Cholesterol Distribution between High-Density-Lipoprotein Subfractions HDL₂ and HDL₃ Determined in Serum by Discontinuous Gradient Gel Electrophoresis

Véronique Atger, Denise Malon, Marie Claude Bertiere, Françoise NoDlaye, and Anik Girard-Globa

We used discontinuous gradients of polyacrylamide gel to determine the high-density-lipoprotein (HDL) subfractions HDL₂ and HDL₃ of serum lipoproteins. Serum (40 μL) pre-stained with Sudan Black was electrophoresed in cylindrical tubes over successive layers of 3.5%, 6%, 13%, and 17.5% acrylamide gels in a Tris·glycine buffer (3–4 h, 300 V). Very-low- (VLDL) and low-density lipoprotein (LDL) were retained by the 3.5% and 6% gels. HDL₂ was concentrated at the interface between the 13% and 17.5% gels, and HDL₃ migrated into the 17.5% gel. The distribution between HDL₂ and HDL₃ was obtained by densitometric scanning. Application of the respective percentages to HDL cholesterol assayed after phosphotungstate–Mg²⁺ precipitation of VLDL and LDL gave calculated concentrations of HDL₂ and HDL₃ cholesterol. The calculated values for HDL₂ cholesterol were in excellent agreement with those for HDL₂ isolated by ultracentrifugation (r = 0.920 for n = 120 sera; differences nonsignificant by Student’s paired t-test). Besides being highly discriminating, the method is rapid, easily performed, and economical.

Although epidemiological studies have demonstrated a positive relationship between increased concentrations of serum cholesterol and the incidence of coronary heart disease (CHD), nearly half of the patients with CHD are not obviously dyslipemic. The inverse correlation between risk and high-density lipoprotein (HDL) concentrations was pointed out many years ago (1) and has been clearly confirmed (2). However, human HDL is a heterogeneous population of particles, currently identified as having two main subfractions, HDL₂ and HDL₃ (3, 4), which seem to have distinct metabolic roles. Concentrations of HDL₂ are directly related to efficiency in the clearance of triglyceride-rich fractions (5), and it is this subfraction that exhibits the strongest negative correlation with both the advent and the extent (6, 7) of coronary atherosclerosis.

Besides ultracentrifugation, several other techniques for determining HDL subfractions have been developed in recent years, based either on precipitation (8–10) or on gradient gel electrophoresis (11–14). Gradient gel electrophoresis presents the advantage of separation on the basis of particle size and circumvents the inconvenience of precipitation, in which the fine balance between lipoprotein components and precipitating agents is easily upset by minute variations in reagent proportions, timing, or environment. The main problem of electrophoresis is quantifying the results, which involves either staining the protein after a preliminary ultracentrifugal isolation of the lipoproteins (11, 12), or micro-determination of cholesterol in the bands cut out after lipid staining (13, 14).

We have previously validated the technique of densitometry of Sudan Black stain for estimating cholesterol distribution among the lipoprotein fractions (15) and have now applied this method to determination of HDL subfractions. We have, moreover, selected discontinuous gel concentrations that constitute barriers for defined particle sizes, ensuring the concentration of HDL₂ in a sharp band at the interface. Finally, we have replaced the unwieldy and expensive gel slab with rod gels, which are easily manufactured and can be used in the exact number needed.

Materials and Methods

Serum was obtained by low-speed centrifugation of pooled samples of freshly drawn blood allowed to clot in the cold. Total lipoproteins were floated by ultracentrifugation of serum at d = 1.21 for 48 h at 140 000 × g in a 50 Ti rotor (Beckman Instruments, Brea, CA). After fractionation by gradient ultracentrifugation (16), lipoproteins were collected through a gradient fractionator (ISCO, Lincoln, NE) with automatic recording of absorbance at 280 nm.

We analyzed the fractions corresponding to HDL individually and estimated their density of flotation by measuring the resistivity of the KBr solution in the fraction. By using a discriminating density of 1.125 kg/L, we divided the fractions into HDL₂ and HDL₃ subclasses. Each subclass was again centrifuged in a shallower gradient (d = 1.040–1.210) and collected into 30 fractions each. Systematic ultracentrifugal determination of HDL₂ and HDL₃ cholesterol was performed as follows: very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) were precipitated from 2-mL samples of serum with dextran sulfate–MgCl₂ (17). The supernate (1.5 mL) was adjusted to 1.125 kg/L with KBr.

---

1 Address correspondence to this author.

2 Nonstandard abbreviations: CHD, coronary heart disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.

3 Received April 24, 1990; accepted March 26, 1991.
solution, topped with 1 mL of 1.125 g/L KBr solution, and centrifuged for 24 h at 140,000 × g in a 50 Ti Beckman rotor. Cholesterol was assayed in the top 1 mL (HDL₃) and in the bottom layer (HDL₄).

Composition analysis of the subfractions of HDL₄ and HDL₃ centrifuged separately on shallow gradients (Table 1) shows that the cholesterol/protein ratio decreased progressively with increasing density, suggesting a spectrum of particles within HDL₂ and HDL₃. On the basis of density and the cholesterol/protein ratio, we pooled the particles into six classes, A to F. Some overlap was evident because a small amount of the fraction with a cholesterol/protein ratio of 0.40 (class B) was found in HDL₃.

