Insufficient Accuracy and Specificity of Polyanion Precipitation Methods for Quantifying Low-Density Lipoproteins

Rödiger Siekmeyer, Winfried März, and Werner Groß

Recently, polyanion precipitation assays for low-density lipoprotein (LDL)-cholesterol have been found to underestimate their analyte in normolipidemic samples (Siekmeier et al., Clin Chim Acta 1988;177:221–30). Therefore, accuracy, specificity, and interference by nonesterified fatty acids have been studied for three precipitants (obtained by heparin, dextran sulfate, or polyvinyl sulfate precipitation). At normal concentrations of LDL, precipitation is incomplete, whereas it is nearly quantitative at high concentrations of LDL. The polyvinyl sulfate reagent markedly responds to variations in the amount of non-LDL protein present in the precipitation mixture. In the dextran sulfate and the polyvinyl sulfate method, but not in the heparin method, the percentages of LDL precipitated notably increase as the concentration of the polyanion compound is decreased. In either assay, very-low-density lipoproteins, but not high-density lipoproteins, are significantly coprecipitated (dextran sulfate 28%, polyvinyl sulfate and heparin 66%) in a concentration-independent fashion. Increased concentrations of nonesterified fatty acids markedly interfere with the dextran sulfate and polyvinyl sulfate assay, but do not much affect results with the heparin reagent.

Additional Keyphrases: intermethod comparison · cholesterol · analytical error

Hypercholesterolemia represents one of the primary risk factors for the premature development of atherosclerosis. Whereas increased concentrations of low-density lipoproteins (LDL) confer a high cardiovascular risk, high-density lipoproteins (HDL) are thought to exert protective effects (1–3). Hence, individual risk profiles should include assays for HDL and LDL. A common approach to the quantification of lipoprotein classes has been the measurement of HDL-C after precipitation of apolipoprotein (apo) B-containing lipoproteins (4–7) and subsequent calculation of LDL-C according to Friedewald et al. (4). In the meantime, methods aimed at the selective precipitation and direct quantification of LDL-C have been designed and are commercially available (8–10).

Starting from our recent observation that concentrations of LDL-C after precipitation with polyanion compounds are lower than those obtained with a combined ultracentrifugation–precipitation procedure (11), we have evaluated the accuracies and specificities for LDL-C of three assays (8–10) based on precipitation of LDL with either heparin, dextran sulfate (DS), or polyvinyl sulfate (PVS).

Materials and Methods

Apparatus. For preparative ultracentrifugation we used a Model L 8-70 (Beckman Instruments, Fullerton, CA) or a Model TGA 75 (Kontron AG, Analytical Division, Zürich, Switzerland) ultracentrifuge with fixed-angle rotors (Kontron types TPT 50.38 and TPT 45.6). Densities were monitored with a DMA 55 digital precision density meter (A. Paar KG, Graz, Austria).

Reagents and other materials. Reagent kits for cholesterol (Monotest CHOD-PAP) and triglycerides (GPO-PAP) were purchased from Boehringer Mannheim, Mannheim, F.R.G. The "NEFAC" test for the enzymatic determination of nonesterified (free) fatty acids was obtained from Wako Chemicals, Neuss, F.R.G. Celite 545 (analytical grade), palmitic acid, stearic acid, and Triton X-100 were from Serva Feinbiochemica, Heidelberg, F.R.G.; other chemicals were from E. Merck, Darmstadt, F.R.G.

Blood collection. Blood samples from ostensibly healthy donors, mostly women, ages 20 to 30 years, were obtained by venipuncture after an overnight fast and drawn into tubes containing K₂EDTA at a final concentration of 1.5–2 g/L (Sarstedt, Nümbrecht, F.R.G.). Plasma was recovered by centrifugation (1500 × g, 30 min) and stored at 4°C. Lipid analyses were completed within no more than five days after blood collection.

 Quantification of LDL-C. LDL-C was determined either after precipitation with heparin (LDL-Cₜₚₜ), dextran sulfate (LDL-C₉), polyvinyl sulfate (LDL-C₉), or by a combined ultracentrifugation and phosphotungstic acid/MgCl₂ method (LDL-Cₚₜₚₜ). For LDL-Cₜₚₜ (8), we added 100 μL of sample to 1.0 mL of precipitant (sodium citrate, 64 mmol/L, pH 5.04, and heparin, 50 000 U/L), swirled, and incubated the mixture...
for 10 min at room temperature. The precipitate was pelleted by centrifugation at 3500 × g for 15 min. We determined the cholesterol in 0.5 mL of the supernate within 1 h.

