Improved Direct Determination of Serum Cholesterol in Low-Density Lipoproteins with Use of Polycations

C. C. Heuck, G. Middelhoft, and G. Schlierf

The previously described procedure of direct determination of LDL-cholesterol after selective extraction of VLDL and HDL with polycations and a polycation-exchange resin has been improved by using dodecylated poly(ethyleneimine) instead of poly(ethyleneimine). The acyl derivative has a higher affinity for lipoproteins. Disc-electrophoretic patterns of lipoproteins separated by this procedure correspond to patterns of apolipoproteins in VLDL and HDL, a finding that corroborates the conclusion that the mechanism of the selective binding of certain lipoproteins to lipophilic water-soluble polycations is based on hydrophobic interaction between the polymer and lipids located at the surface of lipoprotein particles. The procedure offers a simple possibility for direct LDL-cholesterol and LDL-triglyceride determination and for the isolation of VLDL and HDL. The mechanism of the reaction has a biochemical correlate in the action of phospholipase A₂ on phospholipids.

Additional Keyphrases: heart disease • polycation resin • hyperlipoproteinemia

Certain cationic polymers exhibit a high affinity towards lipids. This behavior has been recently studied with respect to lipoproteins. Under certain conditions the polymer selectively binds to VLDL and HDL. They can be extracted from serum in the presence of a cation-exchange resin. We have described a procedure for direct determination of cholesterol in LDL with poly(ethyleneimine) (1). From chemical investigations (2) we predicted that polycations with lipophilic side-chains should be even better reagents, increasingly so as the number of lipid-binding sites is increased. The purpose of this investigation was therefore to test acylated poly(ethyleneimine) for LDL cholesterol determination and thereby to confirm the assumption that the principle of the reaction is based both on hydrophobic and ionic interactions between the polymer and polar lipids located at the surface of VLDL and HDL.

Material and Methods

General procedures for lipoprotein isolation, lipid and apolipoprotein analyses. Serum was sampled from apparently healthy volunteers with normolipemia and from untreated persons with hyperlipoproteinemia of type IIa, type IIb, and type IV after an overnight fast. The specimens were analyzed within 12 h.

Triglyceride and cholesterol concentrations were determined with a Technicon AA II AutoAnalyzer, using isopropanol extracts treated with zeolite.² The quantitation was exactly according to the procedure of the U.S. Lipid Research Clinic Program (3). The determination of cholesterol and triglyceride concentrations in LDL was performed after serum lipoproteins were fractionated by ultracentrifugation (d = 1.006, 4 °C, 20 h) and precipitation with heparin/MnCl₂ as previously described (3). Analytical recovery of cholesterol from isolated lipoproteins was 94 ± 4% (SD). For triglyceride it was 85 ± 6%. The purity of lipoproteins after ultracentrifugation and polyanion precipitation was controlled by agarose gel electrophoresis.

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2 Technicon AutoAnalyzer II (1972), clinical method No. 24, Technicon Instruments, Tarrytown, N.Y., 10591.
The composition of apolipoproteins in lipoproteins isolated by ultracentrifugation or separated with polycations was analyzed by alkaline disc electrophoresis (pH 8.2) as described by Kane (4).

Preparation of dodecoylated poly(ethyleneimine). An aqueous solution of poly(ethyleneimine) (Polymin P; BASF, Ludwigshafen, G.F.R.) was lyophilized for 72 h to remove the solvent. 1 g of the residue was dissolved in 50 ml of 99.6% methanol, and 1 g of dodecoyl chloride (Fluka, Ulm, G.F.R.) was added dropwise with stirring to the solution in a 200-ml round-bottom flask at 25 °C. The mixture was stirred for 24 h at 50 °C. Subsequently, 100 ml of benzene (p.a. grade; Merck, Darmstadt, G.F.R.) was added and the solution was concentrated by evaporation under reduced pressure. The precipitating acylated polymer was washed three times with 100-ml portions of benzene to remove free dodecanoic acid and unreacted acy chloride. The solvent was completely evaporated and the reaction product was dissolved in distilled water to give a 50 g/liter solution of dodecoyl poly(ethyleneimine). Details on the synthesis and studies of various poly(ethyleneimine) derivatives will be presented elsewhere.

Determination of LDL-cholesterol and LDL-triglycerides after treatment of serum with dodecoylated poly(ethyleneimine) and polycation-exchange resin. Cholesterol was measured as recently described (1). Forty microliters of solution of dodecoyl poly(ethyleneimine) was mixed with 1.4 ml of each serum sample which then were simultaneously fractionated by ultracentrifugation. The mixtures were rotated in 2-ml tubes at 60 rpm for 5 min, 0.4 g of Amberlite IRC 50 granules (SERVA, Heidelberg, G.F.R.) was added, rotated for 10 min at low speed, and then for 2 min at 1500 rpm to clear the solution of granules. The supernatant fluids were used for cholesterol and triglyceride determination. Triglyceride concentrations were calculated by subtracting the serum blank values from triglyceride values for the corresponding supernatates from treatment with the Amberlite resin.

