Quantitation of High Density Lipoproteins

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ABSTRACT

The demand for high density lipoprotein (HDL) quantitation has dramatically increased with the renewed awareness of the importance of HDL as a negative risk factor for coronary heart disease. HDL is usually estimated by specific precipitation of the non-HDL apoB-containing lipoproteins by polyanions and divalent cations followed by measurement of cholesterol in the supernatant. A common procedure involves precipitation with sodium heparin at 1.3 mg/ml and $MnCl_2$ at 0.046 M (final concentrations). This method is appropriate for serum but less than ideal for plasma because of incomplete precipitation and sedimentation of the apoB-containing lipoproteins. A two-fold increase in Mn^{2+} to 0.096 M improves precipitation of the apoB-associated lipoproteins from plasma without excessive precipitation of HDL. This modified heparin-Mn^{2+} procedure gives results nearly identical to the results with the ultracentrifugal reference method (cholesterol in the d>1.063 fraction corrected for losses and the presence of apoB-associated cholesterol). The dextran sulfate 500-Mg^{2+} and the sodium phosphotungstate-Mg^{2+} procedures give results consistently 2-4 mg/dl lower than does the reference method. In contrast, a heparin-Ca^{2+} method gives results 5-8 mg/dl higher than does the reference method. Immunochemical analysis of apoA-I in the precipitate and apoB in the supernatant indicates that lower values for the phosphotungstate-Mg^{2+} procedure is due to partial precipitation of the A-I-containing lipoproteins, while higher values by the heparin-Ca^{2+} method are due to incomplete precipitation of the apoB-containing lipoproteins. Quantitation of the principal apoproteins of HDL, A-I and A-II, represent an important additional index of HDL concentrations and composition. Quantitation of plasma A-I and A-II concentrations by radial immunodiffusion indicates that women generally have higher HDL concentrations than men (women, A-I, 135 ± 25, A-II, 36 ± 6; men, A-I, 120 ± 20, A-II, 33 ± 5; mean ± S.D., in mg/dl). A-I and A-II do not increase with age in men but show a slight increase with age in women. Estrogen increases HDL cholesterol and protein and may in part account for the higher HDL in women. The lighter density HDL subclass has a higher A-I/A-II ratio than the denser HDL subclass, with women generally having significantly more of the lighter HDL subclass. Density-gradient ultracentrifugation in $CsCl_2$ gradients indicates that HDL contains subpopulations of differing hydrated density which vary in the A-I/A=II ratio. Immunoassay of A-I and A-II when used in combination with HDL cholesterol analysis is a powerful tool for studies of HDL structure, epidemiology and metabolism.

INTRODUCTION

High density lipoproteins (HDL) are generally defined in terms of one or more of their physico-chemical properties; i.e., hydrated density, flotation characteristics in the analytical ultracentrifuge, electrophoretic mobility, and apolipoprotein composition. The fraction of plasma lipoproteins with d 1.063-1.21 has been operationally defined as the high density lipoproteins (1,2). Since HDL have alpha-electrophoretic mobility, they are often alternatively called alpha lipoproteins. More precisely, HDL can be defined in terms of apoprotein composition as the apolipoprotein A (apoA)-containing lipoproteins which are free of apolipoprotein B (apoB) (3). However, the HDL ultracentrifugal fraction contains some apoB-containing lipoproteins, principally the Lp(a) lipoprotein found primarily in the d 1.050-1.090 plasma fraction (4,5) and may also contain some non-Lp(a) apoB-containing lipoproteins (6). Thus, if we define HDL (or more appropriately the alpha lipoproteins) in terms of their apoprotein composition, then the apoB-containing lipoproteins in the d 1.063-1.21 fraction must be excluded from our definition of HDL. For the purpose of this treatise, HDL will generally be considered synonymous with the alpha lipoproteins, but special emphasis will be placed upon techniques which optimize the quantitation of lipoproteins that contain apoA and are free of apoB.