For LDL-C<sub>PS</sub> (9), we added 1.0 mL of precipitant (Quantitop lipoprotein LDL cholesterol; Immuno AG, Vienna, Austria) containing Tris·HCl, 0.2 mmol/L, pH 7.4, and dextran sulfate (M<sub>B</sub> 500 000) to 100 μL of sample. After 10 min of incubation at room temperature and centrifugation at 3500 × g for 10 min, we measured the cholesterol in the supernate.

For LDL-C<sub>PVS</sub> (10), we mixed 200 μL of sample and 100 μL of precipitant (LDL cholesterol PVS; Boehringer Mannheim) containing, per liter, 5 mmol of Na<sub>2</sub>EDTA, pH 4.2, 1 g of PVS, and 169 g of polyethylene glycol methyl ether as an accelerator. After 15 min of incubation at room temperature and 15 min of centrifugation at 1500 × g, we removed 150 μL of the supernate for the cholesterol determination.

For LDL-C<sub>C</sub> (9), we layered 2 mL of plasma with 1 mL of a solution containing 100 mg of Na<sub>2</sub>EDTA and 11.42 g of NaCl per liter (solvent density, d<sub>o</sub> = 1.0063 kg/L) in thick-walled polycarbonate tubes and centrifuged the gradient in the TFFT 45.6 rotor (2.35 × 10<sup>8</sup> g · min⁻¹, 15°C). The supernatant VLDL were collected by aspiration with a hypodermic syringe and the fraction volume was made up to 2 mL with isotonic saline (NaCl 150 mmol/L) before cholesterol was determined.

HDL-C was quantified after precipitation of apolipoprotein B-containing lipoproteins with a solution of 3.6 g of sodium phosphotungstate and 45 mmol of MgCl<sub>2</sub> per liter (supplementary reagent for HDL cholesterol, Boehringer Mannheim). LDL-C<sub>U</sub> was calculated as the difference of total cholesterol minus the cholesterol in the supernates after LDL precipitation.

Preparation of lipoproteins. Lipoprotein fractions were isolated from pooled sera of normolipidemic and healthy donors of both sexes, ages between 20 and 30 years. Before fractionation, the sera had been supplemented with Na<sub>2</sub>EDTA and Na<sub>3</sub> to final concentrations of 0.4 and 0.5 g/L, respectively. Trace amounts of "chylomicrons and particles" were removed after centrifugation for 30 min at 30 000 × g (d<sub>o</sub> = 1.0063 kg/L). Subsequently, VLDL, LDL, and HDL were prepared by sequential ultracentrifugation at solvent densities (d<sub>o</sub> = 1.0063 kg/L (2.3 · 10<sup>8</sup> g · min⁻¹), 1.063 kg/L (2.6 · 10<sup>8</sup> g · min⁻¹), and 1.21 kg/L (3.4 · 10<sup>8</sup> g · min⁻¹), respectively. Lipoprotein preparations were washed once by recentrifugation under identical conditions, stored at 4°C, and dialyzed against freshly prepared Krebs–Ringer solution (per liter: 119 mmol of NaCl, 4.7 mmol of KCl, 2.5 mmol of CaCl<sub>2</sub>, 25 mmol of NaHCO<sub>3</sub>, 1.2 mmol of KH<sub>2</sub>PO<sub>4</sub>, and 1.2 mmol of MgSO<sub>4</sub>, pH 7.4) just before use. Unless otherwise indicated, this solution was also used to adjust the concentrations of lipoprotein fractions.

Enrichment of plasma with fatty acids. Fatty acids were added to ordinary plasma according to Spector and Hoak (12). In brief, 0.5 mmol of stearic acid and 0.5 mmol of palmitic acid were dissolved in 10 mL of hexane. We spread 10 g of Celite in a beaker, the thickness of the solid phase not exceeding 5 mm. The FFA–hexane solution was added, and the organic solvent was evaporated under a stream of N<sub>2</sub>. Weighed amounts (ranging from 25 to 650 mg) of dry FFA-coated Celite particles were added to 4 mL of freshly drawn fasting plasma, and the suspension was carefully stirred for 30 min. Finally, the Celite was removed by two centrifugations at 2°C and 15 000 × g for 10 min, and the supernate was passed through a membrane filter (pore size 1.2 μm). Adsorption of LDL to the Celite particles was ruled out by a control experiment in which 600 mg (hexane-treated) Celite without FFA was added to 4 mL of serum: LDL-C measured with either precipitation method was not affected by exposure to Celite particles alone.

