Reagent and Procedure for Immunoprecipitation of Apo B-containing Lipoproteins

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ABSTRACT

Background: Immunoprecipitation (IP) with antisera provides the most specific method available for separation of lipoproteins. IP is simple to perform, does not alter lipoprotein particle composition and allows for more robust precipitation than chemical methods. Here, we describe an IP reagent and procedure for isolation of HDL particles in human sera. Methods: IP reagent was delipidated and stabilized goat anti-apo B antisera. Dose-response study indicated that equal volumes of sample and reagent completely precipitated all apo-B containing lipoproteins to 300 mg/dL with no effect on HDL. Incubation time (1 – 60 min), centrifugation speed (8,000-14,000 rpm) and centrifugation time (5 – 15 min) had little effect on results. 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 room temperature (RT), and centrifuged at 12,000 rpm for 10 minutes. Precision was assessed by IP 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 IP. Results: Total imprecision was 5.0%. Analytical imprecision, determined by combining supernatants after IP and measuring apo AI in the supernatant pool 10 times, was 2.8%. By difference, the imprecision attributable to IP was 2.2%. HDL-C by IP (Y) gave excellent agreement to dextran sulfate/$MgCl_2$ precipitation (Figure). The mean recovery of apos AI and B after IP was 98.3% and 1.0%, respectively; all apo B results were < LOD. Conclusion: The IP reagent and protocol is a simple, effective and highly specific tool for isolating HDL particles in human serum.

INTRODUCTION

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 (1). 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 protective 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 (2). 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.***

METHODS

IP reagent was prepared from delipidated and stabilized goat anti-apo B antisera (LipoSep IP™, Sun Diagnostics). 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 1). Incubation time (1 – 60 min), centrifugation speed (8,000-14,000 rpm) and centrifugation time (5 – 15 min) had little effect on results. 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 min at RT, and centrifuged at 12,000 rpm for 10 min (Eppendorf microcentrifuge). Dextran sulfate precipitation was performed essentially as described by Warnick (3); 30 µL reagent was added to 300 µL sample, vortexed, incubated for 10 min at room temperature, and then centrifuged at 10,000 rpms for 5 min (Eppendorf microcentrifuge). Cholesterol (Wako Chemicals USA) and apos AI and B (laboratory developed tests) were measured in supernatants using a Cobas Fara II analyzer. Precision was assessed by immunoprecipitation of 10 replicates of a serum pool. HDL-C results for 25 normolipidemic serum samples were compared after IP and dextran sulfate/$MgCl_2$ precipitation. Robustness of IP and chemical precipitation was determined with 32 turbid and/or high triglyceride specimens to compare the frequency of incomplete precipitation.

RESULTS

Total imprecision was 2.2% for HDL-C after immunoprecipitation. HDL-C by IP gave excellent agreement to dextran sulfate/$MgCl_2$ precipitation (Figure 2). 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. IP successfully removed all apo B-containing lipoproteins in samples with triglycerides up to 1002 mg/dL, while dextran sulfate/magnesium chloride was successful only up to 561 mg/dL triglycerides, and only with additional reagent or sample dilution (Table 1).

Figure 1: Immunoprecipitation of Apo B Lipoproteins with Various Sample and Reagent Volumes

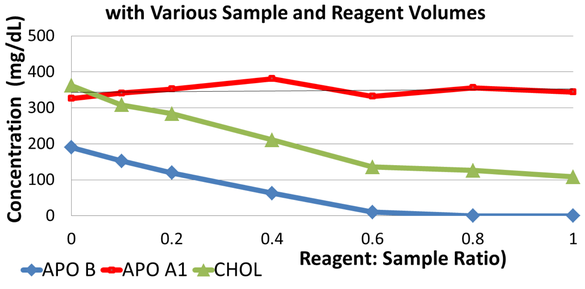
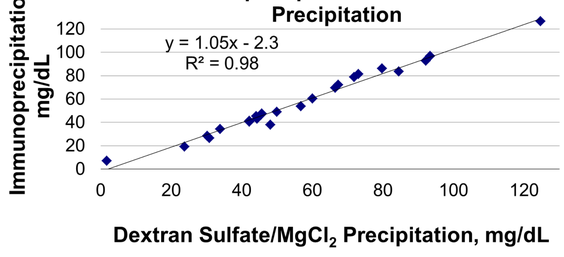


