Cholesterol Determination in Serum after Fractionation of Lipoproteins by Immunoprecipitation

Claus-Chr. Heuck,1 Ingeborg Erbe,2 and Dietger Mathias1

We investigated the immunoprecipitation of apolipoprotein B-binding lipoproteins, or of apo A-I and apo C-binding lipoproteins, by delipidated antiserum for measuring cholesterol in the nonprecipitated lipoprotein fractions. After immunoprecipitation of serum with delipidated anti-apo B, we determined by immunoelectrophoresis that no β- or pre-β lipoproteins were present, whereas α-lipoproteins remained in the supernate. Conversely, after immunoprecipitation with an antiserum against apo A-I + apo C, only lipoproteins with β-mobility were detected and no apo B from β-lipoproteins was in the precipitate. The concentration of cholesterol in the supernate after immunoprecipitation with anti-apo B correlated highly (r = 0.93, n = 118) with cholesterol measured after precipitation with phosphotungstic acid/MgCl2. The cholesterol concentration after immunoprecipitation with anti-apo A-I and anti-apo C correlated similarly well (r = 0.94, n = 145) with the LDL-cholesterol calculated by Friedewald's formula in serum specimens reflecting moderate hyperlipoproteinemia.

Additional Keyphrases: apolipoproteins • immunoelectrophoresis • HDL-cholesterol • LDL-cholesterol • dyslipoproteinemia • immunoassay • precipitation with polyanion comparison

Measurement of cholesterol in lipoprotein fractions of human plasma or serum is routinely used for the differential diagnosis of dyslipoproteinemia and the evaluation of risk for cardiovascular disease. The reference method for the separation of lipoproteins is ultracentrifugation (1) or ultracentrifugation combined with precipitation with polyanion (2). For normolipemic and moderate hyperlipoproteinemic serum, polyanion precipitation alone is the most common method of fractionation (3-5). Also, electrophoretic separation in agarose with subsequent polyanion precipitation for densitometry (6), for quantification of cholesterol in lipoprotein fractions by gas-liquid chromatography (7) or by enzymic determination (8), has been described.

Apolipoprotein (apo) B is the major protein constituent in low-density (LDL) and very-low-density lipoproteins (VLDL) (9), whereas apo A-I and apo C are predominantly bound to high-density lipoproteins (HDL) and VLDL. The hydrated densities of the three major lipoprotein classes are closely related to the distribution of these peptides. Some investigators, however, have also detected apo B in HDL (10) and apo A-I in LDL (11).

Immunoprecipitation has been claimed to successfully separate subfractions of major lipoprotein classes (12). In view of the small quantity of HDL particles binding apo B, relative to the total amount of HDL, one should expect a high correlation for cholesterol in the supernatant solution after polyanion precipitation with that after immunoprecipitation with delipidated anti-apo B. Immunoprecipitation should also be suitable for the subsequent measurement of cholesterol in lipoproteins closely resembling β-lipoproteins.

Materials and Methods

Materials

Antisera against human apo B (lot no. 3766), apo A-I (lot no. 8857), and apo A-II (lot no. 4046) were purchased from Behringwerke AG, Marburg, F.R.G. as was Lipoclean® (perfluoroperclooroethylen). Reagents for cholesterol and triglyceride determination and the phosphotungstic acid/MgCl2 reagent for precipitation of VLDL and LDL were obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G. The other reagents were from Merck GmbH, Darmstadt, F.R.G. Controlled-pore-size glass (Daltosil 1200) was obtained from Serva GmbH, Heidelberg, F.R.G.

Serum samples were obtained from normolipemic and hyperlipoproteinemic children (ages four to 16 years) after overnight fasting and adults (ages 18 to 56 years) without secondary hyperlipoproteinemia, such as liver disease. Serum triglycerides were between 0.5 and 4.5 mmol/L, serum cholesterol concentration was between 2.5 and 15 mmol/L.

Procedures

General assay procedure. We routinely measured triglyceride and cholesterol in the serum samples by enzymic analysis (13, 14) and by the chemical method of the Lipid Research Clinics (2). HDL-cholesterol was measured enzymically after precipitation of VLDL and LDL with phosphotungstic acid/MgCl2 (8). LDL-cholesterol concentrations in serum were calculated by the formula of Friedewald et al. (15). HDL was isolated from human serum by ultracentrifugation, as described by Havel et al. (1). Apo B was measured by immunonephelometry of 30 µL of the supernate after immunoprecipitation with 200 µL of anti-apo B as previously described (16).

