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A comprehensive evaluation of the heparin–manganese precipitation procedure for estimating high density lipoprotein cholesterol

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Abstract The accurate quantitation of high density lipoproteins has recently assumed greater importance in view of studies suggesting their negative correlation with coronary heart disease. High density lipoproteins may be estimated by measuring cholesterol in the plasma fraction of d > 1.063 g/ml. A more practical approach is the specific precipitation of apolipoprotein B (apoB)-containing lipoproteins by sulfated polysaccharides and divalent cations, heparin–Mn^{2+} being the most commonly used combination. The present heparin–Mn^{2+} procedure was found to be reasonably specific and not often subject to large errors; however, 9% (primarily hypertriglyceridemic samples) of the 966 plasma samples treated with heparin–Mn^{2+} had obvious supernatant turbidity, indicating incomplete sedimentation of apoB-associated lipoproteins. Furthermore, 48% of the nonturbid supernates contained more than 1 mg/dl (mean 2.5 mg/dl) of apoB-associated cholesterol when measured by a radial immunodiffusion procedure, indicating slight overestimation of HDL cholesterol. Determination of the extent of the unprecipitated apoB-associated lipoproteins by sensitive radioimmunoassay and of the amount of precipitated high density lipoprotein by radial immunodiffusion assay of apolipoproteins A-I and A-II at various heparin and Mn^{2+} concentrations indicated that the usual heparin level (approximately 1.3 mg/ml) was adequate. However, a twofold increase in Mn^{2+} concentration to 0.092 M improved precipitation of the apoB-associated lipoproteins without excessive precipitation of high density lipoprotein from plasma. This increased Mn^{2+} level also provided improved sedimentation of the apoB-associated lipoproteins from hypertriglyceridemic plasma. Additional observations suggested that, for convenience, the heparin and Mn^{2+} can be added simultaneously as a combined reagent, that samples can be incubated for 10 minutes at room temperature before centrifugation, and that turbid supernates from hypertriglyceridemic samples can usually be made free of apoB-associated lipoproteins by centrifugation at 12,000 *g* for 10 minutes.

Supplementary key words coronary heart disease · immunoassay · lipoprotein quantitation

An inverse correlation has been demonstrated between HDL levels and coronary heart disease risk (1, 2). High HDL cholesterol levels have been associated with increased longevity as well as with a lower incidence of cardiovascular disease (3). In the past, HDL quantitation has been performed primarily as part of an indirect procedure for quantitation of LDL (4–6), a known risk factor for coronary heart disease, and has generally been confined to specialized lipid research laboratories. However, with more general awareness of the importance of HDL as an independent negative risk factor in heart disease, the demand for this measurement will undoubtedly increase.

Classically the lipoproteins are defined operationally in terms of hydrated densities, the principal lipoprotein classes being VLDL of d < 1.006 g/ml, LDL of d 1.006–1.063 g/ml, and HDL of d 1.063–1.21 g/ml. ApoB is the primary protein component of VLDL and LDL as well as of Lp(a), a relatively minor lipoprotein of d 1.050–1.090 mg/ml, which corresponds to the "sinking pre-beta" lipoprotein observed by electrophoresis. Lp(a) overlaps the density range of HDL which contains apoA-I as the major protein.

HDL may be estimated as cholesterol in the plasma fraction of d > 1.063 g/ml after ultracentrifugation (4). A more practical approach, however, is the specific precipitation of apoB-containing lipoproteins, primarily VLDL and LDL, by sulfated polysaccharides with divalent cations followed by measurement of cholesterol in the supernate (7, 8). A common procedure involves precipitation with sodium heparin at 1.2–2.0 mg/ml and $MnCl_2$ at 0.046 M (final concentrations) (4, 6, 9). A recent study (10) showed partial precipitation of isolated HDL by heparin–Mn^{2+}

Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoB, apolipoprotein B; EDTA, ethylenediamine tetraacetic acid disodium salt; HDL, high density lipoprotein of d 1.036–1.21 g/ml; LDL, low density lipoprotein of d 1.006–1.063 g/ml; RID, radial immunodiffusion; VLDL, very low density lipoprotein of d 1.006 g/ml.

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treatment, suggesting possible underestimation of HDL by this method. More recently, however, two groups reported reasonable agreement between cholesterol concentrations in d > 1.063 g/ml plasma fractions and heparin–Mn^{2+} supernates (11, 12). In these studies precipitation of LDL and VLDL was reportedly complete, but the relatively insensitive techniques used to measure unprecipitated apoB-containing lipoproteins could not detect apoB at levels lower than 5 mg/dl. Furthermore, these studies did not directly determine the extent of HDL precipitation by the heparin–Mn^{2+} procedure. Finally, none of these studies dealt with grossly hypertriglyceridemic samples, from which the removal of apoB-associated lipoproteins is often incomplete.

In the present study we compared the ultracentrifugal technique with the heparin–Mn^{2+} procedure for the estimation of HDL cholesterol in plasma samples. We determined by sensitive immunochemical procedures the extent of incomplete LDL–VLDL precipitation and the amount of HDL precipitated at various heparin and Mn^{2+} concentrations. We examined procedures for quantitating HDL in hypertriglyceridemic samples and studied other reaction conditions, including incubation time, temperature, and centrifugation force. As a result of this study, we recommend a number of modifications to the currently used heparin–Mn^{2+} procedure.

MATERIALS AND METHODS

Samples

Plasma samples were obtained from subjects participating in population, genetic, and coronary prevention studies conducted by the Northwest Lipid Research Clinic as well as from referral patients and healthy volunteers. Subjects reported in the morning, after a 12–14 hr fast, for blood collection from the antecubital vein into Vacutainer tubes containing 1 mg/ml of dry EDTA according to Lipid Research Clinic protocol (6). Immediately, after thorough mixing, samples were cooled to 4°C, and cells were removed by centrifugation within 2 hr. Plasma with the level of EDTA stated above was used in all experiments except those noted. Most experiments were performed with plasma or serum from a single subject; in some cases, however, plasma samples were prepared with predetermined lipid levels by pooling plasma containing the appropriate levels approximately 1 week after collection. For the comparisons of plasma and serum, blood was drawn into tubes without anticoagulant; it was then pooled, mixed thoroughly, and divided between two tubes. One tube (plasma) contained EDTA at 1 mg/ml; the other (serum) had no anticoagulant. After 30 min at room temperature both tubes were centrifuged.

Heparin–manganese precipitation procedures

Sodium heparin (Lipo-Hepin, 40,000 USP units/ml, 282 mg/ml, from Riker Laboratories, Northridge, CA) was diluted 1:4, 1:8, and 1:16 with 0.15 M NaCl. Solutions of 1.0, 2.0, 3.0, and 4.0 M were prepared with $MnCl_2 \cdot 4\ H_2O$ from Matheson, Coleman, and Bell, Norwood, OH. All manganese reagents were found to be within 2% of calculated values by titration with EDTA (13). Heparin–manganese precipitations were generally performed within 4 days of sample collection. In the usual procedure, a modification (6) of the original method of Burstein and Samaille (9), 0.12 ml of sodium heparin (1:8 dilution, 35.2 mg/ml) and 0.15 ml of 1.0 M $MnCl_2$ were added sequentially to 3.0 ml of plasma, with thorough mixing by a Vortex mixer. The final concentrations of heparin and Mn^{2+} in the plasma were 1.29 mg/ml and 0.046 M, respectively. Volume measurements were made with Pipetman manual pipets (West Coast Scientific, Oakland, CA), which were tested monthly for accuracy (<1% at 3.0 ml, <2% at 0.12 ml). After reagent addition, samples stood for 30 min at 4°C before centrifugation at 1,500 *g* for 30 min. Supernatant solutions were recovered for analysis by pipetting.

