Quantitation of Beta Lipoprotein (LDL) Cholesterol by Densitometric Evaluation of Disc Electropherograms

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This paper describes a rapid, facile, and accurate new procedure for determination of marginal Type IV or Type II hyperlipoproteinemias, in which discontinuous (disc) electrophoresis on polyacrylamide gel is used to quantitate LDL (beta) cholesterol without ultracentrifugation. Fasting sera, previously assayed by ultracentrifugation and chemical technique, were prestained with Sudan Black, and the lipoproteins were separated by disc electrophoresis. The beta-lipoprotein fraction of each electrophoretic pattern was quantitated by densitometry. The values for the integrated densitometric peak areas for the beta fraction and the chemically assayed cholesterol of the ultracentrifuged LDL fraction correlated well ($r = .90$). Results were determined from a curve relating chemical concentration of the integrated peak values by densitometry to the known LDL cholesterol of a series of standards.

Additional Keyphrases  Types II and IV hyperlipoproteinemias  • densitometry  • polyacrylamide gel

Estimation of plasma lipoproteins and their lipid moieties is a subject of increasing concern in many laboratories today. Many laboratories assign lipoprotein status by the method of Fredrickson et al. (2). Because most abnormal patients fall into Fredrickson's Type II or Type IV categories, and the therapy of each type is necessarily different, these types must be accurately differentiated. We present here an alternative method, which does not involve ultracentrifugation, for determination of LDL cholesterol ("beta" cholesterol). The validity of this alternative is described, including data on precision and on correlation of the results with those obtained by ultracentrifugation.

The definition of phenotypes, as described by Fredrickson et al. (2), is based on precise knowledge of the concentration in plasma of different lipid moieties as well as medical and family history and clinical indications. Assignment to Type II is based on an increased beta cholesterol concentration, and to Type IV is indicated by increased fasting triglyceride concentration after it has definitely been established, from the appearance of the fasting serum stored overnight at 4°C, that no chylomicrons are present.

Although the fasting triglyceride concentration can be determined easily by automated techniques such as that of Kessler and Lederer (3), the beta (LDL) cholesterol has only been determined precisely by the longer procedure of preparative ultracentrifugation plus chemical measurement of the cholesterol in the fractions so separated. LDL cholesterol may be approximated mathematically by use of values for total cholesterol, alpha cholesterol, and triglycerides (LDL cholesterol = total cholesterol - alpha cholesterol - triglyceride/5), but the estimate is not accurate enough for precise determination of LDL cholesterol (6).

Our primary purpose in developing this technique was to have a precise measure of LDL or beta cholesterol that did not require preparative ultracentrifugation but was based on direct measurement of LDL material. A modified commercial system for polyacrylamide gel electrophoresis afforded the opportunity to do this. Certain details of these modifications appear to be important.

Materials and Methods

Materials

The disc electrophoresis system supplied by Canalco Model 12 CB (Canalco, Inc., 5635 Fisher
Lane, Rockville, Md. 20852) was used in this evaluation, together with a densitometer (Canalco Model K) and a lipoprotein reagent kit (Canalco QDL, modified). An interference filter with a nominal wavelength value of 623 nm was used. Disposable capillary pipets (Drummond Scientific Co., 500 Parkway, Broomall, Pa. 19008) were used to measure aliquots of the serum samples.

Methods

All samples were quantified by the Fredrickson methods of ultracentrifugation, triglyceride analysis (3), precipitation, and cholesterol assay of the separated fractions by the Kendall-Abell technique, as well as by the disc electrophoretic method.