Isolation of apolipoprotein and the production of antisera. Apo A-I and apo C peptides from HDL and VLDL were purified as described in detail elsewhere (17). We injected subcutaneously into rabbits 0.3 mg of purified apo A-I mixed with Freund's adjuvant. Other animals were immunized with a mixture of apo C-I, C-II, C-III or a mixture of apo A-I + apo C-I, C-II, C-III. At two- to three-week intervals we administered booster injections of the antigen mixture in incomplete Freund's adjuvant. Ten days after the fourth injection the animals were bled for the isolation of antisera. Testing with immunoelectrophoresis and immunodiffusion (18) showed that the various antisera reacted against apo A-I, apo C-IIIβ, C-III1, C-IIIα, and apo C-II, but not against apo C-I, apo B, or human serum albumin. Serum from animals immunized with apo A-I reacted only with apo A-I. Immunization with a mixture of apo C-I, C-II, C-III produced immunoreactivity against apo C-II and apo C-III, but not against apo C-I.

1 Universitätskinderklinik, Im Neuenheimer Feld 150, 69 Heidelberg, F.R.G.
2 Medizinische Universitätsclinik, Bergheimer Str. 58, 69 Heidelberg, F.R.G.
3 Nonstandard abbreviations: apo, apolipoprotein; HDL, LDL, and VLDL, high-, low-, and very-low-density lipoprotein(s).

Received December 20, 1983; accepted November 2, 1984.
Preparation of lipoprotein-free antiserum. Method A: We mixed 1 mL of rabbit antiserum against human apo B for 10 min at room temperature with 0.1 g of controlled-pore glass (solid phase). After centrifugation for 10 min at 30,000 × g at room temperature, we mixed the supernate with controlled-pore glass, as above. The resulting concentration of cholesterol in the antiserum was 50 μmol/L. We diluted this delipidated antiserum with an equal volume of isotonic saline and used this as the immunoprecipitant.

Method B: We vigorously mixed 1 mL of antiserum against apo A-I and apo C with 2.5 mL of Lipoclean and n-hexylamine (95/5 by vol) at room temperature for 15 min. After centrifugation at 13,000 × g for 5 min, we removed the upper phase by pipetting to extract its lipids by this same procedure two more times. For routine analysis we used this delipidated antiserum after diluting it threefold with isotonic saline. This dilution, which contained 50 μmol of cholesterol per liter, was used as the immunoprecipitant. In alternative experiments we extracted the lipids with Lipoclean and n-hexanol or octanol (75/25 by vol).

Immune precipitaton of lipoproteins. We mixed 10, 20, 30, or 40 μL of fresh human serum with 100 μL of diluted delipidated antiserum to apo B, anti-apo A-I, or a mixture of anti-apo A-I + apo C. After letting the mixture stand for 1 h at room temperature, we centrifuged the immunocomplexes for 10 min at 15,000 × g. For routine measurements we economized by using only 10 μL of serum with 50 μL of the diluted delipidated anti-apo B, or 5 μL of serum with 50 μL of antiserum against apo A-I + apo C. We measured the cholesterol concentrations in the supernates enzymically.

Immunoelctrophoresis studies. After immunoprecipitation we subjected 10 μL of the supernates to immuneelectrophoresis in agarose as previously described (18), using antisera against apo B, apo C, apo A-I, and apo A-II to detect lipoproteins. The lipids in the immunoprecipitates were stained with Sudan black (19). In additional experiments we washed the precipitates from the reaction with anti-apo A-I and anti-apo C twice with 0.5 mL of isotonic saline, then dissolved the precipitates in 20 μL of urea solution (6 mol/L of Tris HCl buffer, 0.1 mol/L, pH 9.6) and checked their immunoreactivity against apo A-I and apo B by immunoelectrophoresis.

Precision studies. To determine the precision of the method, we measured the cholesterol in a series of 10 supernates after immunoprecipitation and daily in eight aliquots of a serum with normal cholesterol and of another serum with a high cholesterol concentration.

Calculations. We calculated residual cholesterol in human serum as the difference between the cholesterol in the supernate after immunoprecipitation and that in the antiserum as determined after delipidation, then multiplied this value by the dilution factor.