Various modifications of this procedure were evaluated as indicated in Results. In some experiments heparin and Mn^{2+} were added to plasma diluted 1:3 with 0.15 M NaCl, isolated plasma lipoprotein fractions, or serum. A range of final heparin and Mn^{2+} concentrations were evaluated by adding the same proportionate volumes, as above, of reagent solutions at the appropriate concentrations. Heparin–Mn^{2+} precipitates were prepared for analysis by resuspending them twice sequentially in 0.15 M NaCl containing heparin and Mn^{2+} at the original precipitating concentration, resedimenting by centrifugation, and suspending the washed precipitate in 0.1 M EDTA. As noted, samples after heparin–Mn^{2+} treatment were incubated at room temperature for various time periods before centrifugation. Certain samples were subjected to centrifugation at 6000 *g* or 12,000 *g* for 10 min. Centrifugation of heparin–Mn^{2+}-treated hypertriglyceridemic samples often produced a layer of aggragated lipoprotein above a clear subnatant solution. An uncontaminated sample of the subnatant solution was obtained with a fine-tipped Pasteur pipet.

A procedure for heparin–Mn^{2+} precipitation incorporating the following modifications was evaluated. A working heparin–manganese solution was

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prepared by adding 0.6 ml of sodium heparin solution (Riker Lipo-Hepin, 40,000 USP units/ml, ca. 280 mg/ml) to 10.0 ml of 1.06 M $MnCl_2$ solution. To 2.0 ml of plasma was added 0.2 ml of the heparin–manganese solution with thorough mixing, which produced a final heparin concentration of approximately 1.4 mg/ml and a Mn^{2+} concentration of 0.092 M. Samples were incubated at least 10 min at room temperature and centrifuged at 1500 *g* for 30 min. Clear supernatant solutions were recovered for lipid analysis by pipetting. Supernatant solutions that remained turbid after centrifugation were again subjected to centrifugation at 12,000 *g* for 10 min, and the clear subnatant solution was recovered by pipetting with a fine-tipped Pasteur pipet.

Lipoprotein isolation by ultracentrifugation

The d > 1.006 g/ml fraction was obtained by a tube slicing technique from 5 ml of plasma that had been overlayered with 0.15 M NaCl and centrifuged for 18 hr at 105,000 *g* in the 40.3 rotor (6). Similarly the d > 1.063 g/ml fraction was obtained from 5 ml of plasma adjusted to d 1.063 g/ml by addition of 415 mg of dried KBr and overlayered with d 1.063 g/ml KBr solution followed by centrifugation at 105,000 *g* for 24 hr. These fractions, including the d > 1.063 g/ml fraction after dialysis against 0.15 M NaCl, were adjusted to the original plasma volume. Recovery of cholesterol from the top and bottom fractions after centrifugation at d 1.006 g/ml was 97% and at d 1.063 g/ml was 94%.

HDL was isolated from fresh donor plasma as the d 1.063–1.21 g/ml fraction. The d > 1.063 g/ml fraction obtained after centrifugation at 100,000 *g* for 24 hr in the 60 Ti rotor was recentrifuged under the same conditions. After density adjustment to 1.21 g/ml with KBr and 44 hr of centrifugation at 100,000 *g*, the top fraction was recentrifuged at the same density. Fractions were dialyzed against 0.15 M NaCl and adjusted to a concentration equivalent to that in the original plasma.

Lipid analysis

Cholesterol and triglycerides were quantitated by Lipid Research Clinic procedures with a Technicon AutoAnalyzer II. The triglyceride method employed 2,4-pentanedione fluorometric quantitation, and cholesterol was measured by a Liebermann-Burchard reagent method after extraction into isopropyl alcohol with zeolite mixture. Standard solutions and quality control samples were provided by the Lipid Standardization Laboratory of the Center for Disease Control, Atlanta. Analysis conditions were designed to maximize precision and accuracy at the low lipid levels of the heparin–Mn^{2+} supernatants. Coefficients of variation of 0.38%, 0.90%, and 1.7% were obtained for pools with cholesterol concentrations of 280, 154, and 60 mg/dl, respectively, after analysis four times daily on the AutoAnalyzer for 12 months. The corresponding coefficients of variation for triglyceride in two pools with triglyceride concentrations of 156 and 69 mg/dl were 1.3% and 2.9%, respectively. Accuracy was within 2% of the Center for Disease Control's target values for all pools. To minimize sample interaction (carryover), which was a significant source of imprecision in the AutoAnalyzer II measurement of heparin-Mn^{2+} supernates, each sample in the comparison experiments was analyzed twice, sequentially, and the second value was recorded. This resulted in better precision than indicated by the quality control pools. Addition of sodium heparin at 2.5 mg/ml and $MnCl_2$ at 0.092 M did not significantly affect the cholesterol analysis. Because KBr at 1.063 g/l produced a 2.8% reduction in the plasma cholesterol measurement, all fractions containing KBr were dialyzed against 0.15 M NaCl before analysis. Supernatant cholesterol values were adjusted for the dilution due to the addition of heparin and Mn^{2+} solutions.

Lipoprotein analysis

Lipoprotein apoproteins were quantitated by RID and/or by double antibody radioimmunoassay. ApoB in the heparin–Mn^{2+} supernates was routinely quantitated by RID to test for completeness of LDL and VLDL precipitation (14). Supernatant solutions were added to 1.8-mm wells in 0.5% agarose (Sigma Chemical Co., St. Louis, MO) gels. The gel contained 0.02 M Tris pH 8.0 buffer with 0.15 M NaCl, 0.05% EDTA, 0.05% NaN_3, 0.5% bovine serum albumin (Sigma), and rabbit anti-human LDL determined to be specific for apoB (14). Dilutions of a d > 1.006 plasma fraction pool of known LDL cholesterol concentrations were used to standardize the assay. Precipitin ring diameters were measured after 72 hr of incubation at 37°C in a humidity chamber. Results were reported as apoB-associated cholesterol in mg/dl. Concentration was linear with the square of the ring diameter for apoB-associated cholesterol concentrations of up to 20 mg/dl. The assay's lower limit of sensitivity was LDL cholesterol of approximately 1 mg/dl. The coefficient of variation of a 2 mg/dl sample was 6% after 50 measurements over 2 months.

To confirm and provide a more sensitive measure of incomplete apoB-associated lipoprotein precipitation, the apoB in selected supernates was also quantitated by double antibody radioimmunoassay. By this technique the sample apoB displaces radiolabeled

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apo-B antigen from its antibody. After antigen–antibody complex precipitation by a second antibody, precipitated label is inversely related to sample apoB concentration. Rabbit anti-human LDL was used as the first antibody, sheep anti-rabbit immunoglobulin as the second antibody, and ^{125}I-labeled LDL as antigen (14, 15). Samples were diluted to the optimum concentration range. LDL of d 1.019–1.063 g/ml was used as standard.

HDL apolipoproteins A-I and A-II in resolubilized heparin–Mn^{2+} precipitates were quantitated by an RID technique (16, 17) similar to that described for apoB RID (14). However, the samples were diluted 1:1 with tetramethylurea (Burdick and Jackson Laboratories, Muskegon, MI), further diluted 1:5 with 0.01 M Tris pH 8.0 buffer containing 8 M urea, and then incubated at 37°C for 30 min before being applied to the wells. The immunoassay gel plates were incubated 72 hr at 37°C in a humidity chamber. Isolated apoA-I and A-II were used as standards.

