Electrophoretic Separation of Serum Lipoproteins in Polyacrylamide Gel

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A method is described for electrophoresing lipoproteins in polyacrylamide gels, in which separations depend both on electrophoretic mobility and molecular size. The sample is prestained with Sudan Black B in a sample gel and then resolved by electrophoresis in a discontinuous pH system consisting of a sample gel, concentrating gel, and separating gel. This method was compared with the paper electrophoretic method of Lees and Hatch [J. Lab. Clin. Med. 61, 518 (1963)] and was found to be equally useful in phenotyping lipoproteinemia, with two distinct improvements over paper electrophoretic methods: lipoproteins are more quickly and discretely separated.

The technique of paper electrophoresis described by Lees and Hatch (1) has been widely used to qualitatively separate lipoproteins, and has represented the basis for classification of dyslipoproteinemia by Fredrickson et al. (2). The poor separation of lipoproteins by this method has prompted the evaluation of electrophoresis on other media, such as cellulose acetate, agarose, and polyacrylamide.

Narayan et al. (3) first described electrophoresis of lipoproteins on polyacrylamide gels. Disc electrophoresis sharply delineates lipoprotein bands and is more rapid than other electrophoretic methods for separating lipoproteins. Since the paper electrophoretic technique (1) does not possess these advantages, we undertook the studies reported here. We have evaluated parameters of the technique of disc electrophoresis of lipoproteins in polyacrylamide gels, developed an optimal system, and compared our results with those for the standard paper electrophoretic technique.

Materials and Methods

Reagents

Solution A. Dissolve 36.6 g of Tris (tris (hydroxymethyl)aminomethane, “Trizma base,” Sigma Chemical Co., St Louis, Mo. 63118) and 0.23 ml of “TEMED” (N,N,N’,N’-tetramethylethylenediamine, cat. no. 8178, Eastman Organic Chemicals, Rochester, N.Y. 14650) in about 100 ml of water. Add 48 ml of 1n HCl and dilute to 200 ml with water. The pH should be 8.9. Stable for three months at 4°C in a brown bottle (as are solutions B through F).

Solution B. Dissolve 5.98 g of Tris (Trizma base) and 0.46 ml of TEMED in water. Add 48 ml of 1n HCl, and dilute to 100 ml with water. The pH should be 6.7.

Solution C. Dissolve 15.0 g of acrylamide (Eastman, cat. no. 5521) and 0.40 g of bisacrylamide (N,N’-methylenbisacrylamide, Eastman, cat. no. 8383) in water, and dilute to 100 ml with water.

Solution D. Dissolve 20.0 g of acrylamide and 5.0 g of bisacrylamide in water and dilute to 200 ml with water.

Solution E. Dissolve 8.0 mg of riboflavin (Merck & Co., Rahway, N.J.) in 100 ml of water.

Solution F. Dissolve 200 g of sucrose (Merck) in water and dilute to 500 ml with water.

Solution G. Dissolve 0.14 g of ammonium persulfate (no. 0762, J. T. Baker Chemical Co., Phillipsburg, N.J. 08865) in water in a 100-ml volumetric flask and dilute to volume with water. Stable for one week at 4°C in a brown bottle.

Dye solution. Add 250 mg of Sudan Black B (Eastman, no. C8690) to 30 ml of absolute ethanol, mix well, and dilute to 100 ml with water. Stable for one month at room temperature in a brown bottle.

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Separating gel solution. Remove the desired amount of solutions A, C, and G from their refrigerated bottles, place in suitable containers, and bring to room temperature before combining them. Combine solutions A, C, and G in the ratio of 1:1:2, by volume. Prepare approximately 1.2 ml of the separating gel solution per separating gel required. The separating gel solution should be prepared just before use.

Concentrating gel solution. Mix solutions B, D, E, and F in the ratio 1:2:1:4, respectively, by volume. Stable for one month at 4°C in a brown bottle.

Sample gel solution. Just before use add 8 parts of concentrating gel solution to 1 part of dye solution and mix by gentle inversion.

Reservoir buffer. Dissolve 6.0 g of Tris (Matheson, Coleman & Bell, no. 7060) and 28.8 g of glycine (Sigma) in about 850 ml of water. If necessary, adjust the pH to 8.3 with Tris or glycine. Dilute to 1 liter with water. The reservoir buffer may be used for one week (based on two runs a day).

Equipment

The Model 1200 "Quick-Disc" Bath (Canalco, Inc., Rockville, Md. 20852) and Duostat Power Supply (Beckman Instruments, Inc., Palo Alto, Calif. 94304) are used. The gel tubes are 7 × 75-mm glass tubes (Canalco Inc., Rockville, Md. 20852).

Specimens

Sera from normal persons and from patients with hyperlipidemia were examined by the new method. Serum was obtained, after the blood had clotted for 1 h, by centrifuging (2000 rpm, 15 min).

Procedure

Add 1.0 ml of freshly prepared separating gel solution to each tube. Carefully add enough water on top of the gel solution to prevent a curved meniscus from forming as the gel polymerizes. The separating gel solution polymerizes if undisturbed for 30 min at room temperature. After photopolymerization is complete, invert the tubes and blot on absorbent paper. Add 0.1 ml of concentrating gel solution to each tube. Layer the gel solution with water as described above and allow the tubes to remain undisturbed for 10 min under intense light. After photopolymerization is complete, invert the tubes and blot on absorbent paper. Add 20 μl of serum or plasma and 0.2 ml of sample gel solution to each tube. Mix each tube by inversion and layer each tube with reservoir buffer. Allow the tubes to remain undisturbed for 30 min under intense light. After photopolymerization is complete, the gel tubes are ready to be inserted into the electrophoretic cell. About 200 ml and 800 ml of reservoir buffer should be in the upper and lower parts of the electrophoretic cell, respectively. The electrophoresis is for 35 min at 5 mA per gel tube.