HDL CHOLESTEROL QUANTITATION

With the new awareness of the importance of HDL as a negative risk factor for coronary heart disease (7-9), the demand for HDL quantitation has dramatically increased. HDL is generally estimated by measuring its cholesterol content. A practical approach which avoids ultracentrifugation is the selective precipitation of the non-HDL apoB-associated lipoproteins with sulfated polysaccharides and divalent cations and the measurement of cholesterol in the supernatant solution. A common procedure involves precipitation of the apoB-associated lipoproteins with sodium heparin at 1.3 mg/dl

TABLE I

Heparin-Mn^{2+} Precipitation Procedure

Standard Method	Modified Method
1. 3 ml plasma	1. 2 ml plasma
2. 0.12 ml 5000 units heparin/ml	2. 0.2 ml combined heparin-Mn^{2+} reagent (0.6 ml 40,000 units heparin/ml, 10 ml 1.06M $MnCl_2 \cdot 4\ H_2O$)
3. 0.15 ml 1 M $MnCl_2 \cdot 4\ H_2O$	3. Incubate 10 min at 23 C
4. Incubate 30 min at 4 C	4. Centrifuge 1500 X g for 30 min at 4 C
5. Centrifuge 1500 X g for 30 min at 4 C	

and $MnCl_2$ at 46 mM (10-12).

This method (Table I), originally applied to serum (10), appears reasonably specific and not often subject to large errors, precipitating generally less than 2% of the HDL (13,14), yet producing nearly complete removal of the apoB-associated lipoproteins. However, if this precipitation method is applied to plasma containing EDTA, it is less than ideal, since about 10% of the plasma samples so treated have supernatants with obvious turbidity, indicating incomplete sedimentation. Furthermore, about half of the samples with nonturbid supernatants still contain ca. 2 mg/dl apoB, leading to slight overestimation of HDL cholesterol. A two-fold increase in Mn^{2+} concentration to 92 mmol Mn^{2+}/liter improves precipitation of the apoB, without substantial precipitation of HDL, and sediments better the apoB associated lipoproteins from hypertriglyceridemic plasma (14). Therefore, a more accurate and more convenient modified version of the heparin Mn^{2+} precipitation technique is recommended for plasma, namely a smaller sample volume, 2 ml instead of 3 ml, a two-fold increase in the Mn^{2+} reagent concentration, addition of heparin Mn^{2+} as a combined reagent, followed by incubation at room temperature for 10 min instead of 30 min at 4 C (Table I). A collaborative study by eight laboratories to evaluate the modified heparin Mn^{2+} precipitation procedure also shows the modified procedure to be both more specific and more convenient. By the standard method, 32 of 375 (9%) of the samples had turbid supernatants, and 72% of the samples had detectable apoB in nonturbid samples. On the other hand, of the plasma samples precipitated by the modified procedure, only 2% had turbid supernatants and only 15% had detectable apoB in nonturbid supernatants (15).

With hypertriglyceridemic samples, the density of the heparin-Mn^{2+} lipoprotein aggregate frequently is too low for it to sediment. These lipoproteins thus remain suspended in the solution, producing obvious turbidity. A number of procedures have been described for circumventing the problem of turbid supernatants (Table II). Method 1 is expensive and time consuming. Method 2 magnifies the imprecision of the measurement of cholesterol in the heparin-Mn^{2+} supernatant. Method 3 is satisfactory for the majority of samples, but requires high speed centrifugation (14). Method 4 removes essentially all of the apoB-associated lipoproteins without removing appreciable amounts of HDL (16). It appears to be the simplest and most convenient procedure for the clinical laboratory.