Lipoprotein electrophoresis (18) was performed using the Biogram A system (BioRad, Richmond, CA). Slides were stained with Fat Red 7B. The presence of "sinking pre-beta lipoprotein" was indicated by a band with pre-beta mobility in the d > 1.006 fraction (19). The Lp(a) lipoprotein was quantitated in heparin–Mn^{2+} supernates by an RID procedure (20).

^{125}I-Labeled HDL precipitation experiments

HDL isolated as the d 1.063–1.21 g/ml plasma fraction from each of three donors was labeled with ^{125}I by the ICl procedure (21) to a sp act of 10^7 cpm/mg with approximately 1% of the label in lipid. Labeled fractions were dialyzed to remove free ^{125}I until less than 1% free iodine remained. Bovine serum albumin was added (final concentration 1%), and the solution was filtered through a 45 μ Millipore filter. Within a week after labeling, trace quantities of ^{125}I-labeled HDL (ca. 1 μg) were added to the plasma samples, to plasma diluted 1:3 with 0.15 M NaCl, to d > 1.006 g/ml plasma fractions, and to serum samples. To aliquots of each were added heparin to a final concentration of 1.29 mg/ml and Mn^{2+} to either 0.046 or 0.092 M. Alternatively heparin and Mn^{2+} were added to give final concentrations of 2.58 mg/ml and 0.184 M, respectively. After the total amount of radioactivity in each tube was determined, the precipitate was removed by centrifugation at 45,000 g-min. The radioactivity in an aliquot of the supernatant solution in each tube was measured to determine the percentage of ^{125}I-labeled HDL in the precipitate. Anti-LDL quantitatively precipitated from 2 to 5% of the radioactivity from the ^{125}I-labeled d 1.063–1.21 g/ml fraction of plasma. For each preparation this amount was subtracted from the percentage of radioactivity found in the heparin–Mn^{2+} precipitates to correct for the small amount of apoB-associated radioactivity present in the labeled preparation. By the same procedure ^{125}I-labeled HDL was added to isolated d 1.063–1.21 g/ml fractions, d < 1.21 g/ml fractions, d > 1.063 g/ml fractions, and d 1.063–1.21 g/ml fractions with human serum albumin at 4, 2, and 1 g/dl. Heparin and Mn^{2+} were added to the same final concentrations as in the previous experiment and the proportion of radioactivity in the precipitate was determined.

RESULTS

Cholesterol in d > 1.063 g/ml fractions vs. heparin–Mn^{2+} supernates

Cholesterol levels were compared in plasma fractions of d > 1.063 g/ml and heparin–Mn^{2+} supernates (1.29 mg/ml and 0.046 M, respectively) in 10 samples with and 25 samples without sinking pre-beta lipoproteins. The lipid distributions (mean ± SD, range in mg/dl) were as follows: sinking pre-beta positive group cholesterol 225 ± 72, 152–400; triglyceride 125 ± 57, 52–248; sinking pre-beta negative group cholesterol 208 ± 55, 120–344; triglyceride 120 ± 41, 46–328. In the 10 samples with high Lp(a) levels, sinking pre-beta positive, the d > 1.063 g/ml fraction cholesterol was 2.8 mg/dl higher than the heparin–Mn^{2+} supernates, 52.6 vs. 49.8 mg/dl. In sinking pre-beta negative samples, heparin–Mn^{2+} supernatant cholesterol was 0.6 mg/dl higher than the cholesterol in the d > 1.063 g/ml fraction, 47.0 vs. 46.4 mg/dl.

Precipitation by heparin–Mn^{2+}

During a 3-month period beginning in June 1976, all heparin–Mn^{2+} supernates from samples submitted to the Lipid Research Clinic Laboratory for HDL cholesterol quantitation were monitored for incomplete removal of apoB-associated lipoproteins (**Table 1**). In 9% of the samples (85 of 966), obvious supernatant turbidity (**Fig. 1**, tube b) indicated incomplete sedimentation of apoB-associated lipoproteins. Furthermore, of the nonturbid supernates (Fig. 1, tube a), 48% (424 of 881) contained at least 1 mg/dl of apoB-associated cholesterol assessed by the RID procedure of which 10% (41 of 424) had more than 5 mg/dl of apoB-associated cholesterol. Analysis of 12 of these by a RID assay for Lp(a) indicated that the unprecipitated apoB-associated material was not the Lp(a) lipoprotein.

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TABLE 1. Incomplete precipitation of apoB-associated lipoproteins from plasma by the present heparin–manganese procedure as indicated by supernatant turbidity and apoB (RID) levels[a]

Type of Sample	Number of Samples	Percent of All Samples	Plasma Concentration: Cholesterol	Plasma Concentration: Triglyceride	Supernatant Concentration: Cholesterol	Supernatant Concentration: ApoB-associated Cholesterol
			mg/dl ± SD		*mg/dl ± SD*	
All samples	966	100	241 ± 48	154 ± 87	48.8 ± 13.1[b]	
Turbid supernates	85	9	262 ± 44	315 ± 131		
Nonturbid supernates but apoB positive by RID	424	44	249 ± 46	147 ± 62	47.3 ± 12.5	2.5 ± 2.6

[a] Final heparin concentration 1.29 mg/ml, final Mn^{2+} concentration 0.046 M.
[b] Turbid supernates excluded from cholesterol and apoB analysis.

Optimum heparin and Mn^{2+} levels for HDL estimation

Heparin titrations of plasma pools similar to those reported previously (12) suggested that heparin concentrations above approximately 1 mg/ml and as high as 5 mg/ml in the presence of 0.046 M Mn^{2+} did not decrease supernatant cholesterol concentrations. In addition, at lower heparin concentrations, incomplete precipitation of the apoB-associated lipoproteins was indicated by obvious supernatant turbidity. Similarly heparin–Mn^{2+} supernatant cholesterol was determined as a function of Mn^{2+} concentration over the range from 0 to 0.18 M with heparin at 1.29 mg/ml (**Fig. 2**). In each titration, a Mn^{2+} concentration of approximately 0.02 M was required before any significant lipoprotein precipitation occurred. As the Mn^{2+} concentration was increased from 0.02 to 0.04, supernatant cholesterol decreased markedly. Further increases in Mn^{2+} concentration produced a further slight but consistent reduction in supernatant cholesterol. Significantly, at limiting Mn^{2+} concentrations below 0.04 M, the supernatant solutions were clear even though the cholesterol levels indicated grossly incomplete precipitation of the apoB-associated lipoproteins.

The optimum Mn^{2+} concentration for precipitating the apoB-associated lipoproteins without removing the apoA-carrying lipoproteins (HDL) was determined by comparing their precipitation at various Mn^{2+} levels (**Table 2**). The proportion of samples with turbid supernates was at a minimum at 0.092 M Mn^{2+} with heparin at 1.29 mg/ml, with only 3% (2 of 68) having turbid supernates. Among the samples with nonturbid supernates, supernatant cholesterol and apoB levels decreased with increasing Mn^{2+} levels, with a slight increase in precipitated apoA proteins. Supernates precipitated at 0.046 M

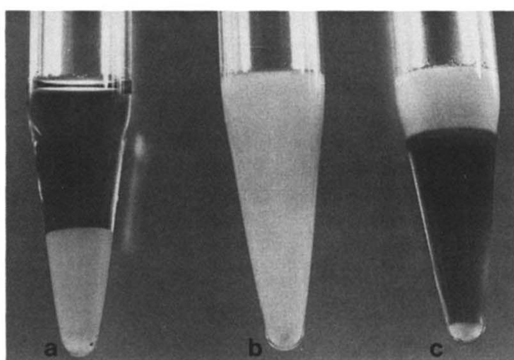

Fig. 1. Precipitation of lipoproteins from plasma samples by heparin (1.29 mg/ml) and Mn^{2+} (0.046 M). Plasma lipid levels, in mg/dl, were: *a*, cholesterol 198, triglyceride 116; *b*, cholesterol 250, triglyceride 870; and *c*, cholesterol 480, triglyceride 2980.

