

## Multilaboratory evaluation of an ultrafiltration procedure for high density lipoprotein cholesterol quantification in turbid heparin-manganese supernates

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**Summary** High density lipoprotein (HDL) can be quantitated by measurement of cholesterol in supernates after precipitation of low and very low density lipoprotein (LDL and VLDL) with heparin and Mn<sup>2+</sup>. Supernatant turbidity, often observed with hypertriglyceridemic specimens, indicates incomplete sedimentation of LDL/VLDL and precludes accurate quantitation of HDL. Ten Lipid Research Clinic Laboratories compared an ultrafiltration technique for clearing turbid heparin-Mn<sup>2+</sup> supernates to current methods involving repeat precipitation of either the original specimen after dilution or the  $d > 1.006$  g/ml fraction after removal of VLDL from the initial specimen by ultracentrifugation. Results for ultrafiltration of 429 turbid supernates averaged only slightly higher (1.0–1.1 mg/dl) than results by the dilution or ultracentrifugation methods on the same specimens, but this difference was

Abbreviations: HDL, LDL, VLDL, high, low, and very low density lipoproteins, respectively; apoA-I, apolipoprotein A-I, the major protein constituent of HDL; apoB, apolipoprotein B, a major protein constituent of LDL and VLDL.

found to be significant ( $P < 0.005$ ). The agreement of the ultrafiltration method with the other two methods is indicated by the following linear regression equations: a), ultrafiltration =  $(0.964 \times \text{ultracentrifugation}) + 2.4$  mg/dl, and correlation coefficient = 0.926; and b), ultrafiltration =  $(0.936 \times \text{dilution}) + 3.3$  mg/dl, and correlation coefficient = 0.933. We conclude that ultrafiltration of turbid heparin-Mn<sup>2+</sup> supernates is a convenient alternative to precipitation after either dilution or removal of VLDL.—**Warnick, G. R., J. J. Albers, P. Bachorik, J. Turner, C. Garcia, C. Breckinridge, K. Kuba, S. McNeely, G. Hillerman, P. King, R. Muesing, B. Most, and K. Lippel.** Multilaboratory evaluation of an ultrafiltration procedure for high density lipoprotein cholesterol quantification in turbid heparin-manganese supernates. *J. Lipid Res.* 1981. **22**: 1015–1020.

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HDL is frequently quantitated as cholesterol remaining in the supernatant solution after precipitation of VLDL and LDL with heparin-Mn<sup>2+</sup> (1, 2). A major source of error associated with this method has been incomplete sedimentation of the insoluble lipoprotein complex which is manifest by turbidity in the supernatant solution. Incomplete sedimentation occurs in approximately 10% of plasma specimens, primarily those with hypertriglyceridemia (3). That this turbidity in supernates is associated with contamination of the HDL fraction by LDL/VLDL is indicated by the presence of apoB, the major protein of VLDL and LDL. The insoluble heparin-Mn<sup>2+</sup> lipoprotein complex of a specimen with elevated triglyceride has a low protein to lipid ratio and hence a low density. When the density of the complex is near that of the solution, the complex may not sediment under the usual centrifugation conditions. In specimens with substantially elevated triglyceride values, the heparin-Mn<sup>2+</sup> lipoprotein complex may actually float and form a layer at the surface of a clear infranatant solution. Previous studies have shown that such a clear infranatant solution is essentially free of VLDL and LDL (4). Therefore, the turbidity observed in some supernates is primarily a result of incomplete sedimentation of the insoluble lipoprotein complex rather than of incomplete lipoprotein precipitation.

Various methods have been used to circumvent the problem of supernatant turbidity. One approach has been to dilute the hypertriglyceridemic specimen prior to heparin-Mn<sup>2+</sup> precipitation (1). This increases the concentrations of heparin and Mn<sup>2+</sup> relative to that of VLDL/LDL while simultaneously decreasing the solution density, thus facilitating sedimentation. A second approach has been to remove the triglyceride rich-VLDL (and chylomicrons, if

present) by ultracentrifugation prior to heparin-Mn<sup>2+</sup> precipitation (2). A third alternative is to remove the insoluble lipoprotein complex by subjecting the turbid supernates to an appropriate ultrafiltration method (3). This paper describes an evaluation completed in ten Lipid Research Clinics Laboratories of such an ultrafiltration technique using 0.22  $\mu$ m filters. This ultrafiltration procedure is compared with the dilution method and with the method involving removal of VLDL prior to centrifugation. In addition, the overestimation of HDL cholesterol associated with various degrees of supernatant turbidity is quantified.