Although the commercially provided electrophoretic reagents and techniques were satisfactory for the qualitative phase of lipoprotein phenotyping, the system was modified for quantitative use. Except for the changes noted, the qualitative and quantitative methods are identical. The gels were cast to a height of 40 mm, instead of by volume, and then allowed to stand for at least 48 h before use. Serum aliquots of 5 μl, smaller than those used in the standard qualitative technique, were added to the loading gel. The loading gel itself was modified to contain a higher concentration of Sudan Black stain (50 mg of dye per 100 ml of solution in the modified technique as contrasted with 15 mg/100 ml of gel solution as supplied by the manufacturer for the qualitative method.) After polymerization of the loading gel-serum mixture was complete, the tubes containing gel were inserted into the electrophoretic apparatus so that the origin ends of all the tubes were at about the same height, and subsequently they were electrophoresed at a current of 5 mA per tube for about 50 min, or until the alpha-lipoprotein bands were about 3 mm from the bottom of the tube. After electrophoresis, each tube containing the separated serum samples was scanned on the densitometer at 623 nm, and the area under the peak was read from the logarithmic integrator.

Several procedures were used to evaluate the system. First, reproducibility of the system was determined by replicate quantitation of each of two sera containing different known concentrations of LDL cholesterol. Each replicate serum was carried through the entire procedure (loading, electrophoresis, and densitometry), and results were compiled from data obtained in several runs. Next, the LDL cholesterol was determined in duplicate for several other samples by using the ultracentrifugation technique of Fredrickson et al. (2). Specifically, by ultracentrifuging plasma in a Model L preparative ultracentrifuge (Beckman Instrument Co., Spinco Division, Palo Alto, Calif. 94304) at its own density, at 40,000 rpm for 16 h. Cholesterol was then measured in the fraction of density >1.006. Alpha-lipoprotein cholesterol in each sample was determined by assay of the supernatant fluid from a heparin-manganese chloride precipitation of undiluted plasma. LDL cholesterol was then determined by difference.

A standard curve was then constructed by using the values obtained for these samples, which related LDL cholesterol concentrations determined chemically to the microdensitometer readout for the beta-lipoprotein bands after scanning and integration. The latter was determined by summing integrator counts under the curve of the beta-lipoprotein bands. The demarcation point between counts for the beta band and the pre-beta band was ascertained by constructing a vertical line between the lowest point of the curve between the two peaks and the line of the integrator markings (Figures 1, 2). Finally, LDL

![Fig. 1. Trace of a typical scan of a sample separated by quantitative disc electrophoresis](https://example.com/fig1)

The dotted line indicates the demarcation between "beta" and "pre-beta" segments of trace. Extension of the line vertically to the integrator trace allows summation of the area under the "beta" band alone.
ch For cholesterol was determined by the ultracentrifuge method on 33 further samples, and the betalipoprotein band was subsequently quantified by the densitometric method described. These 33 samples were chosen to test the system with a wide range of both cholesterol and triglyceride concentrations.

Results

Replicate determinations of beta lipoprotein with this system indicate that its precision is comparable to that of the reference technique (Table 1). The correlation appears to be best at concentrations of LDL corresponding to the concentration of beta-lipoprotein cholesterol that is of greatest clinical interest, 180-200 mg of LDL cholesterol per 100 ml, or 150-175 integrator units for the beta band (Table 1 and Figure 3). Although any system used for quantitatively typing the hyperlipidemias must have good precision in this range, precision throughout a wide range of values, as this system provides, is also highly desirable.

The curve relating the integrated density of the electrophoretically separated beta band to the chemically determined LDL cholesterol was smooth and substantially linear up to a concentration of about 200 mg of beta cholesterol per 100 ml. At greater concentrations, densitometric response to increased concentrations of beta lipoprotein decreased regularly, but gradually enough to allow precise determination of LDL cholesterol (Figures 2 and 3).

When we evaluated the 33 samples, for which beta lipoprotein bands had been quantitated by densitometry, for beta cholesterol with this standard curve, the results correlated well with those for LDL cholesterol determined by ultracentrifugation (Figure 4). The densitometric values for beta cholesterol obtained from the constructed curve

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**Table 1. Precision of Methods of Beta Lipoprotein Quantitation**

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>n</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
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<td>24</td>
<td>5.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Densitometry</td>
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<td>24</td>
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<tr>
<td>Ultracentrifugation</td>
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<td>40</td>
<td>5.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Arbitrary units—integrator counts (beta band measured).  
* mg of LDL cholesterol per 100 ml of serum.  
* Six replicates in each of four runs.  
* Ten replicates in each of four runs.