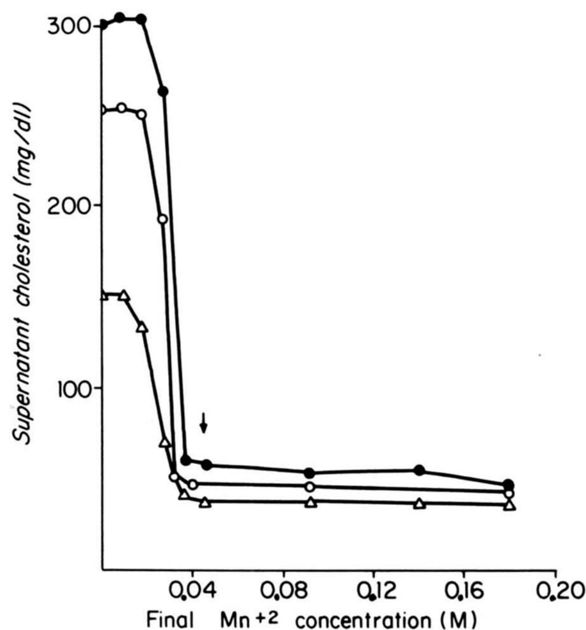

Fig. 2. Titration of plasma pools with $MnCl_2$ at a sodium heparin concentration of 1.29 mg/ml. △——△, Pool with plasma triglyceride of 44 mg/dl. ○——○, Pool with plasma triglyceride of 264 mg/dl. ●——●, Pool with plasma triglyceride of 184 mg/dl. Arrow indicates Mn^{2+} concentration used in current procedures.

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TABLE 2. Effect of heparin and manganese concentration on lipoprotein precipitation

	Heparin Concentration, mg/ml							
	0.046 M Mn^{2+}			0.092 M Mn^{2+}			0.138 M Mn^{2+}	0.184 M Mn^{2+}
	0.65	1.29	2.58	0.65	1.29	2.58	1.29	1.29
Number of samples tested	24	68	7	7	68	7	13	10
(a) Number of samples with turbid supernatant solution	13	7	0	1	2	0	2	5
(b) Analysis of nonturbid supernates[a]								
Normalized cholesterol[b]	3.4 ± 1.1 (11)	2.2 ± 1.2 (61)	3.8 ± 1.3 (4)	4.7 (3)	0 (66)	0.8 ± 1.9 (4)	−0.4 ± 2.3 (11)	−0.5 ± 1.1 (5)
Apolipoprotein B (RIA)	3.4 ± 2.0 (9)	2.5 ± 1.5 (44)	7.9 ± 4.6 (4)	0.3 (2)	0.5 ± 0.6 (44)	0.2 ± 0.2 (4)	0.2 ± 0.1 (5)	0.2 ± 0.2 (4)
(c) Analysis of precipitates								
Apolipoprotein A-I	0.9 ± 0.4 (4)	0.9 ± 0.5 (14)	1.3 ± 0.6 (4)	1.9 ± 0.9 (4)	1.6 ± 0.9 (14)	2.6 ± 0.8 (4)	2.8 ± 0.8 (4)	2.8 ± 1.0 (4)
Apolipoprotein A-II	0.3 ± 0.1 (4)	0.4 ± 0.1 (4)	0.4 ± 0.1 (4)	0.4 ± 0.1 (4)	0.5 ± 0.2 (4)	0.5 ± 0.2 (4)	0.6 ± 0.2 (4)	0.6 ± 0.2 (4)

[a] Results expressed as mean ± SD in mg/dl. The number in parentheses indicates number of nonturbid supernates and precipitates analyzed. Cholesterol analyses were performed on all supernates, apolipoprotein analyses were performed on a subset of supernates and precipitates selected at random.
[b] To allow direct comparison of results at each heparin/Mn^{2+} level, supernatant cholesterol was normalized by expressing the concentration as the difference between the value obtained at each heparin/Mn^{2+} level and that obtained at 0.092 M Mn^{2+}/1.29 mg per ml heparin.

Mn^{2+} averaged 2.5 mg/dl of apoB-associated cholesterol, whereas samples precipitated from plasma with 0.092 M Mn^{2+} had only 0.5 mg/dl of apoB-associated cholesterol and averaged 2.2 mg/dl lower cholesterol levels. At higher Mn^{2+} concentrations only a slight further reduction in supernatant cholesterol and apoB was produced. At 0.092 M Mn^{2+} the precipitation of apoB was nearly complete and the amount of apoA precipitated was negligible, equivalent to less than 0.5 mg/dl HDL cholesterol.

To confirm that the present heparin concentration is adequate, heparin concentrations of half and twice the usual levels were tested at 0.046 and 0.092 M Mn^{2+} concentrations (Table 2). With 0.046 M Mn^{2+} and 0.65 mg/ml heparin, more than half the samples tested (13 of 24) were incompletely sedimented as assessed by turbidity in the supernate. At 1.29 mg/ml heparin, however, only 10% (7 of 68) were incompletely sedimented. At 0.092 M Mn^{2+} with 0.65 mg/ml heparin, 25% (1 of 4) were turbid whereas with 1.29 mg/ml heparin, only 3% (2 of 68) were turbid. At 1.29 mg/ml heparin supernatant cholesterol and apoB levels were minimal and precipitate A-I–A-II levels were not excessive.

TABLE 3. Precipitation of ^{125}I-labeled high density lipoprotein[a] from plasma and serum samples by heparin–manganese treatment

Sample	Number of Samples	Heparin 1.29 mg/ml Mn^{2+} 0.046 M	Heparin 1.29 mg/ml Mn^{2+} 0.092 M	Heparin 2.58 mg/ml Mn^{2+} 0.184 M
Plasma	18	0.5 ± 0.6[b]	1.5 ± 1.1	2.2 ± 1.4
Plasma diluted 1:3 with 0.15 M NaCl	5	0.8 ± 0.4	2.3 ± 0.4	2.7 ± 0.7
d > 1.006 Plasma fraction	2	0.2	0.8	1.2
Serum	15	1.2 ± 1.2	2.9 ± 1.6	5.7 ± 2.1

[a] Tracer amounts (approximately 1 μg) of ^{125}I-labeled HDL, d 1.063–1.21 g/ml plasma fraction, were added to each sample.
[b] Percent of ^{125}I-labeled HDL in precipitate, mean ± SD.

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Precipitation of ^{125}I-labeled HDL

Tracer levels of ^{125}I-labeled HDL were added to representative types of samples to determine the extent of HDL precipitation at various heparin and Mn^{2+} levels (**Table 3**). At 1.29 mg/ml heparin and 0.046 M Mn^{2+}, 0.2–0.8% of the ^{125}I-labeled HDL was precipitated from plasma or plasma fractions. With 0.092 M Mn^{2+} more HDL precipitated, but was equivalent to less than 1 mg/dl from plasma and the d> 1.006 fraction. Slightly more was precipitated from plasma diluted 1:3. At the highest heparin and Mn^{2+} levels, only slightly more HDL was precipitated from plasma samples. Approximately twice as much HDL was precipitated from serum as from plasma at each heparin/Mn^{2+} level. Furthermore, in another experiment, serum heparin–Mn^{2+} supernatant cholesterol levels were consistently 1–5 mg/dl lower than in paired plasma supernates after precipitation at 0.092 M Mn^{2+} and 1.29 mg/ml heparin (mean, 54.0 mg/dl vs. 56.2 mg/dl; n = 13, $P < 0.1$). Also apoB-associated cholesterol was detected by RID in only 3% (2 of 65) of serum supernates precipitated at 1.29 mg/ml heparin and 0.046 M Mn^{2+} compared to 48% RID positive under the same conditions in plasma samples.

