New Method for Isolating and Quantifying Intermediate and β-very-Low-Density Lipoprotein Cholesterol

Regina L. W. Wikinski, Laura E. Schreier, and Silvia B. Rosental

We describe a new method, useful to clinical laboratories, for assessing intermediate density (IDL) or β-very-low-density (β-VLDL) lipoprotein cholesterol. The technique involves selective precipitation properties of the qualitative Wieland and Seidel post-electrophoretic method that immobilizes IDL and β-VLDL in the β-zone of an agarose slide (Clin Chem 1973;19:1139–41). In our method, we separate low-density lipoprotein (LDL) in a second electrophoretic step, in which LDL moves toward the anode, and then quantify the cholesterol of the above lipoproteins remaining in the precipitate band at the β-zone. Replicate within-run precision (CV) of 15 aliquots of a sera pool was 10.1%. The correlation with sequential ultracentrifugation of 30 samples was \( r = 0.96 (P < 0.001) \). Serum reference values for 30 normal individuals are 57 ± 7.0 mg/L. Seven phenotype III hyperlipoproteinemic patients had the highest concentrations of IDL or β-VLDL cholesterol in serum, 1620 ± 346 mg/L.

Additional Keyphrases: electrophoresis, agarose • hyperlipoproteinemia • atherosclerosis

Measurement of intermediate-density (IDL) and β-very-low-density lipoproteins (β-VLDL) is useful for diagnosis of phenotype III hyperlipoproteinemia, either primary (dysbetalipoproteinemia) or secondary (hypothyroidism or diabetes) (1). Also, an IDL increase in familial hypercholesterolemia due to failure in apo B:E receptors, which are common catalytic sites for low-density lipoproteins (LDL) and IDL, has been described (2). On the other hand, high concentrations of IDL and β-VLDL are associated with coronary heart disease (3). Dysbetalipoproteinemia is detected in clinical laboratories by the presence of a broad β-band in the serum electrophoretogram and by post-electrophoretic precipitation with heparin–MgCl\(_2\)–NaCl. The latter is selective for β-VLDL, including IDL particles (4). So far, many laboratories still measure the cholesterol transported by these lipoproteins after separation by sequential ultracentrifugation, but this technique requires expensive apparatus.

Here we describe an application of the principle of Wieland and Seidel (4) for the post-electrophoretic precipitation of IDL or β-VLDL. We combine the method with a further electrophoretic step, in which LDL is separated from the previous β-zone while the precipitated lipoproteins remain immobilized. The cholesterol in IDL or β-VLDL is extracted with organic solvents and measured as suggested by Camejo et al. (5).

Materials and Methods

Patients and Sample Preparation

We studied 30 normal individuals and 39 dyslipemic patients, the latter including seven patients with phenotype III hyperlipoproteinemia (four patients with primary dysbetalipoproteinemia and three with diabetes).

After subjects fasted for 12 h, 35 mL of blood was drawn, placed in clean tubes, and allowed to stand for 45 min at room temperature until clotted completely. The sample was then centrifuged at 1500 × g for 15 min at 4 °C and the serum was transferred to a storage tube. Serum was then centrifuged again at 16 000 × g at 8 °C to dispose of chylomicrons, if any. The infranate was stored at 4 °C for no more than three days. An aliquot was used to test the new assay procedure and another aliquot was assayed by the comparison sequential ultracentrifugation technique (6). In a third serum aliquot we measured total cholesterol concentration by the enzymatic method of Röschlau et al. (7), LDL cholesterol by the method of Wieland and Seidel (8), and HDL cholesterol in the supernate after precipitation of other lipoproteins by the phosphotungstic–MgCl\(_2\) procedure (9, 10). The quality-control procedure was performed according to Naito’s criteria (11). We measured the concentration of serum triglycerides by the enzymatic method of Bučol and David (12) in an ABA/VP automated analyzer (Abbott Labs., North Chicago, IL). We phenotyped the hyperlipoproteinemias according to Beaumont et al. (13). The distribution of hyperlipoproteinemias is shown in Table 1.

Assay Procedure

First electrophoretic step for separation of lipoproteins:

We melted 3.5 mL of 1% agarose gel (A-6013 Type I; Sigma Chemical Co., St. Louis, MO) in a sodium barbitral buffer, pH 8.6, ionic strength 0.05 (Solution 1), then cooled the solution to 50 °C and poured it over clean microscope slides. To make the application slots, we placed 50 mm\(^3\) brass cylinders on the agarose surface. After the gel set, the cylinders were removed and 50 μL of the sample serum, previously mixed with a drop of a bromophenol blue (1000 mg/L) ethanolic solution, was placed into the slot. Samples were run in quadruplicate. The slides were placed in an S-6q electrophoretic chamber (Sebia, France) with a cooling
plate, making the electrical contact through wetted filter paper. The electrode buffer was Solution 1. The electrophoretic run was at 5.0 mA/slide for 60 min at 16 °C.

