Phenotyping of Hyperlipoproteinemias

Effect on Electrophoretic Pattern of Serum Storage at Ambient, Refrigerator, or Freezing Temperatures

James Winkelman, Donald R. Wybenga, and Frank A. Ibbott

The stability of serum specimens collected for cellulose acetate electrophoresis of lipoproteins has been studied for each of the hyperlipoproteinemia phenotypes. In general, samples kept at room temperature for three days are still suitable for analysis. On longer standing, artifacts can cause misinterpretation of strips, or render them completely unreadable. If specimens are stored at refrigerator or freezer temperatures, deterioration is retarded but the period of stability after they are returned to room temperature is unaltered. A second freeze-thaw cycle makes specimens unsuitable for analysis. Samples can be stored at refrigerator temperatures for at least 28 days and at freezer temperatures for at least 14 days if one freeze-thaw cycle is used.

Additional Keyphrases freeze-thawing of serum • lipoprotein denaturation • cellulose acetate electrophoresis

Electrophoresis of serum lipoproteins has recently become a widely used laboratory procedure, since important clinical correlations have been established between specific hyperlipoproteinemia phenotypes and characteristic appearance of the electrophoretograms (1). The empirical nature of the interpretation of these patterns makes it necessary to establish the stability characteristics of serum samples submitted for this procedure, for aging or other artifacts of denaturation can produce electrophoretic patterns that are different from those obtained with fresh specimens. Thorough evaluation of the varieties of aging artifacts is of particular interest to those reference laboratories receiving specimens that have spent various periods in transit or storage.

In this study we evaluate the effects of time and temperature on specimens from patients with the five hyperlipoproteinemia phenotypes as well as the frequently encountered patterns seen in normal individuals.

Materials and Methods

Most specimens were obtained from hyperlipidemic and nonhyperlipidemic patients, and some from normal volunteers. Blood was drawn at
the laboratory and processed within 2 h. Some specimens of the well-defined hyperlipoproteinemia types were taken from the routine laboratory, if no more than 8 h had elapsed between collection and electrophoresis of the serum. As far as possible, we used specimens from individuals who were at ideal body weight, had fasted 12 to 14 h, and had been on a normal diet for two weeks.

Lipoproteinemia was phenotyped according to the technique we described recently (2). Lipoproteins were separated by cellulose acetate electrophoresis (CAE). Cholesterol (3) and triglycerides (4) were determined on the same specimen and these values were considered in the final interpretation of the phenotype according to previously established criteria (2).

Experimental Protocols

Ambient temperature. Specimens were kept at 25 ± 2°C during the study. Aliquots were electrophoresed on days 0, 1, 2, 3, 4, 5, and 7.

Refrigerator temperature. Specimens were placed in a refrigerator (4°C) on day 0, and aliquots were electrophoresed on days 0, 1, 2, 3, 5, and 7. Once an aliquot was removed from the refrigerator, it was kept at room temperature and rerun each day for seven days. Thus we evaluated both the effect of holding a specimen for various periods at 4°C, and the stability of samples at room temperature after having been refrigerated for those intervals.

Freezer temperature. This protocol was the same as the one above, except that specimens were stored at −10°C. In addition, some specimens were taken through more than one freeze-thaw cycle and the effect on the CAE pattern was determined.

Results

The number of specimens with CAE patterns typical of hyperlipoproteinemia Types I to V (T) and the two patterns frequently encountered with serum from normal individuals (N) (specimens kept at room temperature) are shown in Table 1. The lipid fractions were measured in each specimen; the results were consistent with the electrophoretic pattern. The percentage of samples of each type that showed a pattern essentially identical to that obtained on day 0 is given for each day studied.

Certain CAE patterns that were not identical in appearance to that on day 0 could still be accurately read for phenotype. These are also shown in Table 1 (in parentheses). The artifacts of aging that developed with specimens stored constantly at room temperature also illustrate almost all the artifacts seen with specimens kept first at lower temperature and subsequently at room temperature.

Type II (D4) patterns become unreadable for several reasons. The prominent β band migrated too far and appeared in the pre-β region in several instances. This is illustrated by the appearance on day 7 of the D4 electrophoretogram shown in Figure 1. On the basis of the CAE pattern alone, this would be mistakenly read as a D4. Some samples became difficult to apply and ran in uneven bands; in one case a prominent pre-β and a lesser α component disappeared (Figure 1, day 5). Different artifacts can thus appear sequentially with a single specimen.

The situation with Type III (D4) is complicated by the fact that two different CAE patterns can be consistent with that phenotype (5). Several specimens with a broad abnormal β band on days 0 to 2 or 3 later developed a discrete, prominent band in the pre-β region. The same results were seen in two specimens in which both β and pre-β bands were at first abnormally prominent, but in which aging produced an apparent loss of the β band and

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**Table 1. Stability of Serum Samples at Room Temperature**

<table>
<thead>
<tr>
<th>Code*</th>
<th>Phenotype*</th>
<th>No. specimens</th>
<th>% Samples with CAE pattern visually identical (or equivalent)* to that on day 0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>D1</td>
<td>I</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>D2</td>
<td>II</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>D3</td>
<td>III</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>D4</td>
<td>IV</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>D5</td>
<td>V</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>ND1</td>
<td>Nondefinitive</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>ND2</td>
<td>Nondefinitive</td>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>

* These codes, and the criteria for their identification, are described in detail elsewhere (2, 3, 5).
* Certain alterations in CAE pattern were either so slight or of a kind that no misreading of the phenotype would result. In such cases, the number in parentheses is the total percentage of strips that would have been interpreted as the same type as on day 0, although the appearance was not identical.