In contrast to the insignificant HDL precipitation by heparin–Mn^{2+} treatment of plasma, isolated HDL was precipitated (**Table 4**), confirming an earlier report (10). ^{125}I-Labeled HDL added in tracer amounts to isolated HDL, dialyzed d 1.063–1.21 g/ml plasma fractions, was precipitated to the extent of 11–20% at various heparin–Mn^{2+} concentrations. Addition of VLDL and LDL (d < 1.21 g/ml fraction) did not prevent HDL precipitation; however, the addition of other plasma proteins (d > 1.063 g/ml fraction) almost completely prevented precipitation of HDL. Human serum albumin also prevented HDL precipitation with a concentration-dependent effect. HDL precipitation assessed by cholesterol measurements was similar to that indicated by tracer ^{125}I-labeled HDL precipitation.

The previous experiments suggested 0.092 M Mn^{2+} and 1.29 mg/ml heparin to be optimum for removing apoB-associated lipoproteins from EDTA plasma without excessive HDL precipitation. Eighty-nine routine samples in which apoB-associated lipoprotein removal was incomplete at 0.046 M Mn^{2+} were reprecipitated at 0.092 M (**Table 5**). Twenty-two supernates were turbid at 0.046 M Mn^{2+}, compared to four at 0.092 M. Among 48 samples with apoB-associated cholesterol levels between 1.0 and 5.0 mg/dl at 0.046 M Mn^{2+}, only five had detectable apoB at 0.092 M. Of 19 samples with apoB-associated cholesterol greater than 5 mg/dl at 0.046 M, none had detectable apoB when precipitated with 0.092 M Mn^{2+}. The reduction in supernatant cholesterol from 0.046 to 0.092 M Mn^{2+} concentrations corresponded to the reduction in apoB-associated cholesterol as measured by the RID procedure.

HDL quantitation in hypertriglyceridemic samples

The treatment of hypertriglyceridemic samples with heparin–Mn^{2+} frequently produces a supernate that is turbid after centrifugation for 45,000 *g*-min (Fig. 1, tube b). Plasma pools with elevated triglyceride levels were treated with 0.046, 0.092, 0.138, and 0.184 M Mn^{2+} with 1.29 mg/ml heparin (**Table 6**). The lowest supernatant cholesterol and apoB-associated cholesterol levels were obtained with 0.092 M Mn^{2+}. In all pools at the highest Mn^{2+} level and in the pool of the highest triglyceride level at all Mn^{2+} levels, supernatant turbidity indicated incomplete sedimentation of the apoB-associated lipoproteins. With 0.046 M Mn^{2+}, the pool with 406 mg/dl triglyceride exhibited a layer of aggregated lipoprotein over a turbid subnatant solution. At 0.092 M aggregated

TABLE 4. Effect of heparin and manganese levels on ^{125}I-labeled high density lipoprotein[a] precipitation from isolated lipoprotein fractions[b]

Plasma Fraction Density	Added Human Serum Albumin, g/dl	Number of Samples	Heparin 1.29 mg/ml, Mn^{2+} 0.046 M	Heparin 1.29 mg/ml, Mn^{2+} 0.092 M	Heparin 2.58 mg/ml, Mn^{2+} 0.184 M
1.063–1.21 g/ml		2	15[c]	20	11
<1.21		2	26	26	14
>1.063		2	1	2	1
1.063–1.21	4	1	0	2	0
	2	1		6	
	1	1		9	

[a] Tracer amounts (approximately 1 μg) of ^{125}I-labeled HDL, d 1.063–1.21 g/ml plasma fraction, were added to each sample.

[b] Lipoprotein fractions isolated by ultracentrifugation and dialyzed against 0.15 M NaCl with 1 mM EDTA. Concentration is equivalent to that in the original plasma except as noted.

[c] Percent ^{125}I-labeled HDL in precipitate, mean ± SD.

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TABLE 5. Improved precipitation of apoB-associated lipoproteins by the heparin–manganese technique at 0.092 M manganese vs. 0.046 M as indicated by supernatant turbidity and apoB-associated cholesterol levels[a]

Samples	0.046 M Mn^{2+}[a]			0.092 M Mn^{2+}[a]			
	Number	Cholesterol	ApoB-associated Cholesterol[c]	Number Turbid	Cholesterol	Number ApoB Positive	ApoB-associated Cholesterol[c]
		mg/dl ± SD[d]			*mg/dl ± SD*[d]		*mg/dl ± SD*[d]
Turbid supernatant[b] at 0.046 M Mn^{2+}	22			4	39.3 ± 7.8	5	1.7 ± 0.9
Nonturbid with apoB-associated cholesterol of:							
1–5 mg/dl	48	45.3 ± 12.7	3.1 ± 1.2	0	41.8 ± 12.7	5	1.4 ± 0.5
≥5 mg/dl	19	52.7 ± 8.3	8.8 ± 3.3	0	45.2 ± 7.2	0	0

[a] Heparin at 1.29 mg/ml.
[b] Incomplete sedimentation indicated by turbidity in heparin–Mn^{2+} supernate. Samples excluded from cholesterol and apoB analysis.
[c] In apoB positive samples.
[d] Concentration in heparin–manganese supernate.

lipoproteins sedimented with slight turbidity in the supernatant solution. At 0.138 M more of the aggregated lipoproteins remained suspended in the supernate, and at 0.184 M Mn^{2+} aggregated material was again layered over a slightly turbid subnatant solution.

An approach to obtaining sedimentation of apoB-containing lipoproteins from hypertriglyceridemic samples has been to remove the triglyceride-rich lipoproteins by 18 hr ultracentrifugation of plasma before heparin–Mn^{2+} treatment (6). In a comparison on 22 normolipidemic samples, the mean supernatant cholesterol in the d > 1.006 g/ml fractions was only 0.1 mg/dl lower than in the corresponding plasma samples after precipitation with 0.046 M Mn^{2+}. Also, precipitation of ^{125}I-labeled HDL by the heparin–Mn^{2+} procedure from d > 1.006 g/ml plasma fractions was negligible (Table 3).

A second approach has been to dilute the hypertriglyceridemic sample with 0.15 M NaCl before heparin–Mn treatment (6, 9). Fourteen plasma samples with triglyceride levels from 207 to 880 mg/dl that produced turbid supernates after precipitation with 1.29 mg/dl heparin and 0.046 M Mn^{2+} were repeated after 1:3 dilution. Supernates obtained after centrifugation at 45,000 *g*-min were clear with no measurable apoB-associated lipoproteins. Supernatant cholesterol levels in 24 undiluted normolipidemic plasma samples were compared with levels in the same samples diluted 1:3. With 0.046 M Mn^{2+}, the supernatant cholesterol (corrected for dilution) in the 1:3 dilutions averaged 1.1 mg/dl higher than in the undiluted plasmas. With 0.092 M Mn^{2+} supernatant cholesterol levels in the 1:3 dilutions were 2.5 mg/dl lower than in the same samples precipitated without dilution. As might be expected, dilution reduced the precision of the cholesterol measurement; with 0.046 M Mn^{2+}, the coefficient of variation for the un-

TABLE 6. Effect of manganese concentration on lipoprotein precipitation in hypertriglyceridemic pools: plasma heparin–manganese supernatant lipid and apoB-associated cholesterol levels (RID)[a]

Plasma Lipid Concentration, mg/dl		Manganese Concentration, M											
		0.046			0.092			0.138			0.184		
		Concentration in Supernate, mg/dl											
CH[b]	TG[c]	CH	TG	ApoB-CH[d]	CH	TG	ApoB-CH	CH	TG	ApoB-CH	CH	TG	ApoB-CH
252	269	37	225	11	31	21	<1	37	27	5	203	225	64[e]
284	269	38	15	12	33	15	<1	32	15	<1	70	53	33[e]
257	351	181	234	57[e]	44	33	6	116	140	60[e]	239	329	60[e]
221	406	182	344	64[e,f]	66	87	20[e]	194	375	66[e]	55	70	21[e,f]

[a] Heparin at 1.29 mg/ml.
[b] CH, cholesterol.
[c] TG, triglyceride.
[d] ApoB-CH, cholesterol associated with apoB.
[e] Incomplete sedimentation indicated by turbidity in supernatant solution.
[f] Heparin–manganese lipoprotein aggregate partially layered above subnatant solution after centrifugation at 1,500 g for 30 min.