To evaluate particle size, we used nondenaturing continuous 4–18% acrylamide gradients topped with a 3.5% stacking gel (18). Samples contained 30–40 μg of protein; the stain was Coomassie Blue. Particle diameter was estimated by comparison with the Stokes diameter of calibrated proteins (high-M₉ electrophoresis calibration kit; Pharmacia, Uppsala, Sweden).

HDL classes A to F ranged in size from 12.0 to 8.3 nm. By considering density, composition, and size, we identified fractions A and B as HDL₂ and fractions D to F as HDL₃. Fraction C contained particles characteristic of both HDL₂ and HDL₃ and was therefore not utilized further.

Judging from the migration distance in the gradient gel, we estimated the approximate pore-size limits. The optimum was established at 13% for fractions A and B and at 18% for fractions D to F. At such concentrations, HDL₂ and HDL₄ penetrated into their respective discriminating section of the gel but remained concentrated (Figure 1). A mixture of B and E was efficiently separated. LDL was stopped by a 6% gel (Figure 1).

Discontinuous gradients were constructed in cylindrical tubes (7 mm i.d.) from dilutions of stock solutions of 300 g/L acrylamide and 8 g/L bisacrylamide (in Tris 7.5 mmol/L, glycerol 120 mmol/L). Each layer was topped with butanol and allowed to polymerize before the following layer was poured: successively from bottom to top, these yielded 17.5%, 13%, and 6% gels. We took great care to make flat interfaces to minimize barrier effects. An additional layer (0.2 mL) of 3.5% acrylamide served to eliminate VLDL, which, when abundant, can hinder the migration of the other lipoproteins.

Electrophoresis was performed in the Tris-glycine buffer at 300 V for various times, with plasma samples prestained with Sudan Black (5 g/L in ethylene glycol). Absorbance was scanned at 590 nm.

Cholesterol was assayed enzymatically with the Boehringer-Mannheim kit (Meylan, France) and proteins were measured by Peterson's procedure (19). We measured HDL cholesterol by phoephoptongstate precipitation, using Boehringer-Mannheim kits.

Statistical significance of differences was estimated by Student's t-test or, when comparing two determinations on the same samples, by paired t-test.

Results

Optimizing Operating Conditions

Migration. We systematically studied migration time to determine the minimal time needed for particles to come to equilibrium. In five successive experiments, six identical samples were electrophoresed at 300 V for 1 to 18 h. The migration was stabilized after 3 h and did not progress further, except that HDL₃ tended to spread into the lower gel.

Relative proportions of serum and Sudan Black. Because of the possibility that HDL₂ and HDL₃ cholesterol were not equally susceptible to Sudan Black staining, we checked whether the amount of stain applied might be limiting, causing a distortion. Conversely, excess stain has a tendency to clog the top of the 3.5% stacking gel, where, in addition to being unaesthetic, it might impede migration. Therefore, we studied the influence of the proportion of stain in two ways: first, by varying from 10 to 40 μL the amount of stain added to 40 μL of the serum from two different patients; second, by comparing the results obtained for 50 patients by adding 40 μL of Sudan Black to either 40 or 60 μL of serum.

As Table 2 shows, the relative amounts of stain and serum had no impact on the results, whether the proportion of HDL₂ was low (patient A), medium (patient B), or high (patient C). Using 60 μL of serum instead of 40 μL increased the percentage of measured HDL₂ insignificantly, from 35.7 (SD 14.3) % to 36.3 (SD 13.1) %.

Standardized conditions. We determined that the following conditions gave the best results: assay 40 μL of serum with 25 μL of staining solution followed by 40 μL of 500 g/L sucrose solution, let the migration proceed for 3 to 4 h at 300 V, and densitometrically scan the

---

Table 1. Ratios of Cholesterol:Protein Concentrations in Fractions of the Shallow HDL₂ and HDL₃ Gradients

<table>
<thead>
<tr>
<th>Class name and fraction nos.</th>
<th>Density, kg/L</th>
<th>Chol/protein ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:11–13</td>
<td>1.070–1.100</td>
<td>0.53–0.51</td>
</tr>
<tr>
<td>B:14–16</td>
<td>1.100–1.125</td>
<td>0.44–0.40</td>
</tr>
<tr>
<td>HDL₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B:13–14</td>
<td>1.110–1.130</td>
<td>0.40</td>
</tr>
<tr>
<td>C:15–16</td>
<td>1.130–1.145</td>
<td>0.37–0.36</td>
</tr>
<tr>
<td>D:17–19</td>
<td>1.145–1.160</td>
<td>0.33–0.30</td>
</tr>
<tr>
<td>E:20–23</td>
<td>1.160–1.180</td>
<td>0.28–0.24</td>
</tr>
<tr>
<td>F:24–26</td>
<td>1.180–1.200</td>
<td>0.18–0.14</td>
</tr>
</tbody>
</table>

---

Fig. 1. Gradient fractions A, B, E, F, B+E, and LDL prestained with Sudan Black and analyzed on discontinuous gradient gels (from top to bottom: 6%, 13%, and 17.5% acrylamide)
samples within two days at 590 nm without removing the gel from the glass tube. The scans obtained were easily read (Figure 2).