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increase in the pre-β band. These artifacts could lead to misreading D₄ for D₅, as shown in the days 2 and 3 strips in Figure 2. Lipid fractionation data may not resolve such problems in interpretation in cases where the values are very close to the arbitrarily established criteria used in our system of reporting (2). Peculiarly, three specimens with broad abnormal β bands gradually developed well separated bands in the β and pre-β regions. Although these would still have been called D₅ according to our criteria, the changed behavior of the lipoproteins CAE reflects some denaturation of the lipoproteins of these specimens. The α bands gradually became faint or invisible in other denatured D₅ specimens, or all the bands became hazy, pale, or indistinct (Figure 2, day 7). In one, material staining with oil Red O remained at the origin (Figure 2, day 5).

The artifact that made D₄ patterns unreadable consisted of the gradual disappearance of the pre-β band, with material moving into the α region and further (Figure 3). In one case the β band was no longer visible by day 3, but the resulting pattern, consisting of an even more prominent pre-β band that persisted through day 7, was read as consistent with Type IV.

Figure 4 illustrates patterns with all the different changes that occurred in CAE strips, consistent with Type V. The pre-β band progressively migrated further until it was in the α region, as occurred with some D₄ strips. In the day 5 strip (Figure 4), there is a very irregular, serrated accumulation of stained material in the pre-β region that defies categorization; this has virtually disappeared from the strip by day 7. Since only D₅ patterns have material both at the origin and in the pre-β region, certain gross errors of interpretation can be avoided despite the sometimes striking changes.

The only change seen in the ND₁ pattern was the occasional decrease or disappearance of the α band on days 5 and 7 (Figure 5). In one instance
of NDs, this change in pattern was accompanied by the appearance of material at the origin on day 5, and of almost complete obliteration of the pattern on day 7 (Figure 6).

The objective of the experiments in which specimens were kept at refrigerator temperature or frozen was to simulate the conditions that would exist if a specimen was stored at these temperatures before being transmitted, at ambient temperature, to the laboratory. Therefore, samples were run after 1 to 7 days at colder temperatures and from 1 to 14 days after collection. The length of time that a specimen was kept at refrigerator or freezer temperature had no effect on its subsequent stability at room temperature. Therefore, the data shown in Table 2 represent the combined results with specimens brought to room temperature after 1 to 7 days. The kinds of artifacts responsible for making particular strips unreadable were the same as those described above for each type.

The refrigerated Ds samples were readable after

<table>
<thead>
<tr>
<th>Type</th>
<th>Refrig.</th>
<th>Frozen</th>
<th>Equivalence with day 0*</th>
<th>Day 5b</th>
<th>Day 7</th>
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<tbody>
<tr>
<td>Ds</td>
<td>3</td>
<td>...</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>D2</td>
<td>5</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D4</td>
<td>2</td>
<td>...</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>...</td>
<td>+</td>
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<tr>
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<td>5</td>
<td>...</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ND2</td>
<td>3</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Plus or minus indicates equivalence or nonequivalence, respectively. All samples were equivalent on days 1, 2, and 3.

b Days at room temperature after removal from storage at lower temperatures.
five days at room temperature and many could also be read after seven days. Similarly, the refrigerated specimens of types D₁ and D₂ could be read after three days in all cases, and on days 5 and 7 in some. The D₃ specimen remained readable through day 5. ND₁ and ND₂ patterns could be read without problems seven and five days, respectively, after removal from the refrigerator.

Results with specimens taken through one freeze-thaw cycle and then held at room temperature are also shown in Table 2. In general, the stability is the same after freezing as after refrigeration. In other experiments samples were taken through a second freeze-thaw cycle. This treatment caused almost complete obliteration of all types of patterns. The CÆ strips appeared atypical for any recognizable type, definitive or nondefinitive.

Discussion

Early studies of the stability of lipoproteins for electrophoresis have not dealt with the specific materials and techniques of CÆ for phenotyping. Since the older methods of electrophoresing lipoproteins on paper (7, 8) did not separate and identify hyperlipoproteinemia phenotypes, results of the older stability studies are not strictly comparable to ours.

Adlersberg et al. (9) studied lipoproteins by paper electrophoresis and found that storage in the frozen state for as long as six months did not affect their results. Dangerfield and Smith (10) also found no change in paper electrophoretic patterns after many months of storage at −10°C. Reports on stability of lipoproteins at refrigerator and room temperatures have been somewhat contradictory. Gottfried et al. (11) claimed that the β lipoprotein decreased 10, 20, and 25% after one, two, and three days, respectively, at room or refrigerator temperature. Fredrickson et al. (1), however, stated that samples can be stored at 2°C to 4°C for several months.

Fredrickson et al. (1) reported that freezing irreversibly altered the lipoprotein phenotyping pattern obtained by paper electrophoresis, although it was not stated whether more than one freeze-thaw cycle was used. Our findings with specimens taken through one freeze-thaw cycle, however, are consistent with the older reports mentioned above.

Sample stability was probably a minor concern to those who first developed the technique and established the clinical usefulness of phenotyping of hyperlipoproteinemias, since their patients were usually close by. The widespread use of this technique by many regional and reference laboratories has, in our opinion, required several modifications, including the method itself and the system of reporting. This study establishes new constraints that the physician and laboratory must work within if reliable results are to be obtained. We are now reasonably confident that specimens received within three days of collection or within three days after removal from storage in either a refrigerator or freezer are suitable for analysis. Furthermore, familiarity with the artifacts that can develop in denatured specimens may help in the interpretation of otherwise problematic patterns.

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References