Statistics. Nonparametric linear regression (13) and non-linear curve-fitting (14) were carried out as described. The relationship between the percent proportion of LDL-C recovered (LDL-C<sub>rec</sub>) and LDL-C actually present (LDL-C<sub>p</sub>) was assumed to follow the function

\[ LDL-C_{rec} = LDL-C_{p} \cdot k_1/(LDL-C_{p} + k_2) \]

so that LDL-C measured in plasma samples (LDL-C<sub>mes</sub>) could be corrected for LDL recoveries (%) by the following equation:

\[ LDL-C_{mes} = \frac{LDL-C_{p} \cdot 100}{2 \cdot k_1} + \sqrt{\frac{(LDL-C_{mes} \cdot 100)^2}{4 \cdot k_1^2} - \frac{LDL-C_{mes} \cdot 100 \cdot k_2}{k_1}} \]

Results

In experiments with ultracentrifugally purified LDL, the percentages of LDL precipitated by DS and PVS depended on the LDL-C concentration (cf. Figure 1). For LDL-C at 3.5 mmol/L, both PVS and DS precipitated no more than 70% of the initial LDL content. Quantitative precipitation (>95%) was obtained for LDL-C concentrations >12 mmol/L (about threefold the normal concentration). By contrast, even at low LDL concentrations (LDL-C <2.0 mmol/L), heparin almost quantitatively precipitated purified LDL. Within the same concentration range we obtained complete precipitation of LDL with the phosphotungstate/MgCl<sub>2</sub> reagent.

To study the influence of plasma proteins on the precip-
dilution of LDL, we supplemented reaction mixtures with plasma fractions of $d_{20\text{C}} > 1.063$ kg/L (insert, Figure 1). Thereby, we accounted for coprecipitation of Lp(a) and lipoproteins of $d_{20\text{C}} > 1.063$ kg/L by assaying controls that lacked LDL. This confirmed the dependency of LDL recoveries on the initial concentrations of LDL. However, the presence of LDL- and VLDL-depleted serum markedly improved LDL recoveries with the DS and the PVS reagent: about 90% of LDL was precipitated from LDL-C at 3.5 mmol/L. Consequently, we precipitated LDL either in the absence or in the presence of bovine serum albumin (3.6 or 7.2 g/L), but omitted the $d_{20\text{C}} > 1.063$ kg/L fraction. Intriguingly, the addition of albumin led to an increase in the percentage of LDL precipitated by PVS but not by DS. To rule out the possibility that this difference between PVS and DS was due to the higher dilution of the sample in the DS method, we added albumin directly to the DS reagent. This indeed brought about minor increases in the percentage of LDL precipitated. However, these differences were far too slight to account for the differences observed between precipitations in purified LDL and after supplementation of the $d_{20\text{C}} > 1.063$ kg/L fraction. Hence, factors in plasma other than albumin may favor LDL precipitation, at least by DS.

To verify that the polyanion to LDL ratio also governs the accuracy of the precipitation methods, we divided the polyanion concentrations in half by diluting the heparin, DS, and PVS reagents with citrate buffer (64 mmol/L, pH 5.04), doubly distilled water, and Na$_2$EDTA (5 mmol/L, pH 4.2), respectively. Whereas the heparin method was virtually unaffected by the change in polyanion concentration, decreasing the DS and PVS considerably enhanced the precipitation of purified LDL (Figure 2).

Coprecipitation of VLDL was assessed by adding various amounts of VLDL to a pool of normolipidemic samples with known VLDL-C content (final concentrations 0.2–2.0 mmol/L). Within this range roughly two-thirds of the added VLDL was precipitated with LDL by heparin and PVS, whereas DS coprecipitated only 28%. The percentage of coprecipitated VLDL was independent of the initial VLDL-C concentration. Experiments in which sera with low concentrations of HDL were enriched in HDL disclosed that none of the reagents discernibly precipitated HDL.

Figure 3 summarizes the effect of FFA on the polyanion precipitation of LDL. Evidently, FFA concentrations exceeding 1–1.5 mmol/L decreased the amounts of LDL precipitable with DS and PVS, almost complete inhibition being attained at FFA concentrations of about 4 mmol/L. Although a discernible influence was still present, the interference by FFA with the heparin method was far less pronounced than that observed with DS or PVS. Finally, FFA do not at all affect the Ph/T/MgCl$_2$ precipitation of apolipoprotein B-containing lipoproteins.