TABLE II

Methods for HDL Estimation in Hypertriglyceridemic Samples

1. Centrifuge plasma 105,000 X g for 18 hr. Do precipitation on the d>1.006 fraction.
2. Dilute plasma with an equal volume of isotonic saline before precipitation.
3. Perform precipitation. Centrifuge turbid supernatants 12,000 X g for 10 min. Determine cholesterol on clear subnatant solution.
4. Perform precipitation. Filter turbid supernates with 0.22 μm filter protected by two depth prefilters. Determine cholesterol on clear filtrate.

Since Mn^{2+} interferes with the enzymatic cholesterol procedure, other polyanion-cation combinations including dextran sulfate 500 (Pharmacia, Piscataway, NJ 08854)-$MgCl_2$ (17), sodium phosphotungstate-$MgCl_2$ (18), and heparin-$CaCl_2$ (19), along with an ultracentrifugal method at d 1.063 were evaluated (Table III). The ultracentrifugal approach was improved as a reference method by correcting the cholesterol of the d>1.063 g/ml fraction for losses (5%) and the presence of apoB-associated cholesterol (average 5.7 mg/dl, as determined

TABLE III

Comparison of Mean HDL Cholesterol by Several Techniques[a]

Samples							
		Corrected	Heparin-Mn^{2+}		Dextran		
Source	n	d>1.063 fraction[b]	.046 M[c]	.092 M[d]	Sulphate[e] 500 Mg^{2+}	Phosphotungstate[f] Mg^{2+}	Heparin[g] Ca^{2+}
Plasma							
Women	30	61.7	63.9	60.7	58.1	57.4	66.7
Men	27	44.4	45.7	44.1	43.6	42.8	51.5
Children	8	52.2	53.0	50.8	48.0	49.0	57.2
All Subjects	65	53.4	55.0	52.6	50.9	50.3	59.2
Serum							
Women	10	70.0	70.5	66.9	62.4	64.7	75.0
Men	10	44.7	44.2	42.8	40.0	41.6	53.1
All Subjects	20	57.4	57.4	54.8	51.2	53.2	64.0

[a]Results expressed in mg/dl *

[b]Corrected for losses of cholesterol during ultracentrifugation and the presence of apoB-associated cholesterol.

[c]According to the Lipid Research Clinics Procedure (12).

[d]According to Warnick and Albers (14).

[e]According to Kostner (17).

[f]According to Lopes et al. (18).

[g]According to Srinivanson et al. (19).

TABLE IV

Rid Assay for Human Plasma A-I and A-II

1. 50 µl plasma or test sample.
2. 50 µl tetramethylurea, mix.
3. 400 µl 8 M urea, 10 mM Tris pH 8.0, mix.
4. Incubate 30 min.
5. Add 4 µl of the mixture to the antibody-containing Agarose plate.
6. Incubate 24-72 hr.
7. Measure diameter of precipitation rings.

TABLE V

Cholesterol and A-I and A-II in Ultracentrifuged Subfractions of HDL[a]

	d 1.063 - 1.10		d 1.10 - 1.21	
	Men	Women	Men	Women
Cholesterol	9.9	18.6	32.3	36.1
A-I	10.3	23.8	97.2	104.4
A-II	2.0	3.9	26.6	27.4
A-I/A-II	5.1	6.1	3.7	4.0

[a]Results expressed in mg/dl. Each number is a mean and represents the analysis of six ultracentrifuged fractions from six plasma pools. Each pool consisted of plasma samples from three or four healthy normolipidemic adults.

by radial immunodiffusion with anti-apoB). The dextran-sulfate 500-Mg^{2+} method consistently gave HDL cholesterol values on plasma samples 2-4 mg/dl lower and on serum samples 4-8 mg/dl lower than the HDL values estimated by centrifugation. Similarly, the sodium phosphotungstate-Mg^{2+} procedure consistently gave lower results than did the ultracentrifugal method. In contrast, the heparin-Ca^{2+} method gave results 5-8 mg/dl higher than the centrifugal reference method. Analyses of apoA-I in the precipitates and apoB in the supernatant fluid by immunoassay suggest that lower values by the phosphotungstate-Mg^{2+} procedure are due in part to increased precipitation of the apoA-I-containing lipoproteins, while higher values by the heparin-Ca^{2+} method are due to incomplete precipitation of the apoB-containing lipoproteins.