Results

Delipidation

Determination of lipids in serum supernates after selective immunoprecipitation of lipoproteins is suitable only when an antiserum free of lipids is the precipitating reagent. Lipids can be extracted with a liquid–liquid system or a solid–liquid system. On the basis of previous observations (20, 21), we tested several liquid–liquid systems for this. Adding n-hexylamine to Lipoclean extracts the cholesterol from rabbit antiserum more effectively than does the halogen carbon alone (Table 1). To improve delipidation, we repeat the extraction step twice so that only small amounts of cholesterol are detectable. This remaining portion seems to be tightly bound to the protein moiety, such that additional extractions did not improve the delipidation. By countercurrent immunoelectrophoresis (not shown), we determined that the antiserum remains immunoreactive after delipidation with Lipoclean/n-hexylamine, but not after extraction with Lipoclean/n-hexanol or Lipoclean/n-octanol.

Previous studies document complete extraction of fatty acids and lipids with silica (22). Our results with controlled-pore glass confirm that the solid–liquid system is equally suitable for the delipidation of the antiserum. Both methods obviate the isolation of immunoglobulins from the antiserum as an alternative method for the preparation of an immunoreagent.

Immunoelectrophoresis

After immunoprecipitation with anti-(apo A-I + apo C), we investigated a hyperlipidemic serum (triglyceride 4.8 mmol/L, cholesterol 8.5 mmol/L) and its supernate by immunoelectrophoresis (Figure 1, left). The immunoprecipitates from serum stained positive for lipids when anti-apo A-II, anti-apo B, and anti-apo C were used; the supernate stained for lipid in the β-region when reacted with anti-apo B, but no lipid was stained in the α-region when reacted with anti-apo A-II or anti-apo A-I. Lipid-stained immunocomplexes with anti-apo C were also observed in the pre-β region with serum samples but not in the serum supernates after immunoprecipitation. The limit of detection for β-lipoproteins by Sudan Black staining was 200 ng, corresponding to 45 μmol of cholesterol per liter of serum. This value is within the limits of precision for the determination of cholesterol in LDL by ultracentrifugation, or as calculated with the Friedewald formula. In additional studies with severely hyperlipidemic sera (triglyceride ≥ 4.5 mmol/L), an immunoreaction against anti-apo C in the supernate indicated incomplete immunoprecipitation of VLDL.

To study the immunoprecipitation of apo B-carrying lipoproteins quantitatively, we changed the ratio of volume of human serum and lipoprotein-free antiserum (Table 2), keeping the cholesterol concentration in the supernate constant, for a mixing ratio of less than 3/10 (by vol). Immunoelectrophoresis confirmed the selectivity of immunoprecipitation (Figure 1, right). Sudan Black stained the immunoprecipitates obtained on treatment with anti-apo A-I. Apo A-II was present on the serum sample and in the supernate obtained after treatment with anti-apo B in contrast, precipitates with anti-apo B were visible only in serum but not in the supernate after immunoprecipitation. The serum samples for these experiments contained, at most, 2.05 mmol of triglyceride and 8.8 mmol of cholesterol per liter.

Coprecipitation studies. To verify the possibility of coprecipitation, we added increasing volumes of human HDL (isolated by ultracentrifugation) to the serum samples before immunoprecipitation with anti-apo B. The results did not indicate coprecipitation of HDL with precipitated LDL (Table 3). On the other hand, to exclude the coprecipitation

<p>| Table 1. Extraction of Cholesterol from Rabbit Antiserum |
|---------------------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>Cholesterol concn, mg/L in supernate</th>
<th>Immunoactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>410</td>
<td>+</td>
</tr>
<tr>
<td>Lipoclean</td>
<td>340</td>
<td>+</td>
</tr>
<tr>
<td>Lipoclean/n-hexylamine</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>(95/5 by vol)</td>
<td>Controlled-pore glass</td>
<td>50</td>
</tr>
</tbody>
</table>

*Liquid–solid extraction procedure; all others liquid–liquid.*
of lipoproteins carrying apo B, we immunoprecipitated a type IIa serum (triglyceride 0.5 mmol/L, cholesterol 11.4 mmol/L, LDL-cholesterol 9.7 mmol/L) with anti-apo A-I and anti-apo C. When we washed the immunoprecipitate with isotonic saline before immunoelectrophoresis, only anti-apo A-I caused a reaction; no precipitate was visible with anti-apo B (not shown).

Comparison Studies

These observations stimulated us to compare the immunoprecipitation method with polyanion precipitation for HDL-cholesterol determinations. The data obtained on serum samples from 118 individuals correlated satisfactorily (r = 0.93) within the range 0.31 to 1.85 mmol/L for HDL-cholesterol (Figure 2). The ratios of cholesterol concentration after immunoprecipitation to that after polyanion precipitation neither correlated with serum triglyceride (r = 0.06) nor with serum cholesterol (r = −0.31) with respective upper limits for triglyceride and cholesterol of 4.9 and 12 mmol/L.