Isolation of VLDL and HDL with dodecoyl poly(ethyleneimine). In sera with triglyceride concentrations exceeding 10 g/liter (type IV and type V hyperlipoproteinemia), in treatment with the acylated polymer a creamy phase separated and floated on a clear solution. For these sera, treatment with the ion-exchange resin could be omitted. The clear phase was used for LDL-cholesterol determination. The creamy phase was four times washed with 7-ml portions of saline to remove serum proteins, and the fraction was used for alkaline disc electrophoresis. Delipidation could be achieved without treatment with tetramethyl urea (5).

Precision. The precision was estimated for 16 to 19 replicate determinations of cholesterol and triglyceride in sera from subjects with normolipemia type II or type IV or type V hyperlipoproteinemia after treatment with dodecoyl poly(ethyleneimine) and Amberlite IRC 50.

Results

LDL-cholesterol and LDL-triglycerides as determined by ultracentrifugation and by treatment with dodecoyl poly(ethyleneimine) and Amberlite IRC 50. Correlation of quantification of LDL-cholesterol by both procedures, for sera from subjects with normolipemia and hyperlipoproteinemia, is satisfactory, both for samples with low triglyceride concentrations (normolipemia and type IIa, r = 0.94) and those rich in triglyceride (type IV and type IIb, r = 0.95). The slope of the regression line for the latter is closer to the ideal than for the former (Figures 1 and 2). For triglycerides the correlation is not as good (r = 0.80) as for cholesterol (Figure 3).

Precision. We estimated the precision of LDL-cholesterol and LDL-triglyceride determination by replicate analyses of serum samples from persons with normolipemia or type II, type IV, or type V hyperlipoproteinemia. The CV was between

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3 Heuck, C. C., and Nothhelfer, A., manuscript in preparation.
Fig. 3. Correlation of LDL-triglyceride determination (mg/dl) after extraction of VLDL and HDL with dodecyl poly(ethyleneimine)/Amberlite IRC 50 and after density-gradient fractionation

Serum samples with high triglyceride concentrations (type IV and type llb, A); serum samples with low triglyceride concentrations (type IIA and normolipemia), $\Phi \cdot y = 0.82x + 8.7; r = 0.81$

2.5 and 6.4% for cholesterol and 5.1 to 13.0% for triglyceride. Replicate analyses of the type V serum resulted in values that were about twice as high as concentrations determined after fractionation by ultracentrifugation. For this serum sample the analytical recovery of cholesterol after density-gradient centrifugation was only 60%, 48% for triglyceride (Table 1).

**Disc-electrophoresis of lipoproteins.** The disc-electrophoretic patterns of lipoproteins isolated after treatment with dodecyl poly(ethyleneimine) and washed four times with saline have been compared with those for VLDL and HDL isolated by ultracentrifugation (Figure 4). The bands for lipoproteins that have been separated by aggregation by the polycation are in the same position as the bands of apolipoproteins in VLDL and in HDL. The bands for the apolipoproteins C3_1, C3_2, and C2 appear with the same intensity as do the bands of apolipoprotein A1 and A2 in the polycation extract. Comparison with the apolipoprotein bands in isolated HDL and VLDL clearly shows that both lipoproteins are bound to the polymer. This corroborates the previous observation from agarose electrophoresis (1) that only these lipoprotein classes are extracted while LDL remain in the subnatant fluid. On radial immunodiffusion of the washed polycation extracts, only traces of albumin could be detected.

**Discussion**

Selective extraction of VLDL and HDL from serum has been attributed to a high affinity of lipophilic polycations for

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**Table 1. Precision of Cholesterol and Triglyceride Determination in LDL after Extraction of VLDL and HDL with Dodecyl Poly(ethyleneimine)/Amberlite IRC 50 from Sera of Subjects with Normolipemia and Hyperlipoproteinemia of Types llb, IV, and V**

<table>
<thead>
<tr>
<th></th>
<th>Serum chol RecovYUC</th>
<th>Serum TG RecovYUC</th>
<th>LDL-cholYUC</th>
<th>CV</th>
<th>LDL-cholExt. g/liter</th>
<th>CV</th>
<th>LDL-TGExt. g/liter</th>
<th>CV</th>
<th>LDL-TGExt. %</th>
<th>CV</th>
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<tbody>
<tr>
<td>norm. (19)*</td>
<td>1.78 1.75</td>
<td>1.11 0.98</td>
<td>1.08 0.05</td>
<td>4.6</td>
<td>1.2 0.03</td>
<td>2.5</td>
<td>31 5</td>
<td>16.1</td>
<td>28 4</td>
<td>13.2</td>
</tr>
<tr>
<td>Type llb (16)</td>
<td>2.89 2.87</td>
<td>2.11 1.82</td>
<td>2.05 0.11</td>
<td>5.5</td>
<td>2.1 0.13</td>
<td>6.4</td>
<td>79 5</td>
<td>6.3</td>
<td>83 6</td>
<td>7.7</td>
</tr>
<tr>
<td>Type IV (19)</td>
<td>2.58 2.45</td>
<td>2.99 2.55</td>
<td>1.69 0.07</td>
<td>4.1</td>
<td>1.83 0.05</td>
<td>2.7</td>
<td>81 5</td>
<td>6.2</td>
<td>85 7</td>
<td>8.1</td>
</tr>
<tr>
<td>Type V (16)</td>
<td>11.30 6.89</td>
<td>4.10 19.87</td>
<td>0.59 0.11</td>
<td>19.0</td>
<td>1.32 0.04</td>
<td>3.4</td>
<td>63 12</td>
<td>19.0</td>
<td>153 8</td>
<td>5.1</td>
</tr>
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* No. of replicate analyses on each serum sample is given in parentheses.