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**Fig. 2. Densitometric traces after disc electrophoresis of specimens containing indicated quantities of beta cholesterol (ultracentrifugal fraction) in volumes of (left to right) 3, 5, 10, 15, 20, or 25 µl.**

Peak areas (numbers below each peak) are obtained from densitometer's "pip" trace, as illustrated. The actual gel patterns are shown in insert above.

**Fig. 3. Relationship between beta cholesterol concentration as determined densitometrically and that determined by preparative ultracentrifugation.**

Prepared with standards of known value, this is the reference curve used to determine unknown beta-cholesterol concentration (horizontal) from measured beta band counts (vertical).
were compared with the LDL value obtained by ultracentrifuge, and showed no significant difference ($P = 0.02; t = 2.38$).

**Discussion**

Serum lipoproteins were originally separated by density gradient ultracentrifugation into their three main varieties—HDL, LDL, and VLDL (6). Paper electrophoresis was subsequently adapted (6) to separate the same components by their electrophoretic mobility. The paper electrophoretic separation of the serum lipoproteins resulted in a pattern in which the ultracentrifuged HDL fractions migrated in the "alpha" region, the LDL in the "beta" region, and the VLDL (less the chylomicrons) in the "pre-beta" region (Figure 5). Lees and Fredrickson (7) and Levy and Fredrickson (8) used this electrophoretic method in combination with preparative ultracentrifugation and specific lipid determination (i.e., triglyceride assay, total and fractional cholesterol assays) to develop data leading to the now widely accepted system for laboratory classification of five types of hyperlipidemia.

As noted earlier, the preparative ultracentrifugation necessary for quantitation of the LDL cholesterol is inconvenient for many laboratories, and this prompted our investigation of an alternative method. The qualitative disc electrophoretic system used was only modified after preliminary testing of the techniques of the system showed the modifications to be advantageous.

A column height of 40 mm afforded a somewhat longer distance through which the bands might travel. This, together with the longer running time, which brought the alpha band to within 3 mm of the end of the tube, resulted in better separation. We found that the alpha bands could be allowed to migrate out of the gel tube without affecting results, but there was no advantage in allowing this to occur, because a missing band could cause confusion if the system were to be used by a less experienced operator.

The 5-μl serum samples were used, because aliquots of this volume could be measured easily and precisely, without using too much of the dye from the modified stain. This combination of less sample with greater stain concentration in the loading gel permitted the lipid moiety of the lipoproteins to become saturated by the dye, within a certain range of lipid values, without sacrificing precision of sample measurement or causing difficulties in preparation of the loading gel. The serum must be carefully measured and carefully mixed with the loading gel.

It is noted in the basic Canalco instructions supplied with the QDL system that sharper bands are obtained if the separating gels are stored after preparation for 24 h or longer before electrophoresis. For quantitative use, we found it essential that the separating gels be aged at least 48 h before use. Failure to allow the gels to equilibrate in this manner sometimes caused the lipoprotein bands to be more diffuse than desirable, resulting in variability in the quantitation phase. In practice, it is quite convenient to prepare large numbers of these gels at the same time, and put them away for the required storage period. A
week's stockpile requires little refrigerator space because the storage racks are so compact.

Our studies indicated good run-to-run precision with this modified technique. However, we do think that it is advisable to run one or more serum samples or beta fraction standards of known value in each batch of samples processed, to assure that response of the system is unchanged since preparation of the standard curve.

Thus, results of this preliminary trial indicate that this quantitative disc electrophoretic method is useful for accurate determination of LDL or beta cholesterol values, which is essential for categorizing a subject as Type II hyperlipoproteinemic.

References