On plasma samples, the modified heparin Mn^{2+} method, which uses 0.092 M Mn^{2+}, was in excellent agreement with the estimation of HDL cholesterol by the ultracentrifugal method, while the Lipid Research Clinic heparin Mn^{2+} method using 0.046 M Mn^{2+} gave 1-to-3 mg/dl higher values. However, on serum samples, the heparin Mn^{2+} procedure using 0.046 M Mn^{2+} agreed the best with the ultracentrifugal estimation of HDL cholesterol. Thus, 0.046 M Mn^{2+} appears adequate for precipation of HDL from serum but a higher Mn^{2+} concentration is needed for complete precipitation of apoB-containing lipoproteins from plasma, probably because Mn^{2+} is bound by EDTA (14,15). Yet, EDTA-treated plasma is preferred to serum for lipid and lipoprotein determinations since, during clot formation,

TABLE VI

Mol Wt, Apolipoprotein and Cholesterol Analyses on HDL Subfractions Isolated by Density Gradient Centrifugation[a]

Density of Subfraction	10^{-5} x Mol Wt	A-I (mg/dl)	A-II (mg/dl)	CH (mg/dl)	A-I/A-II (Molar Ratio)	CH/(A-I+A-II) (Wt. Ratio)
(Male)						
1.151-1.200	1.2	16.2	3.9	4.1	2.6	0.21
1.119-1.150	1.4	29.3	8.4	11.9	2.2	0.32
1.106-1.118	2.0	23.4	6.3	11.4	2.3	0.38
1.093-1.105	2.4	14.6	3.4	7.4	2.7	0.41
1.083-1.092	3.0	9.9	1.4	5.9	4.4	0.53
1.077-1.082	3.3	7.3	0.8	4.9	5.7	0.63
1.063-1.076	3.7	3.9	0.4	3.5	6.0	0.82
(Female)						
1.151-1.200	1.2	13.2	3.1	3.0	2.6	0.19
1.119-1.150	1.4	27.9	7.9	10.7	2.2	0.30
1.106-1.118	2.0	22.6	6.7	11.3	2.1	0.38
1.093-1.105	2.4	14.4	3.0	6.5	2.9	0.38
1.083-1.092	3.0	11.1	1.5	5.9	4.6	0.47
1.077-1.082	3.3	8.4	0.9	5.6	5.5	0.61
1.063-1.076	3.7	4.2	0.5	3.5	5.3	0.75

[a]Mean values expressed in mg/dl from six healthy normolipidemic adult volunteers (three male and three female). Mol wt estimated by density gradient electrophoresis. A-I and A-II determined by radial immunodiffusion. HDL of d 1.063-1.25 was placed in a CsCl gradient and centrifuged in a SW 41 rotor at 40,000 rpm at 16 C for 72 hr.

chylomicrons can be removed by the clot, and changes may occur in the lipoprotein distribution. Furthermore, serum contains heavy metals that are known to promote auto-oxidation, whereas EDTA in plasma chelates metal ions, minimizing auto-oxidation. Therefore, the most appropriate procedure for quantitation of HDL cholesterol is precipitation of the apoB-containing lipoproteins from EDTA-plasma by the modified heparin-Mn^{2+} method and determination of the cholesterol in the supernatant fraction. Since Mn^{2+} interferes with the enzymatic cholesterol procedure, other polyanioncation combinations need to be considered for laboratories using the enzymatic cholesterol procedure.