## METHODS

According to standardized Lipid Research Clinic protocols (2), HDL was quantitated by measurement of cholesterol in supernates of EDTA plasma after removal of other lipoproteins by precipitation with heparin and Mn<sup>2+</sup> (4, 5). Specimens giving turbid supernates were again precipitated after dilution of the original specimen with an equal volume of 0.15 M NaCl solution (2). Alternatively, VLDL was removed from the original specimen by centrifugation at 105,000 *g* for 18 hr and the heparin-Mn<sup>2+</sup> precipitation was repeated on the VLDL-free (*d* > 1.006 g/ml) fraction adjusted to the original plasma volume (2). Cholesterol was quantified in isopropyl alcohol extracts of EDTA plasma specimens and HDL supernates by a Lieberman-Burchard reagent method (2) for the AutoAnalyzer II (Technicon Instruments, Inc., Tarrytown, NY). Triglycerides were quantified simultaneously by the Kessler procedure (6).

An alternative approach to HDL quantitation in turbid heparin-Mn<sup>2+</sup> supernates, the evaluation of which is the subject of this report, involved ultrafiltration of turbid supernates as described previously (3). Briefly, a 25-mm Swinnex holder was assembled with a 0.22  $\mu$ m Millipore filter protected by an AP 15 and an AP 20 depth prefilter of 22 mm diameter fitted within the silicone gasket (Millipore Corp. Bedford, MA). Turbid supernates were poured into a 5-ml syringe attached to the top of the filter assembly. The syringe plunger was inserted and the supernate was forced through the filter with moderate pressure.

The ten participating laboratories were each requested to perform the ultrafiltration procedure on approximately 50 turbid heparin-Mn<sup>2+</sup> supernates selected from their routine workload. On approximately 30 of the specimens in each laboratory, ultrafiltration was to be compared to the dilution procedure. The original plasma specimen was diluted

with an equal volume of 0.15 M NaCl solution and then treated with heparin and Mn<sup>2+</sup> solutions as described. Ultrafiltration was to have been compared to precipitation of the VLDL-free (*d* > 1.006 g/ml) specimens on approximately 30 additional specimens by each laboratory. In this instance, the turbid supernate was filtered immediately. The *d* > 1.006 g/ml fraction was obtained after overnight ultracentrifugation of the original specimen and was treated with the heparin and Mn<sup>2+</sup> solutions. Therefore, all three separation methods were compared by each laboratory on approximately ten of the specimens.

In each laboratory, supernates and filtrates for a single specimen were analyzed for cholesterol sequentially in duplicate with the second value of each duplicate used for comparison to minimize the sample interaction effects on the AutoAnalyzer (3). Cholesterol results were corrected for dilution. Analytical performance in quantitation of HDL cholesterol has been reported (7). The results were initially compiled at the Northwest Lipid Research Laboratory and were analyzed statistically by the Central Patient Registry (Chapel Hill, NC) for the Lipid Research Clinics Program.

Each laboratory was requested to indicate the type of supernatant turbidity based on the following categories that are obviously somewhat subjective: 1) small, granular particles floating on the surface of a clear supernatant solution; 2) an oily, chylomicron-like ring or layer of insoluble material at the surface of a clear solution; 3) slight turbidity throughout the supernatant solution; and 4) obvious or pronounced turbidity throughout the supernatant solution. Supernates with an oily layer on the surface but with turbidity throughout the solution were included in category 4. In categories 1–3 most and in category 4 some of the insoluble lipoprotein complex was sedimented, forming a pellet at the bottom of the tube.