Despite this good statistical correlation, certain special

Table 2. Cholesterol Concentrations (mmol/L) in Supernates of Three Sera after Immunoprecipitation with Diluted Lipoprotein-Free Anti-Apo B

<table>
<thead>
<tr>
<th>Serum cholesterol concn</th>
<th>Serum sample, μL*</th>
<th>Calculated</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.7</td>
<td>40</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>7.2</td>
<td>30</td>
<td>0.55</td>
<td>0.65</td>
</tr>
<tr>
<td>4.3</td>
<td>20</td>
<td>0.45</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Volume of sample precipitated with 200 μL of anti-apo B.

Table 3. Cholesterol Concentration in Supernates of Serum Plus HDL, after Immunoprecipitation of Apo B-Carrying Lipoproteins

<table>
<thead>
<tr>
<th>Sample*</th>
<th>HDL added, μL</th>
<th>Cholesterol, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td>Calculated</td>
</tr>
<tr>
<td>Serum HDL</td>
<td>50</td>
<td>1.3</td>
</tr>
<tr>
<td>Serum + HDL</td>
<td>100</td>
<td>2.0</td>
</tr>
<tr>
<td>Serum + HDL</td>
<td>200</td>
<td>2.4</td>
</tr>
<tr>
<td>Serum + HDL</td>
<td>300</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*20 μL of each sample was precipitated with 100 μL of diluted, delipidated antiserum; 20 μL of the supernate was used for cholesterol determination.

![Fig. 1. Immunoelectrophoresis of serum and supernate after immunoprecipitation with a rabbit antiserum against human apo A-I plus apo C (left) or with antiserum against apo B (right) Stain: Sudan Black](image1)

![Fig. 2. Correlation between the determination of cholesterol in serum supernates after precipitation with phosphotungstic acid/MgCl₂ or with anti-apo B: y = 0.83x + 0.18 mmol/L (r = 0.93, n = 118)](image2)

mens showed remarkable differences between results by each method. Because our results were independent of the serum cholesterol and triglyceride concentrations, we excluded incomplete immunoprecipitation of apo B-carrying lipoproteins by investigating three supernates of serum samples for the presence of apo B by immunonephelometry. The cholesterol concentration in these samples after immunoprecipitation was greater than after polyanion precipitation by 0.24 mmol/L. The immunonephelometric method has a detection limit of 30 ng of apo B per 200 μL of incubate (corresponding to 45 μmol of LDL-cholesterol per liter of untreated serum). Subsequent immunoreactions to detect the presence of nonprecipitated apo B lipoproteins gave no positive results that could account for the difference.

Further we investigated four serum samples that had at least 0.24 mmol/L less cholesterol in the supernate after immunoprecipitation than after polyanion precipitation, to determine by counterimmunoelectrophoresis whether the immunoprecipitate contained any apo A-I. The limit of detection for apo A-I was 200 ng (corresponding to 45 μmol of HDL-cholesterol per liter of serum). None of the immunoprecipitates reacted with anti-apo A-I, indicating that HDL was coprecipitated during the treatment of the serum with anti-apo B.

In view of the limitations of cholesterol calculation imposed by hypertriglyceridemia, we restricted our comparison of measurement of cholesterol by immunoprecipitation...
with anti-(apo A-I + apo C) vs LDL-cholesterol determination according to Friedewald to specimens with serum triglyceride less than 4.5 mmol/L. Results by the two methods correlated highly ($r = 0.94$, $n = 145$), although the line of regression deviated slightly from the ideal (Figure 3). The ratio of cholesterol in the supernatant solution after immunoprecipitation to LDL-cholesterol (according to Friedewald et al.) was independent of serum triglyceride ($r = 0.151$) and of serum cholesterol ($r = 0.20$).

**Precision Studies**

The coefficient of variation (CV) for determinations with anti-apo A-I and anti-apo C for a series of 10 aliquots of a hyperlipidemic serum (cholesterol 1.62 mmol/L, triglyceride 1.94 mmol/l) was 3.8%. The CV was 5.5% for estimations with a normolipemic serum (cholesterol 7.3 mmol/L, triglyceride 1.6 mmol/L) stored at 4°C over eight consecutive days and 8.1% for the hyperlipidemic serum sample. Precision decreased for samples stored longer.