RecovYUC: total recovered cholesterol (chol) and total recovered triglyceride (TG) from ultracentrifugation.

LDL-cholYUC: LDL-cholesterol from ultracentrifugation.

LDL-TGExt: LDL-triglyceride from ultracentrifugation.

LDL-cholExt: LDL-cholesterol from extraction of VLDL and HDL with polycations.

LDL-TGExt: LDL-triglyceride from extraction of VLDL and HDL with polycations.

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polar lipids located at the surface of these particles (1). This affinity can be increased by introducing lipophilic residues onto the polymer (6). This supports the idea that poly(ethyleneimine) acylated with long-chain fatty acids should be more efficient than the unaltered polycation. Our data confirm this for dodecyl poly(ethyleneimine). The concentration to be used for complete extraction on VLDL and HDL with this polymer is only a fifth that needed for poly(ethyleneimine) (1), which clearly demonstrates the higher affinity of the acyl derivates for the two lipoprotein classes.

Previously discussed electrophoretic studies revealed that only LDL remain in the supernatant fluid of serum that has been treated with the polycation and the exchange resin (1). The disc-electrophoretic analysis demonstrates that VLDL and HDL are in fact the lipoproteins that are aggregated by the polycation; other serum proteins do not react and can be separated by washing. Thus lipophilic polycations not only can be used for the routine analyses of LDL-cholesterol determination but also to isolate VLDL and HDL. Van Dam-Mieras et al. (7) were able to show that the specific binding site of pancreatic phospholipase A_2 for phospholipids is formed by a sequence of lipophilic amino acids adjacent to the amino acid arginine. Formation of the enzyme/substrate complex was found to be due both to lipophilic forces between the amino acid sequence and acyl residues and ionic forces of oppositely charged groups of the phospholipid at the binding site. The similarity between the action of the lipase and of the polycation is obvious: both polymers have similar structural characteristics, namely closely adjoining lipophilic residues and free amino groups. The specificity of the binding of the phospholipase can be therefore regarded as a biochemical correlate to the action of poly(ethyleneimine) and its derivates.

The distinct electrophoretic mobilities of lipoproteins reflect considerable differences in the negative charge density of the lipoprotein surfaces. This is why the action of poly(ethyleneimine) and poly(ethyleneimine) derivates towards lipoproteins is supposed to occur preferentially with VLDL and HDL, whereas the binding of LDL is minimal because of the lower negative charge density of this class of lipoproteins.

Various factors have to be considered to explain the differences in the determination of LDL-cholesterol in type V sera by ultracentrifugation and by treatment with polycations. On one hand, the recovery of cholesterol and triglycerides from ultracentrifugation is generally not satisfactory. Therefore LDL-cholesterol and LDL-triglycerides may be underestimated by this procedure. On the other hand, VLDL in type V sera may vary with regard to their charge density. The tailing of VLDL of type V serum in agarose electrophoresis may be an expression of these differences. The finding of higher triglyceride concentrations in the supernatant fluid in our type V serum indicates that not all of VLDL have been extracted. The efficiency of extraction was not considerably improved by adding more dodecyl poly(ethyleneimine), thus the polymer concentration was not simply too low for the extraction.

The lower correlation for the triglyceride determination can be explained by the lower recovery of triglycerides after ultracentrifugation. It improves in the case of sera rich in triglycerides. In addition small fluctuations lead to considerable changes in the CV at low triglyceride concentrations.

The use of dodecylated poly(ethyleneimine) instead of poly(ethyleneimine) offers practical advantages. Less is needed and the reagent solution is less viscous. We have also compared the action of other ethyleneimine polymers(“PEI 6” and “PEI 600” from Dow Chemical). The polymers qualitatively show similar characteristics towards lipoproteins, but for quantitative investigations results were satisfactory only with Polymin P. It is highly crosslinked and of higher molecular weight than PEI 600, with which the extraction of VLDL and HDL was incomplete. Supposedly the different behavior may be due to a different affinity of lipoprotein/PEI 600 complexes to the ion-exchange resin. Regarding other ion-exchange materials, we observed similar qualities with Amberlite XAD 2 (Serva, Heidelberg, G.F.R.), but the precision was not as good with Amberlite IRC 50.

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References