1	0
7	23.5	73.2	0
8	24.2	74.0	0
9	23.6	74.2	0
10	24.0	79.2	0
Mean	23.7	73.0	0.0
SD	0.51	3.65	0.0
CV	2.2%	5.0%	