Simultaneous Separation of Serum Creatine Kinase and Lactate Dehydrogenase Isoenzymes by Ion-Exchange Column Chromatography

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Lactate dehydrogenase isoenzymes were partially separated by use of a previously described column technique for creatine kinase [Clin. Chem. 20, 36 (1974)]. Extracts of lactate dehydrogenase-rich tissues were used to evaluate column resolution. Samples layered on mini-columns containing DEAE-Sephadex were eluted with Tris-buffered sodium chloride (100 and 200 mmol/liter). Lactate dehydrogenase activity in column effluents was measured by the Wacker method, and their isoenzyme content was assessed by electrophoresis on polyacrylamide gel. Dehydrogenase isoenzymes 3, 4, and 5 were separated from isoenzymes 1 and 2, and the separation was tissue-specific and reproducible. The electrophoretic technique for isoenzymes 3, 4, and 5 gave values about 20% lower than did the column technique. Sera from 15 healthy laboratory technicians contained total lactate dehydrogenase, isoenzymes 1 and 2, and isoenzymes 3, 4, and 5 in the ranges 94 to 152, 34 to 64, and 38 to 75 U/liter, respectively. Activities of sera from 15 patients with acute myocardial infarction (total lactate dehydrogenase) ranged from 212 to 800 U/liter and lactate dehydrogenase isoenzymes 1 and 2 ranged from 138 to 628 U/liter. Lactate dehydrogenase and creatine kinase isoenzymes were rapidly and easily measured after being simultaneously separated. The procedure is specific and sensitive for following the post-infarct time course of changes in isoenzyme activities.

Previously, I described a column chromatographic technique for separating serum creatine kinase (CK)\(^1\) isoenzymes (1). CK isoenzymes MM, MB, and BB were isolated by ion-exchange chromatography on mini-columns containing DEAE-Sephadex; column effluents were quantified with an assay normally used to determine total serum CK. Recently, the clinical usefulness of this technique in diagnosis of myocardial infarction has been demonstrated by several investigators (2–4).

Lactate dehydrogenase (LD), like CK, is a diagnostically useful enzyme for detecting and monitoring myocardial necrosis. Although LD is an ubiquitous enzyme, the ability to measure the activities of its isoenzymes in serum has greatly improved the clinical usefulness of such measurements (5).

I describe here a partial separation of serum LD isoenzymes with the previously described CK ion-exchange column technique (1). LD isoenzymes 1 and 2 are separated from LD isoenzymes 3, 4, and 5, and column effluents are quantitatively assayed with a conventional serum LD procedure. This column-chromatographic technique for simultaneously assessing CK and LD isoenzymes is evaluated with respect to sera from healthy individuals and from patients with recent myocardial infarction.

\(^1\) Nonstandard abbreviations used: CK, creatine kinase (EC 2.7.3.2); LD, lactate dehydrogenase (EC 1.1.1.27); Tris, tri(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; MM, skeletal-muscle isoenzyme of CK; MB, cardiac-muscle isoenzyme of CK; BB, brain isoenzyme of CK; LD 1 and 2, cardiac lactate dehydrogenase isoenzymes; LD 3, 4, and 5, liver and skeletal muscle lactate dehydrogenase isoenzymes; LD 1.2, cardiac lactate dehydrogenase isoenzymes in effluent fractions 7, 8, and 9; LD 3.4.5, liver and skeletal muscle lactate dehydrogenase isoenzymes in effluent fractions 1, 2, and 3.
Materials and Methods

Tissue and Sample Preparation

Human tissue used in this study was taken from autopsy material in which no gross anatomical changes were evident. Homogenates (2 g diluted to 20 ml) were prepared in Tris-HCl buffer (50 mmol/liter, pH 8.0) containing sodium chloride (100 mmol/liter). After the homogenate was centrifuged (12 000 × g, 10 min), the pellet was discarded and the supernate used in subsequent chromatographic experiments.

Sera obtained from a general hospital population were kept at room temperature until isoenzyme analysis, which usually was performed just after centrifugation of the blood sample.

Enzyme Activity Analysis

An Abbott ABA-100 (Abbott Biochromatic Analyzer; Abbott Laboratories, Diagnostic Division, Pasadena, Calif. 91030) set to operate in the rate mode at 37 °C with a 340/380 nm filter carriage, was used to assay total serum CK and column-isolated CK and LD isoenzymes. The SMA-12/60 (Technicon Instruments Corp., Tarrytown, N. Y. 10591) with the Wacker method (6) modified for continuous-flow analysis (7) was used to assay total serum LD.