We determined LDL-C in 113 samples from ostensibly healthy donors with each precipitation method and our combined ultracentrifugation and precipitation method (11). Tables 1 and 2 summarize the results. Both means and regression lines indicate that the precipitation assays underestimate LDL-C. Given the assumption that this was attributable to incomplete precipitation of LDL, we corrected raw LDL-C scores for concentration-dependent recovery, and recalculated the regression lines (bottom half, Tables 1 and 2). For this purpose we estimated $k_1$ and $k_2$ from the curves shown in the insert to Figure 1. Because of the low VLDL content in these samples, we disregarded the coprecipitation of VLDL. This transformation did, in fact, eliminate or at least considerably decrease the negative intercepts of the original regression lines.

![Fig. 3. Influence of free fatty acids on LDL precipitation](image)

Table 1. LDL-Cholesterol as Determined by Polyanion Precipitation and Combined Ultracentrifugation/ Precipitation*

<table>
<thead>
<tr>
<th>LDL-C$_{CUP}$</th>
<th>Mean ± SD</th>
<th>Median ± 68%$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C$_{CUP}$</td>
<td>3.23 ± 0.98</td>
<td>2.93 ± 0.68</td>
</tr>
<tr>
<td>LDL-C$_{HEP}$</td>
<td>2.54 ± 1.10</td>
<td>2.33 ± 0.84</td>
</tr>
<tr>
<td>LDL-C$_{OS}$</td>
<td>2.45 ± 0.82</td>
<td>2.35 ± 0.68</td>
</tr>
<tr>
<td>LDL-C$_{PVS}$</td>
<td>2.81 ± 0.87</td>
<td>2.67 ± 0.67</td>
</tr>
</tbody>
</table>

Corrected for LDL recovery

| LDL-C$_{CUP}$ | 2.90 ± 1.04 | 2.71 ± 0.82 |
| LDL-C$_{HEP}$ | 2.78 ± 0.77 | 2.69 ± 0.85 |
| LDL-C$_{PVS}$ | 3.22 ± 0.82 | 3.09 ± 0.84 |

$n = 113$ samples with triglycerides < 1.71 mmol/L.

* Part of the data are reproduced with permission of Clinica Chimica Acta (11).

$^a$ 68th percentile of distances from the median.
Table 2. Regression Parameters for LDL-C Determinations by Various Procedures*

<table>
<thead>
<tr>
<th>Methods</th>
<th>Mean (and upper/lower limits, 95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw scores</td>
<td></td>
</tr>
<tr>
<td>LDL-C_{HPR} vs UC</td>
<td>1.16 (1.06/1.27)</td>
</tr>
<tr>
<td>LDL-C_{DS} vs UC</td>
<td>1.01 (0.91/1.10)</td>
</tr>
<tr>
<td>LDL-C_{PV5} vs UC</td>
<td>0.99 (0.92/1.06)</td>
</tr>
<tr>
<td>Corrected for LDL recovery</td>
<td></td>
</tr>
<tr>
<td>LDL-C_{HPR} vs UC</td>
<td>1.09 (1.00/1.20)</td>
</tr>
<tr>
<td>LDL-C_{DS} vs UC</td>
<td>0.95 (0.86/1.04)</td>
</tr>
<tr>
<td>LDL-C_{PV5} vs UC</td>
<td>0.94 (0.87/1.01)</td>
</tr>
</tbody>
</table>

Slopes and intercepts have been calculated for n = 113 samples with triglycerides < 1.71 mmol/L.

* Part of the data are reproduced with permission of Clinica Chimica Acta (11). Same samples as in Table 1.

** Slope significantly different from unity (P <0.05).

*** Intercept significantly different from zero (P <0.05).

**Discussion**

Interactions between lipoproteins and sulfated polyanions in the presence or absence of divalent cations have been known for many years and their nature has comprehensively been characterized by several investigators (15–20). Making use of these interactions has allowed the development of precipitation methods for quantitative and selective precipitation of LDL (8–10). A major agreement among the precipitation assays for LDL-C (21–23) as well as between LDL-C after precipitation and composite methods similar to ours (8–10) has been reported. By contrast, we have recently observed that procedures for the precipitation of LDL underestimate LDL-C (11; cf. Tables 1 and 2).

Regression lines relating the precipitation methods to an established method showed pronounced negative intercepts, which became even more severe when hypertriglyceridemic samples were included (11). Here we have demonstrated that this is caused by incomplete precipitation of LDL within the concentration range found in healthy subjects. Consistently, adjustment of the raw data for incomplete recovery eliminated the negative intercepts from the regression lines.