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diluted samples was 1.1%, as opposed to 3.9% for the 1:3 dilutions. Furthermore, precipitation of ^{125}I-labeled HDL was slightly greater from plasma diluted 1:3 than from undiluted plasma (Table 3).

Heparin–Mn^{2+} treatment of samples with triglyceride levels above approximately 1,000 mg/dl often produced a layer of aggregated lipoprotein over a clear subnatant solution after centrifugation for 45,000 *g*-min (Fig. 1, tube c). The same result was obtained with samples of intermediate triglyceride levels (approximately 200–1,000 mg/dl) after centrifugation at higher *g* forces even though centrifugation for 45,000 *g*-min produced a turbid supernate. For example, 14 heparin–Mn^{2+} (0.046 M) supernates from samples with plasma triglyceride levels of 207 to 880 mg/dl, turbid after centrifugation for 45,000 *g*-min, produced clear subnatant solutions under a layer of aggregated lipoprotein when re-subjected to centrifugation for 10 min at 12,000 *g*. The apoB-associated cholesterol levels in the clear solutions averaged 1.7 mg/dl, which is similar to levels obtained after precipitation of normolipidemic samples at 0.046 M Mn^{2+}. The mean cholesterol was 42.2 mg/dl, compared with 40.8 mg/dl for aliquots of the same samples diluted 1:3 prior to heparin–Mn^{2+} treatment. Mn^{2+} at 0.092 M provided improved sedimentation of the apoB-associated lipoproteins from moderately hypertriglyceridemic samples (Tables 2 and 5). Fourteen samples with plasma triglyceride levels between 1,000 and 5,430 mg/dl and nine samples with triglyceride levels between 532 and 1,000 mg/dl were treated with heparin at 1.29 mg/ml and Mn^{2+} at 0.092 M. Centrifugation for 45,000 *g*-min produced clear subnatant solutions that contained less than 1.0 mg/dl of apoB-associated cholesterol. Increased centrifugation force rather than longer time was required to clear supernates turbid after centrifugation at 1500 *g* for 30 min.

Other modifications to the heparin–Mn^{2+} procedure

Burstein and Samaille's original method (9) for HDL cholesterol quantitation specified centrifugation at 6000 *g* for 10 min. We have followed a more recent adaption (6) recommending 1500 *g* for 30 min. A test was made to determine whether the unprecipitated apoB-associated lipoproteins in nonturbid supernates at 0.046 M Mn^{2+} were a result of the decrease in centrifugation force. In a paired comparison of 16 normolipidemic plasma samples, the mean heparin–Mn^{2+} supernatant cholesterol after centrifugation at 6000 *g* for 10 min was 48.6 mg/dl, exactly the same as after centrifugation at 1500 *g* for 30 min. In a second comparison of 15 samples, the mean supernatant cholesterol after centrifugation at 1500 *g* for 30 min was 45.3 mg/dl, compared to 44.9 mg/dl for aliquots of the same samples subjected to centrifugation at 12,000 *g* for 10 min. Nine samples treated with 0.046 M Mn^{2+} and 1.29 mg/ml heparin and each divided into three aliquots, which were subjected to centrifugation at 1500 *g* for 30, 40, and 50 min, respectively, produced mean supernatant cholesterol levels of 56.6, 57.1, and 57.3 mg/dl. Similarly, the same samples treated with 0.092 M Mn^{2+} had supernatant cholesterol levels of 55.1, 54.3, and 55.0 mg/dl, respectively. Also, precipitation of added tracer ^{125}I-labeled HDL was not increased by the higher centrifugation forces.

Heparin–Mn^{2+} supernates, clear immediately after centrifugation, frequently develop a cloudy appearance after overnight storage at 4°C, which is reportedly due to manganese oxide formation (8). Removal of this material from supernates, precipitated at 0.046 M Mn^{2+}, by centrifugation did not significantly reduce supernatant cholesterol. The mean supernatant (at 0.046 M Mn^{2+}) cholesterol of 22 samples was 41.2 mg/dl before the removal of the cloudy precipitate by centrifugation and 40.8 mg/dl after removal. However, in some supernates at 0.092 M Mn^{2+}, a precipitate containing as much as 10–15 mg/dl HDL cholesterol was observed after approximately 2 weeks storage at 4°C. Therefore, it is advisable to resuspend this secondary precipitation by thorough mixing before lipid analysis.

In all experiments described previously, heparin and Mn^{2+} solutions were added to plasma separately. However, combining the two as a single reagent did not affect results. The mean heparin–Mn^{2+} supernatant cholesterol level in 23 samples when the heparin and manganese were added as a combined reagent solution was 41.1 mg/dl compared with 41.0 mg/dl when the reagents were added to the plasma separately. The combined reagent was stable for at least a month; precipitation by solutions over a month old produced the same results as fresh solutions.

A current procedure for HDL quantitation by the heparin–Mn^{2+} technique (6) recommended incubation at 4°C for 30 min after reagent addition. We observed no difference in supernatant cholesterol values with incubation times of 5–60 min. The six pools tested had plasma triglycerides of 122–406 mg/dl. Ten samples incubated at 4°C for 10 min had a mean supernatant cholesterol concentration of 41.4 mg/dl vs. 41.0 mg/dl for aliquots of the same samples incubated for 30 min. A third aliquot of the 10 samples incubated at 23°C for 30 min had a mean supernatant cholesterol concentration of 42.0 mg/dl.

A method was developed from these observations

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that incorporated all the conditions determined to be most suitable. Heparin and Mn^{2+} were added to plasma as a combined reagent to produce final concentrations of 1.4 mg/ml and 0.092 M, respectively, as explained in Methods. Samples were incubated at room temperature for 10 min prior to centrifugation at 1500 g for 30 min. Forty-six plasma samples precipitated according to this procedure had mean supernatant cholesterol concentrations of 40.8 mg/dl, vs. 43.7 mg/dl for the same samples precipitated with 0.046 M Mn^{2+} by following the current procedure (6). The difference reflected incomplete precipitation of apoB-associated lipoproteins at 0.046 M Mn^{2+}; no apoB-associated lipoproteins were detectable by RID in samples precipitated by the modified procedure.

DISCUSSION

HDL quantitation after ultracentrifugation

Due to the implication of HDL as a negative risk factor in cardiovascular disease, a need exists for an accurate but convenient quantitative method. Measurement of HDL after ultracentrifugation in the d > 1.063 g/ml fraction is not practical for routine analysis. The procedure is technically difficult, requires ultracentrifugation for approximately 24 hr, and is limited by the capacity of the ultracentrifugation equipment. In addition, the d > 1.063 g/ml fraction contains some apoB-associated lipoprotein, primarily Lp(a) (20, 22). Thus, when defining HDL in terms of its apoprotein composition, as the apoA-containing lipoproteins without apoB, the HDL cholesterol is overestimated in the d > 1.063 g/ml fraction by the amount of Lp(a) cholesterol, on the average 2–3 mg/dl.