Total serum CK and column-eluted LD isoenzymes were assayed with use of a 1:51 syringe-plate assembly, which automatically dispensed 5 μl of sample and 250 μl of reagent. The lyophilized contents of kinetic test kits for CK and LD (Smith Kline Instruments, Palo Alto, Calif. 94304) based on the methods of Rosalki (8) and Wacker, respectively, were reconstituted with water as specified by the manufacturer. The ABA-100 print-out for total CK (calibration factor, 1130) was in U/liter; the print-out for column-fractionated LD isoenzymes (calibration factor, 6000) was converted to U/liter as follows. A reference sample with known LD activity was assayed in the same manner as the column effluents. A conversion factor was calculated: Factor = LD, U/liter (reference)/print-out (reference). This factor was then used in the following formula to convert the ABA-100 printout (i.e., the raw data) to U/liter. Print-out (column effluents) × factor = LD, U/liter. LD activity was linearly related to print-out to 1700 U/liter. Routinely, only fractions 1, 2, 3, 7, 8, and 9 were assayed for LD. CK isoenzymes MM and MB in column effluents were assayed with the ABA-100 as previously described (9).

Column Chromatography

One milliliter of serum was applied to a mini-column of DEAE-Sephadex A-50 as described by Mercer (1). The modified elution scheme of Mercer and Varat (9) (five 1-ml fractions of 100 mmol/liter sodium chloride and three 1-ml fractions of 200 mmol/liter sodium chloride) was used to simultaneously separate MM, MB, LD-3,4,5, and LD-1,2 isoenzymes.

Electrophoresis

LD isoenzyme electrophoresis was performed on polyacrylamide (70 g/liter) with an analytical vertical-gel apparatus (Ames Co., Elkhart, Ind. 46514). Samples diluted with an equal volume of sucrose (400 g/liter) were applied to the top of the polymerized gel (0.5 × 3.5 cm) and electrophoresed at 2 mA per gel with the Tris-glycine buffer system as originally described by Davis (10). LD isoenzymes were detected by the staining technique of Dietz and Lubrano (11).

Results

The ion-exchange chromatographic system was characterized by chromatographing extracts of CK- and LD-rich tissues. Figure 1 illustrates the behavior of CK and LD isoenzymes on DEAE-Sephadex columns. Extracts of skeletal muscle exhibited prominent MM-CK and LD activity in effluent fraction 1 and 2. Trace amounts of MB-CK and LD activity were found in effluent fractions 7, 8, and 9. Liver extract exhibited 99% of its LD activity in effluent fractions 1 and 2. No CK activity was found in liver extracts. Cardiac extract exhibited about 90% MM-CK in fractions 1 and 2 and about 10% MB-CK in fractions 7, 8, and 9. LD activity was predominantly in fractions 7, 8, and 9.

Electrophoretic identification of column-fractionated LD is shown in Figure 2. In this experiment, aliquots of effluent fractions 1–9 of skeletal muscle, liver, and heart extracts from DEAE-Sephadex col-

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**Fig. 1.** Elution pattern of creatine kinase and lactate dehydrogenase activities in extracts of various tissues (DEAE-Sephadex columns)
umns were applied to polyacrylamide gels. Electrophoresis and subsequent LD staining revealed LD isoenzymes 1 and 2 in fractions 7 to 9 and LD isoenzymes 3, 4, and 5 to be prominent in fractions 1 to 3.

Figure 3 shows the total activity for LD and CK in each fraction of effluent, for a patient 48 h after onset of an acute myocardial infarction. LD activity is predominant in fractions 7–9 (LD-1,2). Significant amounts of MB-CK activity were also evident in fractions 7–9. Table 1 shows results of quantitative analysis for LD-1,2 and MB-CK activities in the sera of 15 patients with recent myocardial infarction. LD-1,2 activities of 138 to 628 U/liter (sixfold normal) were detected. Compared to normal MB-CK values reported previously (9), 130-fold normal MB-CK activities were observed in this group of patients.

For healthy individuals, CK and MB-CK activities similar to those previously described for normal individuals were observed (9). There were nearly equal amounts of LD activity in effluent fractions 7–9 (LD-1,2) and 1–3 (LD-3,4,5). Table 2 summarizes results for MB-CK, LD-1,2, and LD-3,4,5 in sera of healthy laboratory technicians. Recovery of total LD was <100% because LD activity in column fractions 4, 5, and 6 was not included in the recovery calculation.