Figure 2: HDL Cholesterol by Immunoprecipitation vs. Chemical Precipitation



RESULTS

TABLE 1: INCOMPLETE PRECIPITATION OF APO B-CONTAINING LIPOPROTEINS IN SAMPLES WITH ELEVATED TRIGLYCERIDES USING IMMUNOPRECIPITATION VERSUS CHEMICAL PRECIPITATION

TRIG (mg/dL)	IP 1ST ATTEMPT	IP 2ND ATTEMPT	DS 1ST ATTEMPT	DS 2ND ATTEMPT
242	1	-	0	1
253	1	-	1	-
290	1	-	1	-
292	1	-	0	1
312	1	-	0	1
313	1	-	0	1
316	1	-	0	1
341	1	-	0	1
347	1	-	0	1
349	1	-	0	1
359	1	-	0	1
367	1	-	0	1
520	1	-	0	1
534	1	-	0	1
541	1	-	0	1
559	0	1	0	1
561	0	1	0	1
961	0	1	0	0
972	0	1	0	0
1002	0	1	0	0
1085	0	0	0	0
1112	0	1	0	0
1136	0	0	0	0
1170	0	0	0	0
1179	0	1	0	0
1352	0	0	0	0
1356	0	0	0	0
1367	0	0	0	0
1393	0	1	0	0
1469	0	1	0	0
1985	0	1	0	0
SUCCESS RATE:	15/32	**25/32**	**2/32**	**17/32**

Key: 1 = Successful; 0 = Unsuccessful (Incomplete precipitation) 1st attempt: Usual procedure; 2nd attempt: additional reagent and/or sample dilution prior to precipitation. IP: Immunoprecipitation reagent; DS: Dextran sulfate/magnesium chloride reagent; TRIG: triglycerides

CONCLUSIONS

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 other HDL components. Once immunoprecipitated, 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.

These data clearly show that immunoprecipitation is more robust than chemical precipitation in clearing triglyceride-rich lipoproteins. Because of the specificity of anti-apo B antibodies, HDL particles will not co-precipitate with apo B-containing lipoproteins, which may be an issue with chemical precipitation methods.

DISCUSSION

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. The dextran sulfate magnesium chloride precipitation method, using dextran sulfate with an approximate molecular weight of 50,000 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 very high triglycerides presumably because the higher densities of the TRLs make sedimentation by centrifugation difficult. In one study, the percentage of samples requiring additional treatment because of incomplete precipitation was 4%, 7.5%, 10%, 11%, and 12% for PEG (10%), dextran sulfate/magnesium chloride, heparin/manganese, and PEG (7.5%), respectively (5). Warnick et al (3) determined that both dextran sulfate/magnesium and heparin/manganese left small amounts of apo B in the supernate, while precipitating small amounts of HDL. Dextran sulfate precipitated slightly more apo B-containing lipoproteins than heparin, but also precipitated slightly more HDL.

Monospecific antibodies directed against apolipoproteins are the most specific method available for the separation of lipoproteins. 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 (6); Chemical precipitation and ultracentrifugation failed to separate 4% to 20% of HDL particles. Similar results were reported by Heuck and colleagues; no beta or pre-beta lipoproteins were present in the supernatant, with all alpha lipoproteins remaining (7).

Our data are consistent with these previous studies showing that immunoprecipitation is specific in isolating HDL particles, and more robust in sedimenting triglyceride-rich lipoproteins. The availability of a validated, commercially available immunoprecipitation reagent will prove useful to clinicians and researchers with a need to accurately separate HDL particles.

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