APOLIPOPROTEIN A-I AND A-II QUANTITATION

HDL is usually estimated in terms of its lipoprotein cholesterol content. The cholesterol moiety is, however, only 15-20% of the total weight of HDL. Thus, a change in the HDL cholesterol could reflect a change in HDL composition or a change in whole HDL concentration, or both. Assessment of the principal apoproteins of HDL, apoproteins A-I and A-II, together comprising 50% of the weight of HDL, thus respresents an important additional index of alteration in HDL concentration or composition. We developed a simple yet precise and accurate radial immunodiffusion assay for human plasma A-I and A-II (Table IV) (20,21). The diameter of the precipitation rings are measured in 0.1-mm units, using a calibrated viewer. Since usually less than 1% of the total plasma A-I and A-II levels are found in the $d<1.063$ plasma fraction, quantification of total plasma A-I and A-II is representative of total HDL A-I and A-II levels (21). It has been estimated that ca. 10% of the plasma A-I and 3% of the plasma A-II is in the $d>1.21$ fraction (21). However, it is likely that most of the A apolipoproteins found in the $d>1.21$ fraction were those that had become dissociated from HDL during ultracentrifugation. Analysis of ultracentrifuged subfractions of the HDL preparations from normolipidemic adult subjects indicates that men and women have similar A-I, A-II and HDL levels in the d 1.10-1.21 subfraction (Table V). However, women have approximately twice as much A-I, A-II and HDL choelsterol in the d 1.063-1.10 fraction as men. Also for both men and women the A-I/A-II molar ratio of the lighter density HDL subfraction was significantly greater ($p<0.01$) than that in the heavier density HDL subfraction. It is clear, therefore, that apoprotein compositions differ significantly within the HDL hydrated density subclasses. Fractionation of HDL on the basis of hydrated density by CsCl density-gradient centrifugation and subsequent A-I and A-II analysis shows that the A-I/A-II ratio varies with the lipoprotein hydrated density (Table VI). The A-I/A-II molar ratio of HDL lipoproteins banding between d 1.106-1.150 was nearly constant at 2.2 ± 0.2. In the density range 1.51-1.25, the A-I/A-II

TABLE VII

Plasma A-II in Normal Subjects
(mean ± S.D., mg/dl)

Age	Number		Plasma A-II	
	Men	Women	Men	Women
20-29	27	82	34 ± 4	35 ± 6
30-39	53	33	33 ± 5	35 ± 7
40-49	55	44	34 ± 5	37 ± 5
50-59	35	27	33 ± 5	38 ± 6

TABLE VIII

Plasma A-I Levels in Normal Subjects
(mean ± S.D., mg/dl)

Age	Number		Plasma A-I	
	Men	Women	Men	Women
20-29	50	114	117 ± 18	132 ± 26
30-39	77	39	117 ± 19	135 ± 26
40-49	77	62	120 ± 10	137 ± 22
50-59	55	37	125 ± 22	140 ± 32
60-65	4	5	126 ± 20	168 ± 23

TABLE IX

Plasma HDL-Cholesterol, A-I and A-II Levels in Normal Subjects[a]

Subject group	n	HDL CH	A-I	A-II
All men	192	45	120	33
All women	188	55	135	36
Women taking no estrogen[b]	92	54	130	34
Women on estrogen	19	61	149	39
Women on estrogen and progesterone	56	54	140	39

[a]Normal subjects refers to a subset of an industrial population who were selected independently of their lipid levels. Results expressed as mean levels in mg/dl.

[b]Refers to a subset of women from the population who had taken no medication for 2 weeks before blood drawing.

ratio increased as the density increased. However, in the density range between d 1.077 and d 1.105, the A-I/A-II ratio increased as the density decreased. The cholesterol/(A-I + A-II) ratio decreased as the density increased in all six HDL samples examined. Gradient gel electrophoresis of the density-gradient fractions showed that as the density of the HDL particle increased, the apparent mol wt decreased. Thus, HDL contains subpopulations which differ not only in molecular weight but also in the A-I/A-II molar ratio. HDL subfractions with the same hydrated density had comparable A-I/A-II and cholesterol/protein ratios whether isolated from men or from women. These results suggest that the differences between HDL concentrations in men and those in women primarily reflect differences in the relative proportions of HDL subclasses rather than intrinsic male-female differences in HDL structure (22).