The CV for a series for cholesterol determinations in supernates after immunoprecipitation with anti-apo B of serum (triglyceride 1.2 mmol/L, cholesterol 4.2 mmol/L) was 5.0%. The CV was 8.2% in a daily series of determinations over eight days after storage at 4°C (cholesterol concentration after immunoprecipitation, 0.94 mmol/L).

**Discussion**

The fractionation of lipoproteins by immunoaffinity chromatography and immunoprecipitation was successfully applied to isolate subclasses of lipoproteins (12, 23, 24). To date, no investigation exists describing a simple immunological method for separating lipoproteins by immunoprecipitation to determine the lipids in the nonprecipitated fraction. When antisemur is used as an immunoreagent, the lipids of the antisemur must be removed so that falsely high concentrations of lipid will not be measured in the supernate. Our experiments demonstrate that the lipids may be successfully extracted either by liquid–liquid or by solid–liquid extraction without loss of immunoreactivity. This treatment obviates the need to isolate the gamma-globulin fraction of the antisemur, which may be equally suitable for immunoprecipitation (unpublished observation).

The fractionation of lipoprotein classes by immunoprecipitation implies that antigenic binding sites differ from one lipoprotein class to another. Apo A-I and apo A-II are the major apolipoproteins in HDL. All subclasses of HDL have apo A-I, whereas the distribution of apo A-II varies considerably and may not be detectable in certain HDL subclasses (23). Small amounts of the apo A peptides are also detectable in VLDL and in LDL (11). The major protein constituents in VLDL are the apo C peptides and apo B. Apo C is also found in HDL. On the other hand, apo B composes approximately 85 to 95% of the LDL protein, and is also detectable in HDL subclasses (10). The heterogeneous composition of the apolipoprotein moieties in the different lipoprotein classes indicates that the cholesterol concentration in the supernate after immunoprecipitation with anti-apo B need not correspond to the concentration of cholesterol after lipoprotein precipitation by polyanions. This is also true of the cholesterol measurement after immunoprecipitation with anti-apo A-I and anti-apo C in comparison with LDL-cholesterol determinations by ultracentrifugation or by the Friedewald formula.

Our studies indicate a selective immunoprecipitation of apo B-carrying lipoproteins. We did not observe incomplete immunoprecipitation of apo B-carrying lipoproteins or co-precipitation of α-lipoproteins. Kostner, on the other hand, isolated subpopulations of lipoproteins without apo B in the low-density fraction (11). These observations indicate that the immunoprecipitation of apo B-carrying lipoproteins correlates well with, but is not equivalent to, the fractionation of lipoproteins according to their affinity for polyanions.

Several studies demonstrate considerable variations in the precipitation of apo B-carrying lipoproteins by polyanions (24, 25). Recent investigations suggest that the treatment of serum with polyanions and divalent cations may not precipitate only VLDL and LDL, but also small amounts of HDL (26–28). Therefore, the slight divergence of cholesterol concentration after immunoprecipitation and polyanion precipitation may reflect the nonidentity of the mechanism of the two reactions. Interestingly, the correlation between the methods compared is as good as that for the HDL-cholesterol determination after precipitation of VLDL and LDL with use of different polyanions (26). The immunoelectrophoresis studies also confirm the efficacy of the immunoprecipitation of lipoproteins carrying apo A-I and apo C. Thus, either the proportion of the β-lipoproteins with a heterogeneous composition of apolipoproteins is small enough to be within the precision of the photometric determination of cholesterol in the supernatant β-lipoprotein fraction, or the accessibility of antigenic binding sites in apo A-I and apo C bound to LDL may differ from that of the apolipoproteins bound to VLDL and HDL.

The quality of the antisemur undeniably limits its applicability. We are well aware of the limitations of our present immunoreagent. Therefore, we confined our studies to serum specimens without pronounced hypertriglyceridemia. The quality of the reagent is, however, sufficient for analysis of 85 to 90% of the serum specimens received by a routine laboratory. Our efforts have concentrated on the production of an antisemur with a greater immunoreactivity against apo C peptides, which would extend the range of analysis to serum samples exhibiting an even higher degree of hyperlipidemia. In our experience, the described method offers several advantages: only very small sample volumes are required, and, in contrast to the polyanion procedure, results are less susceptible to external influences, thereby allowing a direct determination of cholesterol equivalent to LDL-cholesterol.

**References**