Results and Discussion

Figure 1 shows the position of the major serum lipoprotein bands as determined by the polyacrylamide gel electrophoresis system described above. Each band was further identified by comparison with the paper electrophoretic technique (I): Serum specimens were electrophoresed in duplicate on paper (I), and one paper strip was stained with Oil Red O to determine the position of the β, pre-β, and α bands. The duplicate strip was not stained, and areas corresponding to the major bands on the companion stained strip were cut out and the lipoprotein eluted from them with water or NaCl (9 g/liter). The eluates were then subjected to the polyacrylamide procedure. Note that the position of the β and pre-β lipoprotein bands on polyacrylamide gel are reversed when compared to their positions after electrophoresis on paper. This is because separation on polyacrylamide gel depends on both electrophoretic mobility and molecular size, while separation on paper depends only on electrophoretic mobility. The lipoproteins separate more discretely in polyacrylamide gel than by paper electrophoresis.

With certain serum samples electrophoresed gels may show one to three additional bands, situated between the β and pre-β fractions, which vary in amount from minor to prominent. The
character and significance of these bands are not well understood. However, when present, they are always reproducible. The “mid-band” (a band appearing halfway between the \( \beta \) and pre-\( \beta \) bands) appears to be associated with a slightly or clearly increased concentration of triglycerides in the serum, suggesting that it might be a subfraction of pre-\( \beta \) lipoproteins.

In determining the best conditions for a polyacrylamide electrophoresis system, we tried many experimental designs that may be of interest. It was possible to eliminate the sample (top) gel by carefully applying the prestained serum on top of the concentrating (middle) gel. However, poor reproducibility was the result. The concentrating (middle) gel is necessary. Without it, adequate separation of the lipoprotein fractions was not obtained. When separating (lower) gels with higher concentrations of acrylamide and bis-acrylamide (5 and 7.5 g/100 ml) were tried, \( \beta \) and pre-\( \beta \) fractions would not migrate into the separating gel. The best system for polyacrylamide electrophoresis of lipoproteins apparently requires all three gels: sample (upper), concentrating (middle), and separating (lower) gel.

Figure 2 illustrates typical normal and hyperlipoproteinemic electrophoretic patterns on the polyacrylamide-gel system and the paper strip. [Serum cholesterol was determined by the ethanol extraction method of Franey and Amador (4), and the automated fluorometric method of Kessler and Lederer (5) was used for determining all triglyceride values.] In this discussion, Types II, III, IV, and V hyperlipoproteinemia refer to the classification of Fredrickson et al. (2). In Figure 2c, showing the Type V abnormality, note that the chylomicrons “dip” into the concentrating gel. We postulate that the chylomicrons seen in Type V hyperlipoproteinemia are charged and do exhibit some electrophoretic mobility. Molecular size may prevent deep penetration into the concentrating gel, thus the “dipping” effect is produced.

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**Fig. 2.**
a. Normal electrophoretic pattern; b, Type II hyperlipoproteinemia; c, hyper-\( \beta \) and hyper-pre-\( \beta \) lipoproteinemia; d, Type IV hyperlipoproteinemia; e, Type V hyperlipoproteinemia
Table 1. Comparison of Electrophoretic Separation of Lipoproteins by Polyacrylamide Gel and Paper Electrophoretic Methods

<table>
<thead>
<tr>
<th>Variable</th>
<th>Polyacrylamide</th>
<th>Paper</th>
</tr>
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<tbody>
<tr>
<td>Principle</td>
<td>Electrophoretic mobility and molecular size</td>
<td>Electrophoretic mobility</td>
</tr>
<tr>
<td>Sample volume, μl</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Staining time, min</td>
<td>30</td>
<td>240</td>
</tr>
<tr>
<td>Electrophoresis time, h</td>
<td>0.5</td>
<td>16</td>
</tr>
<tr>
<td>Total analysis time, h</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Stain</td>
<td>Sudan Black B</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>Migration of lipoprotein fractions (fastest to slowest)</td>
<td>α, β, pre-β chylomicrons</td>
<td>α, pre-β, β chylomicrons</td>
</tr>
<tr>
<td>No. of bands</td>
<td>Can have at least 3 bands in addition to above 4 bands (see text)</td>
<td>Maximum of 4 bands</td>
</tr>
<tr>
<td>Separation</td>
<td>Clear-cut separation of β and pre-β lipoproteins</td>
<td>Poor separation of β and pre-β lipoproteins</td>
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</table>

The sample gel stains heavily because excess Sudan Black B is not bound to lipoproteins (Figure 1). In our experiments we have observed two types of chylomicron bands. One is the type that produces the “dip” (Figure 2e), as described above. Chylomicron bands that we have observed in serum from patients with hyperlipoproteinemia other than Type V are usually a discrete band (Figure 2d) just below the interface of the sample and concentrating gels.

Our data show that serum lipoproteins separate better in this new polyacrylamide gel system than by paper electrophoresis (Table 1). The principal advantages of the polyacrylamide-gel procedure are that β and pre-β lipoproteins separate clearly, and that the polyacrylamide-gel procedure can be completed in 2 h, while the paper method requires about 20 h.

With improved resolution of lipoproteins by electrophoresis on this polyacrylamide gel system, the classification of hyperlipidemic patients may become more specific and more meaningful. Experiments are now in progress to characterize the “mid-band” and other “new bands” chemically and by ultracentrifugation. A clinical correlation of “lipoprotein profiles” in terms of these “new bands” is under way.

References