Precipitation methods

Lipoproteins form insoluble complexes with sulfated polysaccharides and divalent cations (7, 8); the larger the sulfated polysaccharide or lipoprotein, the greater the tendency for complex formation. Mn^{2+} forms more stable complexes than Mg^{2+} or Ca^{2+}. Thus by appropriate reagent selection the principal lipoprotein classes, including HDL, can be selectively precipitated (8). One method for HDL quantitation employing sodium dextran sulfate 2000 (mol wt 2×10^6) and Ca^{2+} underestimated HDL by approximately 20 mg/dl compared to the ultracentrifugal method, presumably because of partial HDL precipitation (23).

A more common approach to HDL quantitation uses heparin and Mn^{2+} to precipitate the apoB-associated lipoproteins, primarily VLDL and LDL from plasma or serum with estimation of HDL as cholesterol remaining in the supernate. Recommended heparin concentrations are in the range 1.2–2.0 mg/ml with Mn^{2+} at 0.046 M (4, 6, 9). At these levels, reasonable agreement has been observed between heparin–Mn^{2+} supernatant and fraction d > 1.063 g/ml cholesterol (11, 12, 23). The d > 1.063 g/ml fraction cholesterol is relatively higher in samples with high Lp(a) levels (sinking pre-beta positive) (12). Similarly, we have found reasonable agreement between the ultracentrifugal and the heparin–Mn^{2+} methods. The heparin–Mn^{2+} supernatants in the two more recent studies (11, 12) did not contain measurable LDL. However, the immunodiffusion and electrophoretic methods used to detect unprecipitated apoB-containing lipoproteins were sensitive only to LDL cholesterol concentrations above approximately 5 mg/dl.

Heretofore, we have routinely measured HDL as cholesterol in heparin–Mn^{2+} supernatants of EDTA plasma with heparin at approximately 1.3 mg/ml and Mn^{2+} at 0.046 M final concentration. Using this procedure, we observed that approximately 9% of all plasma samples submitted for HDL cholesterol quantification by the Lipid Research Clinic laboratory during a 3-month period produced turbid heparin–Mn^{2+} supernates that contained high levels of apoB-associated lipoproteins. This was generally but not invariably associated with hypertriglyceridemic samples. Such samples were routinely repeated on a VLDL-free plasma fraction (d > 1.006 g/ml) or a diluted sample. Furthermore, an apoB RID procedure sensitive to LDL cholesterol concentrations of approximately 1 mg/dl indicated that 44% of the nonturbid supernates contained detectable apoB. The levels indicated overestimation of HDL cholesterol by approximately 2 mg/dl on the average.

We determined that increasing the heparin concentration did not improve precipitation of the apoB-associated lipoproteins. Supernatant cholesterol was relatively constant over a wide range of heparin concentrations above a critical level of approximately 1 mg/ml. Therefore, we concluded that with this heparin lot a level of 1.3 mg/ml was suitable. It is advisable, however, to test each lot by titration to determine the appropriate level. In contrast, the heparin–Mn^{2+} supernatant cholesterol and apoB levels were reduced by increasing the Mn^{2+} concentration. A concentration of 0.092 M appeared to be optimum for removing apoB-associated lipoproteins from plasma without precipitating significant amounts of HDL.

The original method for heparin–Mn^{2+} precipitation, which employed 0.046 M Mn^{2+}, quantitated HDL in serum rather than in EDTA plasma (9). Our data suggest that this level may be adequate

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for serum samples, which exhibited much lower levels of supernatant apoB at 0.046 M Mn^{2+} than did plasma samples. EDTA chelation of Mn^{2+} in plasma may reduce the effective concentrations, allowing incomplete apoB precipitation. The higher Mn^{2+} level (0.092 M) produced slight, although not excessive, precipitation of HDL from serum. We conclude, therefore, that although Mn^{2+} at 0.046 M may be adequate for serum samples, the 0.092 M level is preferable for plasma.

HDL precipitation

Srinivasan et al. (10) reported interaction of HDL with heparin in the presence of Mn^{2+} and approximately 25% precipitation of isolated HDL by heparin–Mn^{2+} treatment. Similarly, we found that approximately 20% of isolated HDL was precipitated by heparin–Mn^{2+} from a d 1.063–1.21 g/ml plasma fraction. This HDL precipitation did not appear to be due to the reduction in ionic strength by dialysis, as HDL was not precipitated from dialyzed whole plasma. Addition of the apoB-containing lipoproteins in the $d < 1.063$ g/ml plasma fraction to HDL did not prevent HDL precipitation. However, addition of the other plasma proteins in the $d > 1.21$ g/ml fraction, as well as albumin, virtually eliminated HDL precipitation by heparin–Mn^{2+} treatment. Reportedly, albumin as well as other plasma proteins is complexed by heparin–Mn^{2+} (7). These proteins may exert a protective effect by competing for heparin/Mn^{2+}, thereby lowering the effective concentration. Consistent with these results, we found that HDL precipitation from plasma or serum under the conditions of the heparin–Mn^{2+} procedure is not a significant problem.

HDL quantitation in hypertriglyceridemic samples

The quantitation of HDL in hypertriglyceridemic samples is particularly difficult, because the usual precipitation conditions frequently result in a turbid heparin–Mn^{2+} supernate grossly contaminated by apoB-associated lipoproteins. We have demonstrated that increasing the Mn^{2+} concentration to 0.092 M reduces the proportion of turbid supernates in hypertriglyceridemic samples. In addition, many of the remaining turbid samples can be cleared by recentrifugation at higher *g* forces. Clear heparin–Mn^{2+} subnatant solutions from samples with plasma triglyceride levels as high as 5000 mg/dl were essentially free of apoB-associated lipoproteins. Therefore, the lack of sedimentation in hypertriglyceridemic samples appears to be a function of the lipoprotein–heparin–Mn^{2+} complex density in relation to the solution density and not to an absence of insoluble complex formation.

A previous approach to HDL quantitation in hypertriglyceridemic samples has employed preliminary dilution of the sample with 0.15 M NaCl before addition of the heparin and Mn^{2+} reagents (9). This increases, relatively, reagent concentration and reduces solution density, facilitating sedimentation of the insoluble lipoprotein complex. However, dilution can compensate for only moderately elevated triglyceride levels and magnifies measurement imprecision of the already low supernatant cholesterol. A third disadvantage is a slight increase in HDL precipitation. Another approach has been removal of VLDL and chylomicrons by ultracentrifugation before heparin–Mn^{2+} treatment. While this procedure provides accurate HDL quantitation, provided that the recovery of the $d > 1.006$ g/ml fraction is complete, ultracentrifugation is often impractical and sometimes impossible. Therefore, a short high-speed centrifugation to clear turbid heparin–Mn^{2+} supernates after reagent addition appears to be a useful alternative.

Modifications of the heparin-Mn^{2+} procedure

Our evaluation of the heparin–Mn^{2+} procedure suggests several other changes that may improve convenience without sacrificing accuracy. Heparin and Mn^{2+} can be added to plasma as a single reagent. The reagents need be incubated with plasma for only 10 min prior to centrifugation and incubation may be at room temperature rather than 4°C.