Validation of the Technique

**Stability.** The proportion of HDL$_2$ was evaluated in duplicate samples of 50 different sera treated 24 h apart. The means (± SD) were, respectively, 35.7 (14.3) %, and 36.1 (14.6) %, not significantly different by Student's paired t-test. Preserving the serum for two days at 4 °C before assay did not alter the proportions measured (Table 3). Rapid freezing to −20 °C also ensured adequate preservation for at least eight days.

**Within-run variations.** Three different sera known to have very dissimilar proportions of HDL$_2$ were each treated as 10 distinct samples in the same run. The means (± SD) were, respectively, 22.0 (2.1) %, 35.5 (2.1) %, and 40 (1.2) %; the coefficients of variation (CVs) were 9.5%, 5.9%, and 3.0%, respectively.

**Between-run variations.** In eight separate runs (the maximum that can be performed in two days), four different samples yielded respective HDL$_2$ values of 17.2 (SD 1.9) %, 24.5 (SD 1.4) %, 38.5 (SD 2) %, and 52 (SD 2.2) %. The corresponding CVs varied from 11.0% in the lower HDL$_2$ range to 4% in the upper range. For the two runs performed 24 h apart in the stability test (Table 3), the average CV was 4.3%.

**Comparison with HDL$_2$ results obtained by ultracentrifugation.** To measure absolute cholesterol concentrations in HDL$_2$ and HDL$_3$, we multiplied the percentage obtained by densitometry of the discontinuous gels by the HDL cholesterol concentration obtained by phosphotungstate–Mg$^{2+}$ precipitation. In a systematic study of 120 samples, we compared these values with those for HDL$_2$ isolated by ultracentrifugation. The proportions of HDL$_2$ ranged from 17% to 62% of total HDL concentrations, corresponding to 0.13 to 1.50 mmol of HDL$_2$ cholesterol per liter. The comparison yielded respective means of 0.49 (SD 0.24) and 0.47 (SD 0.24) mmol/L, with a very small systematic bias (0.015 mmol/L, 3% of the measured values), which was statistically significant (P = 0.000). Regression analysis showed an excellent correlation (r = 0.920), which was equally good for low and high values of HDL$_2$.

**Discussion**

The discontinuous concentrations of acrylamide to use in the gels were determined experimentally by using well-characterized purified subfractions of HDL. The results were reproducible and correlated well with those by ultracentrifugation. Moreover, the calculation of cholesterol in the HDL$_2$ from the precipitated HDL cholesterol fraction correlated well with results by ultracentrifugal separation.

Ubbink et al. (13) described a similar technique with discontinuous slab-gel gradients, in which HDL$_2$ and HDL$_3$ were discriminated by their different rates of migration in a 13% gel, the other concentrations serving only to retain VLDL and LDL. Their technique requires delicate timing because both fractions keep migrating and tend to smudge. Moreover, in our hands, the discrimination of their method tended to decrease as the proportion of HDL$_2$ increased and was not usable for sera containing >40% HDL$_2$ cholesterol. In our method

### Table 2. Effect of Proportions of Staining Solution and Serum on Estimates of HDL$_2$ Percentage of Total HDL

| Serum source | Staining solution volume, µL | 10 | 20 | 25 | 30 | 40
|--------------|-----------------------------|----|----|----|----|----
| Patient A    | 16$^b$                      | 16 | 16 | 15.5 | 16 | 16
| Patient B    | 29                         | 28 | 28 | 28 | 28 | 28
| Patient C    | 46                         | 44 | 44 | 45 | 45 | 45

* All serum samples were 40 µL.

### Table 3. Stability of HDL$_2$ in Two Storage Conditions

| Storage temp. | Days of preservation | 0  | 1  | 2  | 8  | 14 | 30
|---------------|----------------------|----|----|----|----|----|----
| 4 °C          |                      | 35.7 | ±14.3 | ±14.6 | | | |
| (n = 50)      |                      | 30.4 | 30.1 | 29.7 | | | |
| 20 °C         |                      | 30.5 | ±9.5 | ±9.2 | ±11.3 | | |

* HDL$_2$, % of total HDL (mean ± SD).
the use of pore-size limiting concentrations renders the final profile independent of migration time and voltage, thus improving reproducibility and discrimination. Prestaining with Sudan Black has the advantage of allowing direct densitometric estimation without resorting to micro-assay of cholesterol by gas–liquid chromatography or requiring prior ultracentrifugation of the lipoproteins, both of which are expensive and time consuming.

The comparison with cholesterol contents assayed after ultracentrifugal separation of HDL₂ and HDL₃ justifies the application of the densitometrically obtained percentages to HDL cholesterol measured by a classical precipitation technique.

Finally, the small amount of sample required is a definite advantage, as is the stability during short-term storage at −20 °C.

We acknowledge the financial support of the Caisse Nationale d'Assurance Maladie (CNAM-TS).

References