Table VII presents total plasma A-I levels by age decade in the population tested. Among the men, the older age groups (5th to 7th decades) had slightly higher A-I values. The women also had slightly higher A-I values in the older age groups, with an average annual increment of 0.33 mg/dl/yr. A-II did not increase with age in men, but showed a slight increase with age in women (Table VIII). HDL cholesterol was highly correlated with A-I (r=0.72) and A-II (R=0.60); also, A-I was highly correlated with A-II (r=0.72). Women had significantly higher HDL cholesterol and apolipoprotein A-I and A-II levels than did men (Table IX). Women taking estrogen-containing medication had significantly higher HDL cholesterol and A-I concentrations and somewhat higher A-II concentrations than those not taking estrogen. The observation that women taking estrogen had significantly higher HDL cholesterol levels and

somewhat higher A-I/A-II ratios suggests that estrogen preferentially increases the lighter density HDL-2 subclass. On the other hand, women on combination contraceptives had the same HDL cholesterol concentrations as those on no medication, but somewhat higher A-I and A-II levels, suggesting that progestins modify the estrogen response and promote an increase in the heavier density HDL-3 subclass (21). The work of Krauss et al. confirms this suggestion (23). Fourteen women studied before and after 14 days of estrogen therapy (ethinyl estradiol 1 μg/kg/day) had a mean A-I increase of 24%, whereas the HDL cholesterol increased by an average of 19%, supporting the concept that estrogen is in part responsible for the higher HDL_2 levels observed in women (20,21).

There are many conditions or diseases in which HDL concentrations or compositions are altered; e.g., persons with existing coronary heart disease and those with many of the conditions associated with increased risk of coronary heart disease have reduced concentrations of HDL cholesterol (24-26).

Quantitation of A-I and A-II in 90 male survivors of myocardial infarction (MI) sampled at least 3 months after an acute MI indicated that MI survivors had significantly lower ($p<0.01$) A-I (112 ± 2 mg/dl, mean ± S.E.M.), A-II (29 ± 1 mg/dl) and HDL CH (39 ± 1 mg/dl) than did a plasma-choelsterol and triglyceride-matched control group, and the HDL cholesterol of the MI survivors was significantly related to log triglyceride (4 = -0.442 lipid matched controls, r = -0.520 MI survivors). These results are consistent with a relative decrease of HDL in MI survivors over and above that attributable to their increased triglyceride levels. Furthermore, the low HDL cholesterol/protein ratio observed in the HDL of MI survivors suggests a relatively greater decrease of the cholesterol-rich HDL_2 subclass than that of HDL_3 (24).

CONCLUSIONS

Recent epidemiological studies have emphasized the importance of HDL as a negative risk factor (7-9). As a result, clinical laboratories have experienced an increased demand for quantitation of this lipoprotein. HDL is usually estimated by quantitation of its cholesterol. Precipitation techniques for estimating HDL cholesterol are used widely. However, they do not give equivalent results. Thus, careful validation of specific precipitation conditions is necessary in order to ensure an accurate estimate of HDL cholesterol.

Quantitation of the principal apolipoproteins of HDL, A-I and A-II, together comprising ca. 50% of the weight of HDL, represents an important additional index of alterations in HDL concentration or composition. The radial immunodiffusion assays for A-I and A-II do not require prior extraction of lipids, and use only 50 μl of plasma. These assays therefore are suitable for mass screening. Therefore, the quantitation of A-I and A-II shows particular promise as methods for complementing the HDL cholesterol procedure.

The concentration, composition and subclass distribution of HDL changes in response to a variety of physiological, pharmacological, pathological and dietary perturbations. Thus, the application of assay methods for HDL are numerous. The functional role of HDL in health and disease will become clearer as accurate and precise assays for HDL become widely available.

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