The major change we recommend is a twofold increase in Mn^{2+} concentration to 0.092 M. Even though the average reduction in supernatant cholesterol between 0.046 M and 0.092 M Mn^{2+} is small (approximately 2 mg/dl), it is significant and, in some samples, the overestimation of HDL is much greater. Mn^{2+} at 0.046 M is very close to the critical concentration below which an inadvertent reduction in Mn^{2+} concentration could cause gross overestimation of HDL cholesterol. Even more importantly, the use of 0.092 M Mn^{2+} allows complete removal of aggregated apoB-associated lipoproteins from more of the hypertriglyceridemic samples, reducing the proportion of samples that require special handling to quantitate HDL. This is in agreement with Ishikawa et al. (11) who also recommended an increase in Mn^{2+} concentration. They observed that, although precipitation was usually complete with 0.046 M Mn^{2+} within their limits of detection for unprecipitated apoB-containing lipoproteins (5 mg/dl for LDL cholesterol), this concentration was at the lower limit for complete precipitation.

The nature of the apoB-associated lipoprotein that is precipitated by heparin–manganese treatment at a Mn^{2+} concentration of 0.092 M but not at 0.046 M is not known; however, it does not appear

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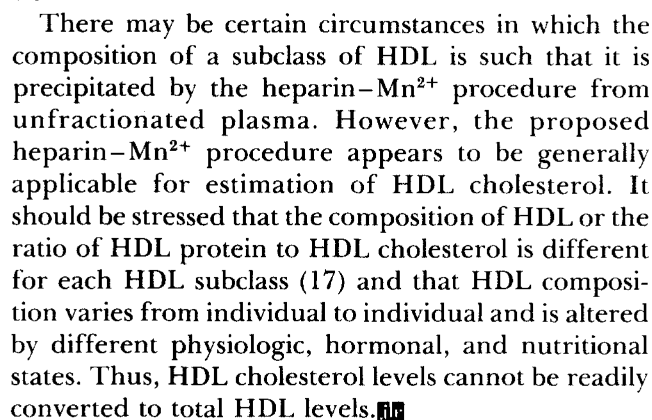
to be the Lp(a) lipoprotein. LDL is heterogeneous in size and composition, and the tendency for insoluble complex formation with sulfated polysaccharides and divalent cations varies with both of these parameters. Therefore, unprecipitated apoB in clear supernates may represent the smaller lipoproteins of the LDL spectrum, which require an increased Mn^{2+} concentration to become insoluble. In contrast, incomplete sedimentation reflects relative densities of the lipoprotein complex and the solution.



There may be certain circumstances in which the composition of a subclass of HDL is such that it is precipitated by the heparin–Mn^{2+} procedure from unfractionated plasma. However, the proposed heparin–Mn^{2+} procedure appears to be generally applicable for estimation of HDL cholesterol. It should be stressed that the composition of HDL or the ratio of HDL protein to HDL cholesterol is different for each HDL subclass (17) and that HDL composition varies from individual to individual and is altered by different physiologic, hormonal, and nutritional states. Thus, HDL cholesterol levels cannot be readily converted to total HDL levels.

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REFERENCES

1. Miller, G. J., and N. E. Miller. 1975. Plasma high density lipoprotein concentration and development of ischaemic heart disease. *Lancet.* **1:** 16–19.
2. Rhoads, G. G., C. L. Gulbrandsen, and A. Kagan. 1976. Serum lipoproteins and coronary heart disease in a population study of Hawaii Japanese men. *N. Engl. J. Med.* **294:** 293–298.
3. Glueck, C. J., R. W. Fallat, F. Millet, P. Gartside, R. C. Elston, and R. C. P. Go. 1975. Familial hyper-alpha-lipoproteinemia: Studies in eighteen kindreds. *Metabolism.* **24:** 1243–1265.
4. Fredrickson, D. S., R. I. Levy, and F. T. Lindgren, 1968. A comparison of heritable abnormal lipoprotein patterns as defined by two different techniques. *J. Clin. Invest.* **47:** 2446–2457.
5. Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. 1972. Estimation of the concentrations of low density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–502.
6. Manual of Laboratory Operations, Lipid Research Clinics Program. 1974. Lipid and lipoprotein analysis, DHEW Publication No. (NIH) 75-628.
7. Cornwell, D. G., and F. A. Kruger. 1961. Molecular complexes in the isolation and characterization of plasma lipoproteins. *J. Lipid Res.* **2:** 110–134.
8. Burstein, M., H. R., Scholnick, and R. Morfin. 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. *J. Lipid Res.* **11:** 583–595.
9. Burstein, M., and J. Samaille. 1960. Sur un dosage rapide du cholesterol lie aux alpha et aux beta-lipoproteins du serum. *Clin. Chim. Acta* **5:** 609.
10. Srinivasan, S. R., B. Radhakrishnamurthy, and G. S. Berenson. 1975. Studies on the interaction of heparin with serum lipoproteins in the presence of Ca^{++}, Mg^{++}, and Mn^{++}. *Arch. Biochem. Biophys.* **170:** 334–340.
11. Ishikawa, T. T., J. B. Brazier, P. M. Steiner, L. E. Stewart, P. S. Gartside, and C. J. Glueck. 1976. A study of the heparin–manganese chloride method for determination of plasma α-lipoprotein cholesterol concentration. *Lipids* **11:** 628–633.
12. Bachorik, P. S., P. D. Wood, J. J. Albers, P. Steiner, M. Dempsey, K. Kuba, R. Warnick, and L. Karlsson. 1976. Plasma high density lipoprotein cholesterol concentrations determined after removal of other lipoproteins by heparin–manganese precipitation or by ultracentrifugation. *Clin. Chem.* **22:** 1828–1834.
13. Flaschka, H., and A. M. Amin. 1953. Micro titrations with ethylene diamine tetraacetate. X. Direct titration of manganese in pure solutions and in the presence of other metals. *Mikrochim. Acta.* **19:** 414–420.
14. Albers, J. J., V. G. Cabana, and W. R. Hazzard. 1975. Immunoassay of human plasma apolipoprotein B. *Metabolism.* **24:** 1339–1351.
15. Rachmilewitz, D., J. J. Albers, and D. R. Saunders. 1976. Apoprotein B is fasting and postprandial human jejunal mucosa. *J. Clin. Invest.* **57:** 530–533.
16. Albers, J. J., P. W. Wahl, V. G. Cabana, W. R. Hazzard, and J. J. Hoover. 1976. Quantitation of apolipoprotein A-I of human plasma high density lipoprotein. *Metabolism.* **25:** 633–644.
17. Cheung, M. C., and J. J. Albers. 1977. The measurement of apolipoprotein A-I and A-II levels in men and women by immunoassay. *J. Clin. Invest.* **60:** 43–50.
18. Hatch, F. T., F. T. Lindgren, G. L. Adamson, L. C. Jensen, A. W. Wong, and R. I. Levy. 1973. Quantitative agarose gel electrophoresis of plasma lipoproteins: A simple technique and two methods for standardization. *J. Lab. Clin. Med.* **81:** 946–960.
19. Albers, J. J., V. G. Cabana, G. R. Warnick, W. R. Hazzard. 1975. Lp(a) lipoprotein: relationship to sinking pre-beta lipoprotein, hyperlipoproteinemia, and apolipoprotein B. *Metabolism.* **24:** 1047–1054.
20. Albers, J. J., and W. R. Hazzard. 1974. Immunochemical quantification of human plasma Lp(a) lipoprotein. *Lipids.* **9:** 15–26.
21. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
22. Albers, J. J., C. H. Chen, and F. Aladjem. 1972. Human serum lipoproteins. Evidence for three classes of lipoproteins in S_f 0–2. *Biochemistry.* **11:** 57–63.
23. Wilson, D. E., and M. J. Spiger. 1973. A dual precipitation method for quantitative plasma lipoprotein measurement without ultracentrifugation. *J. Lab. Clin. Med.* **82:** 473–482.

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