In separate experiments performed at the Northwest Lipid Research Laboratory, the amount of apoB-associated (non-HDL) lipoprotein present in the various categories of turbid supernate was estimated by measuring cholesterol and apoB-associated cholesterol in turbid supernates before and after ultrafiltration. In addition, filtration performance with cellulose acetate filters (Celotate, Millipore Corp.) was compared to that using the usual Millipore filter, which is composed of mixed esters of cellulose acetate and nitrate. ApoA-I and apoB levels were measured by previously described radial immunodiffusion methods with specific antibodies to apoA-I and apoB, respectively. ApoB was quantitated as apoB-associated cholesterol by reference to a standard prepared from pooled human plasma fractions of

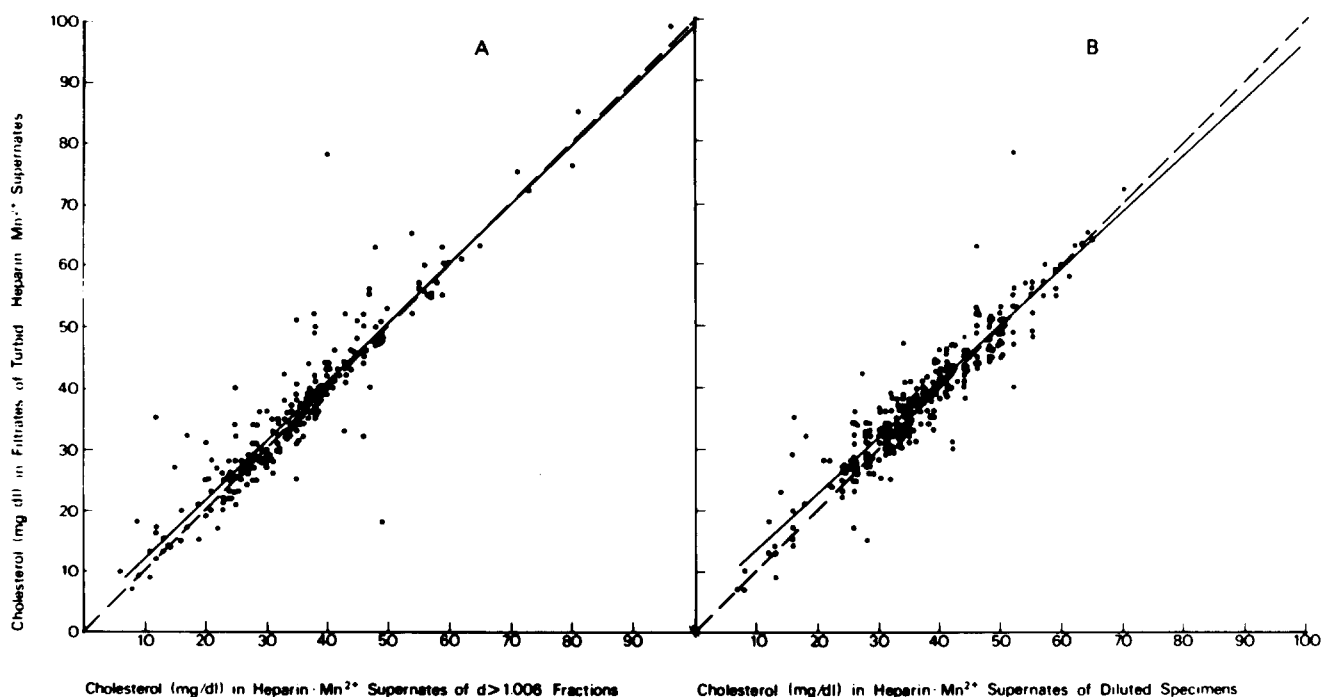
$d > 1.006$  g/ml of known LDL cholesterol content (3, 8, 9).