Precipitation of IDL or β-VLDL: Three slides were placed overnight in a container with a solution of heparin (H-3125; Sigma Chemical Co.) 25 000 USP units/L, containing 20 g of MgCl₂·6 H₂O and 10 g of NaCl per liter of water (Solution 2). The fourth slide was fixed in another container with a solution of ethanol: methanol:isopropanol:water (45:1:1:30 by vol) (Solution 3), dried for 30 min in an oven at 70 °C, and stained with a solution of Sudan Black B (0.4 g) and Zn acetate (4 g) in ethanol (120 mL) and water (80 mL). This slide is considered a control for visually monitoring the β-zone. The precipitate band appeared in the β-zone of the three slides, one of which is shown in Figure 1A. Serum with low concentrations of IDL may not show this band of precipitate and slides must be carefully compared with the control.

Second electrophoretic step for separation of LDL: The three precipitate slides were washed twice for 10 min with Solution 1, and a third wash was performed for 6 h. The slides were placed in the electrophoretic chamber, with Solution 1 as buffer. The electrophoretic run lasted 2.5 h at 8 mA/slide at 16 °C for better results. IDL or β-VLDL remained in the β-zone, but LDL moved toward the anode. The slides were placed in fixing solution, Solution 3. We found LDL to be in a clearly defined band (Figure 1B).

Quantification of cholesterol in the precipitated lipoprotein band: We cut out the precipitate band with a stainless steel blade and placed the band in a Pyrex tube containing 2 mL of water, for analysis by the method of Camejo et al. (5). One, two, or three bands may be used, according to the precipitation intensity. We heated the sample in a boiling water bath for 5 min, then added 2 mL of n-butanol (Merck, Darmstadt, F.R.G.) after the solution cooled to 50 °C. We shook the sample for 1 min in a vortex-type mixer, then centrifuged at 3000 × g for 15 min. We placed 1 mL of the upper (butanolic) phase in a Teflon-lined screw-cap tube and performed a Zak reaction (14). We added 2 mL of a 250 mg/L solution of FeCl₃·6 H₂O in aldehyde-free glacial acetic acid. Then, we pipetted 2 mL of concentrated sulfuric acid (Merck) gently down the side of the tube. The layers were mixed rapidly and the tube recapped and set aside to cool for 30 min. We read absorbance at 540 nm vs a reagent blank, and used crystalline cholesterol (5000 and 1000 mg/L in n-butanol) to prepare the standard curve.

Calculations: The concentration of IDL or β-VLDL cholesterol was calculated by assuming that 1 mL of butanolic phase represents 25, 50, or 75 μL of serum, depending on the number of precipitate bands used in the extraction step.

Comparison Method

We performed sequential flotation ultracentrifugation by the method of Schumaker and Puppione (6). Na₂HEDTA (1.2 g/L) and NaN₃ (0.1 g/L) were added to the serum aliquots. We layered each aliquot carefully with an equal volume of a solution containing 0.01 mol/L Tris, 1.7 mmol/L Na₂HEDTA, 0.154 mol/L NaCl, pH 7.4 (density 1.006 kg/L). The samples were centrifuged in a Sorvall OTD 55B (Du Pont Co., Wilmington,
DE) with a T-865-1 fixed-angle rotor at 105 000 \times g at 16 °C for 17 h. The floating normal VLDL was discarded, and the subnatant solution was adjusted at a density of 1.020 kg/L and centrifuged under the same conditions. The supernatant IDL was quantitatively isolated and cholesterol was measured after extraction with n-butanol as above (14).

Evaluation of the Method

We added increasing volumes of a concentrated IDL fraction (obtained ultracentrifugally) to a serum sample (IDL concentration 18.2 mg/L). Next we assayed IDL cholesterol. We subtracted the concentration of endogenous IDL cholesterol from the measured value and divided the result by the amount of added IDL cholesterol. Each value was obtained in triplicate.

We assessed within-run precision in 15 aliquots of a sera pool and evaluated between-day precision by assaying a sera pool in quadruplicate in a three-day study (a longer study was not feasible because the stability of the lipoproteins without chemical preservatives or antibiotics is limited).