As could be demonstrated for low and normal concentrations of LDL, reducing the PVs and DS concentrations increased the percentage of LDL precipitated, whereas dilution of heparin had no effect. Along with the concentration-dependent behavior of LDL recoveries, this suggests that the assay accuracies, at least of the DS and PVs procedures, are subject to a stoichiometric relationship between LDL and polyanion compound. In LDL solutions the LDL-C_{DS} and LDL-C_{PV5} values were lower than in mixtures of LDL with the ultracentrifugal d >1.063 kg/L bottom fraction. Addition of albumin to an LDL preparation improved percentage of LDL precipitated with PVs, but recoveries with DS remained low. This underscores that the matrix in which precipitations are performed may be of crucial importance and that plasma proteins and (or) other plasma constituents may exert adjuvant effects on the LDL–polyanion interaction. Our findings are in strong disagreement with observations (24) suggesting that, in general, polyanion precipitations of lipoproteins are more readily attained with purified lipoproteins than in the presence of plasma proteins.

In keeping with earlier reports (8–10), we found that neither polyanion reagent coprecipitated HDL. However, all reagents significantly captured VLDL, which is at variance with the results of other investigators (8–10). At present, there is no final explanation for this inconsistency, but coprecipitation of VLDL, e.g., with the heparin reagent, may be due to the well-documented interaction of heparin with the apolipoprotein B or apolipoprotein E moiety (or both) of triglyceride-rich lipoproteins (19, 20, 25, 26), which may occur even in the absence of divalent cations (18–22).

Interferences from FFA have recently been pointed out for the DS reagent (9, 18), but no systematic investigations had been available for the heparin and PVS method. Here we have shown that FFA concentrations above a threshold of 1–1.5 mmol/L significantly influence precipitation with DS and PVS; a moderate effect was seen for the heparin method, and none for the phosphotungstic acid/MgCl₂ reagent.

In conclusion, none of these methods for the direct determination of LDL proved specific for this analyte nor were they accurate in terms of analytical recovery. The formation of insoluble lipoprotein–polyanion complexes depends on various conditions: ionic strength, pH, sulfation grade and molecular mass of the polyanion, and stoichiometric relationships between lipoproteins and polyanion (9, 16, 19, 20). Furthermore, this formation may be modified by the presence of other macromolecular matrix constituents. Perhaps modifications of the present precipitation methods for LDL with respect to these factors will bring sufficient improvements to justify their application in the clinical laboratory.

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**References**

Detection of Carrier Status in Duchenne and Becker Muscular Dystrophies by Quantitative Polymerase Chain Reaction and Allele-Specific Oligonucleotides

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Detection of carriers of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), in the deletion cases, involves calculating gene dosage from Southern blots. We show that the analysis of dosage can also be made from the polymerase chain reaction (PCR) with use of allele-specific oligonucleotides (ASOs). The deletion-prone exons are amplified, transferred to a membrane, and hybridized with ASOs complementary to the exons; the autoradiographic bands are then quantified with a densitometer. After determining the quantitative conditions of the amplification reaction, we were able to identify deletions in a DMD/BMD carrier female. The determination of carrier status via PCR removes several of the technical limitations of Southern analysis and is also cost- and labor-effective.

Additional Keyphrases: gene probes · heritable disorders

Duchenne muscular dystrophy (DMD) is an X-linked recessive progressive muscle-wasting genetic disorder that affects ~1 of 3500 to 5000 newborn males.⁴ Both DMD and the milder allelic Becker muscular dystrophy (BMD) result from mutations in the dystrophin gene. The 14-kb coding sequence of the dystrophin gene has been cloned. Use of cDNA probes has shown that molecular deletions account for ~65% of the mutations in DMD and BMD (3–6). Carrier detection in the deletion cases involves assessing gene dosage by quantitative Southern-blot analysis, whereby one determines whether the female at risk exhibits no reduction (noncarrier status) or 50% reduction (carrier status) in hybridization intensity in those bands that are deleted in the affected male (7). The dosage determinations permit direct analysis for carrier status and eliminate the inherent problems of the restriction-fragment-length-polymorphism technique (e.g., recombinations, noninformative meioses, unavailability of family members, and sporadic mutations). To further increase the accuracy of the dosage analysis, we quantify the autoradiographic bands by scanning with a densitometer (8).

Although dosage analysis has significantly improved carrier studies, particularly in the isolated cases of the disease, there are technical limitations. Dosage analysis of Southern blots requires optimal conditions; very good quality blots are necessary, with uniform transfer and hybrid-

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4 Nonstandard abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; and SDS, sodium dodecyl sulfate.