## RESULTS AND DISCUSSION

Ten North American Laboratories of the Lipid Research Clinics Program participated in the collaborative evaluation of the ultrafiltration technique. The ultrafiltration method was compared on a total of 429 specimens, all of which exhibited turbid supernates as defined above after heparin- $Mn^{2+}$  precipitation. Plasma cholesterol values on these specimens ranged from 144 to 1405 mg/dl with a mean of 290 mg/dl and a standard deviation of 105 mg/dl. Total triglyceride values ranged from 45 to 10,560 mg/dl with a mean of 565 mg/dl and a standard deviation of 880 mg/dl. Only 29.6% (127) had total triglyceride values of less than 300 mg/dl, and 3.7% (16) had total triglyceride values of less than 150 mg/dl. In 50.8% (218 of 429) of the specimens, ultrafiltration was compared to precipitation of the  $d > 1.006$  g/ml fraction, and in 72.3% (310) ultrafiltration was compared to the dilution method. All three methods were compared on 22.8% (98) of the specimens.

Categories of supernatant turbidity were defined as described in Methods. Of the 429 turbid supernates, 313 were categorized by these criteria; some participating laboratories did not categorize turbidity. Thirteen percent (41) were in category 1, 25.9% (81) in category 2, 22.7% (71) in category 3, and 38.3% (120) in category 4. The mean plasma total triglyceride values for specimens with supernates in each category were 283, 660, 309, and 720 mg/dl for categories 1, 2, 3, and 4, respectively.

The results for HDL cholesterol quantification by ultrafiltration of turbid supernates compared to precipitation of the  $d > 1.006$  fractions for the same specimens are presented in **Fig. 1A**. The linear regression relationship was as follows: ultrafiltration =  $(0.964 \times \text{ultracentrifugation}) + 2.375$  mg/dl, with a correlation coefficient of 0.926. The overall cholesterol mean by ultrafiltration was 36.17 mg/dl compared to 35.06 mg/dl for precipitation of the  $d > 1.006$  g/ml fraction. This difference, 1.11 mg/dl, while small, was statistically significant at  $P < 0.005$  ( $t = 3.06$ ) by Student's paired  $t$ -test. The average between-method differences in individual laboratories ranged from 0.1 to 3.2 mg/dl. The ultrafiltration values averaged higher than the ultracentrifugation values in every laboratory with the exception of one, which observed



**Fig. 1.** Panel A, Cholesterol in 218 heparin- $Mn^{2+}$  supernates of  $d > 1.006$  g/ml (VLDL-free) fractions (x) compared to  $0.22 \mu\text{m}$  filtrates (y) of turbid heparin- $Mn^{2+}$  supernates for the corresponding plasma specimens. The linear regression parameters for the solid line are  $y = (0.964 \times \text{ultracentrifugation}) + 2.375$  mg/dl, with a correlation coefficient of 0.926. The dotted line illustrates  $y = x$  or the line of perfect agreement. Panel B, Cholesterol in 310 heparin- $Mn^{2+}$  supernates of specimens precipitated after dilution with an equal volume of 0.15 M NaCl solution (x) compared to  $0.22 \mu\text{m}$  filtrates (y) of turbid heparin- $Mn^{2+}$  supernates for the same specimens without dilution. The linear regression relationship is described by  $y = (0.936 \times \text{dilution}) + 3.323$  mg/dl, with a correlation coefficient of 0.933.

TABLE 1. Comparison of three methods for turbid heparin-Mn<sup>2+</sup> supernates: ultrafiltration (UF), precipitation of d > 1.006 g/ml plasma fraction (B), and precipitation of diluted plasma (D)<sup>a</sup>

	Cholesterol in mg/dl			Paired <i>t</i> <sup>c</sup>	Linear Regression Parameters		
	Mean X	Mean Y	S.D. <sup>b</sup>		Slope	Intercept	Correlation Coefficient
	UF (Y) versus B (X)	35.0	36.4		4.88	1.96 <sup>d</sup>	0.94
UF (Y) versus D (X)	35.5	36.4	4.07	1.54 <sup>d</sup>	0.95	2.50	0.912
D (Y) versus B (X)	35.0	35.5	3.92	0.85 <sup>d</sup>	0.94	2.76	0.910

<sup>a</sup> Comparison on 98 samples tested by all three methods.

<sup>b</sup> Paired Standard Deviation =  $(\sum \text{difference}^2/2n)^{1/2}$ .

<sup>c</sup> Student's paired *t* test.

<sup>d</sup> Difference not significant at  $P < 0.05$ .

an average 1.0 mg/dl difference in the opposite direction.