From 30 individual serum samples, we calculated the correlation between the results of our technique and those of sequential ultracentrifugation.

Results

Analytical variables. Separation of the IDL or \( \beta \)-VLDL electrophoretic band was achieved by means of the procedure and within the conditions described in Figure 1.

Mean analytical recovery of increasing amounts of IDL cholesterol added to a serum sample was 104.5% (Table 2). Within-run precision (CV) for 15 aliquots of a sera pool was 10.1%, at a mean IDL cholesterol concentration of 88.3 mg/L. Between-run precision (CV) was 10.5%. The statistical correlation coefficient of our method with that of ultracentrifugation, in 30 samples obtained from different patients, was \( r = 0.96, P < 0.001 \) (Figure 2).

To evaluate the possible interference of high triglyceride concentrations, we analyzed samples from two Type IV patients who had triglyceridemias of 6330 and 8050 mg/L. The measured IDL cholesterol values were 120 and 160 mg/L, respectively. Neither VLDL coprecipitation nor an increase in IDL cholesterol was found in either subject, compared with the values obtained by sequential ultracentrifugation.

| Table 2. Mean Analytical Recovery of IDL Cholesterol |
|---------------------------------|-----------------|-----------------|
| Added                          | Measured        | Recovery, %     |
| 38.5 mg/L                      | 39.7            | 103             |
| 64.8 mg/L                      | 62.8            | 97              |
| 109.5 mg/L                     | 111.0           | 101             |
| 224.3 mg/L                     | 263.3           | 107             |
| Mean                           |                 | 104.5           |
| \( n = 3 \) each.              |                 |                 |

Fig. 2. Correlation between the ultracentrifugation method and the proposed assay for IDL or \( \beta \)-VLDL separation and quantification

Reference values in normolipemic and dyslipemic patients: Table 1 shows the distribution of cholesterol in VLDL, IDL or \( \beta \)-VLDL, LDL, and HDL. The serum concentration of total triglycerides was also assessed. It should be pointed out that IDL or \( \beta \)-VLDL cholesterol values in dysbetalipoproteinemic patients (Type III) are higher than those in other groups (\( P < 0.001 \)). Also, IDL cholesterol in groups II and IV is statistically greater than that in normal subjects (\( P < 0.001 \)). There is no overlap between the dysbetalipoproteinemic group and types IIb and IV.

Discussion

Development of this two-step electrophoretic technique is based on the selective precipitation method proposed by Wieland and Seidel for qualitative detection of increased \( \beta \)-VLDL lipoproteins in Type III hyperlipoproteinemic phenotype (4), which includes IDL as stated by Havel (1). We found that such a precipitation method is effective to neutralize the electrical charge of IDL or \( \beta \)-VLDL, but not the charge of LDL, despite both lipoproteins being at the \( \beta \) -position at the end of the first electrophoretic step. Therefore, in the second electrophoretic step, LDL moves toward the anode, separating from IDL or \( \beta \)-VLDL, which remains in the precipitate band at the \( \beta \) -zone.

Our technique showed a good correlation (\( r = 0.96 \)) with sequential ultracentrifugation. The experiments performed to evaluate the new method assessed the conditions of the first electrophoretic step, precipitation of IDL or \( \beta \)-VLDL, washing of the slides, the second electrophoretic step, and measurement of cholesterol in the isolated band of these lipoproteins.

The conditions of the first electrophoretic step and the selective precipitation of IDL and \( \beta \)-VLDL are almost the same as those reported by Wieland and Seidel and recommended by the Centers for Disease Control (4, 15). Within-run and between-day precision (CV), the latter performed in a short-term study, were 10.1% and 10.5%,
respectively. Camejo et al. (5) found an average CV of 8%, ranging from 5.1% to 14.8%, for their electrophoretic method for separating the main four lipoprotein classes and measuring VLDL cholesterol (at a serum concentration similar to that of IDL cholesterol).

We found a clear cutoff point for IDL concentrations between the dysbeta lipoproteinemic group (phenotype III) and that of normal individuals. The concentration of IDL cholesterol in Phenotype III patients also differs from that in the other dyslipemic patients, groups IIb and IV. The normal concentration of IDL cholesterol is similar to that reported elsewhere (16-17), and phenotype III shows an increase in IDL cholesterol consistent with recent publications (18). This procedure not only allows measurement of IDL or \( \beta \)-VLDL cholesterol in clinical and population studies for evaluation of this cardiovascular risk factor, but also eliminates the need for expensive ultracentrifugation equipment.

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References