Figure 4 shows typical changes in post-infarct activities of MB-CK and LD-1,2 with time. MB-CK activity was greatest at 24 h, followed by a sharp decline to base values at 72 h. In contrast, LD-1,2 increased slowly to a maximum at 72 h, followed by a slow decline. LD-3,4,5 remained within normal limits in uncomplicated cases of myocardial infarction but patients with progressive congestive failure had abnormally high values.

Column reproducibility for LD-1,2 was demonstrated by daily analysis of an abnormal pool collect-
ed from patients with myocardial infarction. The mean (n = 16), standard deviation, and coefficient of variation in the abnormal pool were 244 U/liter, 17.53 U/liter, and 7.2%, respectively. LD-5 reproducibility was monitored by daily analysis of serum fortified with a commercially prepared LD-5 (Sigma Chemical Co., St. Louis, Mo. 63178) and yielded a mean (n = 14) of 539 U/liter, a standard deviation of 32.4 U/liter, and a coefficient of variation of 6.0%.

Comparison between LD isoenzymes isolated by column and by electrophoresis revealed 20% greater recovery of column-isolated isoenzymes 3, 4, and 5. Recoveries for chromatographed total LD were usually in the 97 to 132% range when all nine column fractions were assayed. Column capacity for LD isoenzymes was not exceeded when samples with LD activity as great as 3000 U/liter were applied to the column.

Discussion

Measurement of LD isoenzymes has greatly improved the clinical usefulness of LD, especially in diagnosis of cardiac and hepatic disease (5, 12). Early isoenzyme determinations, performed with electrophoretic techniques, provided values for five separable isoenzymes (13-16). However, this technique has not been widely adopted as a routine procedure because it is time-consuming and requires highly specialized techniques. Recent developments have been directed toward simplified procedures that provide estimates of LD activity of cardiac and hepatic origin. Currently, these include heat stability (17), selective chemical inhibition (18), and selective substrate (19).

This paper describes a simplified column-chromatographic procedure with which LD isoenzymes 1 and 2 are separated from LD isoenzymes 3, 4, and 5. Although the five isoenzymes can be completely separated by ion-exchange chromatography (20), large columns and long elution times are necessary, which make this technique cumbersome and unreasonable for routine clinical use. The technique described here is readily adaptable for routine use with large numbers of samples. In my laboratory, one technician now performs 30 to 40 such fractionations and assays per day, with report forms usually being returned to the patient’s chart within 8 h of sample collection.

The incomplete separation of LD isoenzymes by this procedure does not appear to impair its useful-

<p>| Table 2. Total Lactate Dehydrogenase and Its Isoenzymes in Sera of Healthy Men and Women |
|----------------------------------|------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Total LD</th>
<th>LD-1,2</th>
<th>LD-3,4,5</th>
<th>LD-1,2</th>
<th>LD-3,4,5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean U/liter</td>
<td>117</td>
<td>54</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>Range</td>
<td>95-152</td>
<td>33-64</td>
<td>32-46</td>
<td>40-54</td>
</tr>
<tr>
<td>% of total</td>
<td></td>
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</tbody>
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ness (Tables 1, 2 and Figures 3, 4). Moreover, because combined LD and CK isoenzyme analysis is easily performed, the procedure enhances patient monitoring, especially in suspected cases of myocardial infarction.

There is evidence elsewhere (21) for the clinical usefulness of combined LD and CK isoenzyme determinations, and this is borne out by comparing time-course curves produced by the present technique (Figure 5) and by the electrophoretic technique (21). Because of the transiency of MB-CK activity in serum, increased MB-CK activity may be missed if samples are collected 48 h after an infarction. In such a situation, the advantage of simultaneous LD-1,2 assays is apparent: increased activity of LD-1,2 appears at a time when normal or only slightly increased MB-CK activity makes interpretation difficult. Thus, combined CK and LD isoenzyme determinations provide the sensitivity and specificity needed to evaluate both early and late stages of post-infarction.

Electrophoretic results for LD isoenzymes 3, 4, and 5 were consistently lower (20%) than those obtained by the column technique. Other investigators have estimated loss of LD activity during electrophoresis to be as high as 70% (23), such loss being most likely related to the heat produced by the resistance to current flow through the electrophoretic support media. LD isoenzymes 4 and 5 are heat labile and probably account for most of the activity loss. Because LD isoenzymes 3, 4, and 5 are better accounted for analytically with the column technique, the values reported here for percentage LD-1,2 are slightly lower than those previously reported with the electrophoretic technique. Analytical recoveries of total LD activity greater than 100% in column effluents probably reflect the dilution or removal of LD inhibitors by the ion-exchange process. Thus, the conditions described here for ion-exchange separation on micro-columns appear to be ideal for the isolation and recovery of both LD and CK isoenzymes.

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References

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