The comparison of ultrafiltration with dilution is presented in Fig. 1B. The agreement between methods is indicated by the linear regression parameters: ultrafiltration =  $(0.936 \times \text{dilution}) + 3.323$  mg/dl with a correlation coefficient of 0.933. The mean cholesterol by ultrafiltration was 37.71 mg/dl compared to 36.74 mg/dl for dilution. The between-method difference was again slight, 0.93 mg/dl, but statistically significant at  $P < 0.005$  ( $t = 4.37$ ). Average differences in individual laboratories ranged from 0.1 mg/dl to 2.0 mg/dl. Values by ultrafiltration averaged higher than those by dilution in every laboratory except one which observed an average difference of 0.6 mg/dl in the opposite direction.

These results suggest excellent correlation and agreement between the ultrafiltration method and either precipitation of the d > 1.006 g/ml fraction or precipitation of the specimen after dilution. The small differences observed between the methods were, nevertheless, statistically significant because of the excellent precision obtained in the cholesterol measurement. Because the between-method differences were statistically significant, results for specimens tested by all three methods were compared to deter-

mine whether the difference between ultrafiltration and either precipitation of the d > 1.006 g/ml plasma fraction or the diluted sample was greater than the difference observed between the latter two methods. Results from this comparison, obtained for a subset of 98 specimens, are shown in **Table 1**. On these specimens, the mean cholesterol by ultrafiltration was 36.4 mg/dl compared to 35.0 mg/dl for precipitation of the d > 1.006 g/ml fraction and 35.5 mg/dl for dilution. None of the between-method differences on this subset were statistically significant at  $P < 0.05$ . The linear regression slopes, intercepts, and correlation coefficients (Table 1) for these comparisons were similar.

In other experiments performed at the Northwest Lipid Research Laboratory, the extent of overestimation of HDL cholesterol associated with the various categories of supernatant turbidity was determined by measuring cholesterol and apoB-associated cholesterol in turbid heparin-Mn<sup>2+</sup> supernates and in clear filtrates of the same supernates (**Table 2**). Supernates in the first category contained relatively little apoB-associated cholesterol and filtration removed on the average only 2.1 mg/dl cholesterol. ApoB-associated cholesterol in filtrates averaged 1.4 mg/dl compared to 1.7 mg/dl in the supernates. Therefore,

TABLE 2. Mean cholesterol and apoB-associated (non-HDL) cholesterol values in turbid heparin-Mn<sup>2+</sup> supernates and in their clear filtrates

Category	Supernatant Appearance <sup>a</sup>	n	Turbid Supernate		Clear Filtrate	
			Cholesterol	apoB <sup>b</sup>	Cholesterol	apoB <sup>b</sup>
1	Granular particles at surface of clear supernate	11	50.4 mg/dl	1.7 mg/dl	48.3 mg/dl	1.4 mg/dl
2	Oily layer or ring at surface of clear supernate	9	49.1	5.1	44.6	1.6
3	Slight turbidity throughout supernatant solution	7	56.6	10.5	43.8	0.2
4	Pronounced turbidity throughout supernatant solution	6	127.2	>30.3 <sup>c</sup>	34.3	1.4

<sup>a</sup> As defined in Methods.

<sup>b</sup> ApoB-associated cholesterol by radial immunodiffusion assay with specific anti-apoB sera.

<sup>c</sup> Some levels exceeded assay range.

filtration of these supernates removed virtually no apoB-associated cholesterol. The cholesterol loss with filtration was comparable to that previously reported for filtration of clear supernates, which is probably explained by lipoprotein adsorption on the ultrafiltration membranes (3). The low apoB-associated cholesterol values in category 1 supernates were similar to those reported for clear heparin-Mn<sup>2+</sup> supernates. Filtration of nine category 2 supernates removed an average of 4.5 mg/dl cholesterol. ApoB-associated cholesterol averaged 5.1 mg/dl in the initial supernates compared to 1.6 mg/dl in their filtrates. These particular specimens had only moderate amounts of apoB-associated lipoproteins at the surface; most of the insoluble lipoprotein complex had sedimented. In other similar supernates, contamination could be much greater depending on the VLDL and chylomicron levels. With extremely hypertriglyceridemic specimens, virtually all of the insoluble lipoprotein complex may layer at the surface during centrifugation.

Supernates in category 3 (Table 2), with slight turbidity throughout the solution, contained an average of 10.5 mg/dl apoB-associated cholesterol and their filtrates averaged 0.2 mg/dl. Filtration removed on the average 12.8 mg/dl cholesterol. Category 4 supernates, with pronounced turbidity, were extensively contaminated with apoB-associated lipoproteins. Some supernates exceeded the linear range of the radial immunodiffusion method (>30 mg/dl) and an average of 92.9 mg/dl cholesterol was removed by the filtration process. Filtrates averaged 1.4 mg/dl apoB-associated cholesterol.

These results confirm that supernatant turbidity indicates contamination by the apoB-associated lipoproteins and demonstrate that the apoB-associated cholesterol in supernates is related to their degree of turbidity. Cholesterol measurement in supernates with turbidity or an oily layer at the surface would generally substantially overestimate HDL. Therefore, supernates that are not clear require further manipulation to separate the apoB-associated lipoproteins. The ultrafiltration technique removed virtually all of the apoB-associated lipoproteins, regardless of the degree of initial supernatant turbidity.

However, the small granular particles that are sometimes observed at the surface of a clear heparin-Mn<sup>2+</sup> supernate appear to contain negligible apoB-associated lipoprotein cholesterol and do not necessitate ultrafiltration for accurate quantitation of HDL. It should also be noted that supernates that are allowed to stand at room temperature may develop a slight cloudiness due to formation of an insoluble (non-lipoprotein) manganese complex (4). It is impor-

tant, therefore, to distinguish between supernates that are turbid immediately upon removal from the centrifuge and those that become turbid after standing at room temperature. This can be done by screening samples for turbidity immediately after centrifugation.

Filtration of turbid heparin-Mn<sup>2+</sup> supernates was tested with the Celotate (cellulose acetate) membranes, which reportedly have less tendency to adsorb protein than the standard Millipore filters. Results on 12 samples indicated that more apoA-I was removed by the Celotate membrane (mean filtrate apoA-I = 90.3 mg/dl) than by the Millipore (mean filtrate apoA-I = 116.5 mg/dl). In addition, the apoB-associated cholesterol level in the Celotate filtrates averaged slightly higher than that of the Millipore filtrates (2.4 versus 1.0 mg/dl). Therefore, separation by the Millipore filter appeared to be more specific than that by the Celotate filter for this application.

One disadvantage of the 25-mm filtration apparatus is the relatively large void volume. This necessitated filtering at least 1.5 ml of supernate to obtain sufficient material for analysis. We tested 13-mm Swinnex holders (Millipore Corp.) that were assembled with 0.22  $\mu$ m filters and two depth pre-filters. This size of filter, unfortunately, had a greater tendency to plug during filtration reducing flow rates and retaining HDL. Therefore, the smaller filter did not appear suitable for filtration of most turbid supernates.

In summary, these results confirm the effectiveness of the filtration technique for clearing turbid heparin-Mn<sup>2+</sup> supernates. Cholesterol results are comparable to those by either the dilution method or the ultracentrifugation procedure, which are presently used for quantitating HDL in hypertriglyceridemic specimens. Ultrafiltration removes virtually all of the apoB-associated lipoproteins, regardless of the degree of supernatant turbidity. The particular ultrafiltration apparatus as described previously and evaluated here appears optimal for clearing turbid supernates.

In this evaluation, ultrafiltration was performed on supernates obtained by heparin-Mn<sup>2+</sup> at 46 mM final concentration as prescribed in Lipid Research Clinics protocol. A modified procedure with Mn<sup>2+</sup> at 92 mM has been demonstrated to give a substantially smaller proportion of turbid supernates (5). In addition, this ultrafiltration procedure was previously demonstrated to similarly remove apoB-associated lipoproteins from supernates turbid after precipitation at the higher Mn<sup>2+</sup> concentration (3). ■■

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