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DUAL-DETECTOR–POST-COLUMN REACTOR SYSTEM FOR THE DETECTION OF ISOENZYMES SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. EVALUATION AND APPLICATION TO LACTATE DEHYDROGENASE ISOENZYMES

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SUMMARY

We describe the separation of lactate dehydrogenase isoenzymes by high-performance liquid chromatography–anion-exchange columns and their quantitation by a computer-controlled, dual-detector post-column reaction system. The recoveries from the separation column were *ca.* 90%. The dynamic range of the system was linear over about three orders of magnitude from 3 to 1500 U/l. The coefficient of variation for isoenzyme peak areas was *ca.* 2%. The method is compared to the classical electrophoresis measurement and shows increased speed, resolution, precision and accuracy.

INTRODUCTION

We have reported the separation of isoenzymes (chemically distinct variants of an enzyme that catalyze the same reaction) by high-performance liquid chromatography (HPLC). We first reported the separation of creatine kinase isoenzymes (CK) on an anion-exchange column[1]. Later, we described[2] the separation of lactate dehydrogenase isoenzymes (LD) on DEAE-Glycophase™, which is an anion-exchange material patended by Regnier and Chang[3]. In both papers we demonstrated that isoenzymes could be separated by HPLC on incompressible anion-exchange supports. The separations were rapid (10–20 min), but subsequent fraction collection with either manual[1] or automated analysis with standard AutoAnalyzer techniques[2], proved to be the rate-limiting step (hours), and resulted in reduced resolution. However, the resolution of this technique was superior to electrophoretic methods. We also observed that the effect of optically interfering, nonreacting peaks was eliminated, because discrete samples (fractions) were determined by rate methods.

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To avoid the tedium of fraction collection and discrete analysis, we devised[4] a post-column reaction (PCR) detector system to measure the enzymatic activity continuously. The first use of a PCR to detect isoenzymes after separation by HPLC was reported by Chang *et al.*[5]. It was observed that PCRs with single detectors were satisfactory for purified enzyme preparations. However, we found that optical interferences in serum samples and baseline drift, especially at the end of the gradient, caused many problems in data interpretation.

To overcome these problems, we used[6] a serial-stream, dual-detector–PCR with computerized-data acquisition and reduction. This paper describes the application of that system to the determination of LD isoenzymes in serum. We evaluated the accuracy, precision, and recovery of the LD isoenzyme analysis. Further, we demonstrated experimentally that our dispersion model[6] can be used to correct for blanks and baseline drifts directly.

MATERIALS AND METHODS

The chromatographic apparatus and computer programs have been described previously[6]. The chromatographic column, 18 × 0.5 cm, was made from precision-bore stainless-steel tubing (Altech, Houston, Texas, U.S.A.). It was slurry-packed with microparticulate DEAE-Glycophase™ (Corning Glassworks, Corning, N.Y., U.S.A.), using a Micromeritics, Model 705 slurry packer (Micromeritics, Norcross, Ga., U.S.A.) and isopropanol as the solvent. This material may be commercially available from Pierce (Rockford, Ill., U.S.A.), or a comparable material is available from SynChrom (Linden, Ind., U.S.A.). The column was sealed at both ends with 2-μm stainless-steel frits (Altech). The pre-column, 4.0 × 0.5 cm, was similarly prepared using Vydac™ 211AX (The Separations Group, Hesperia, Calif., U.S.A.) and placed directly before the analytical column.

The buffers for gradient elution were 20 m*M* Tris–HCl, pH 7.8 (buffer A), and 20 m*M* Tris–HCl containing 150 m*M* NaCl (pH 7.8). Unless otherwise indicated in the figures, the gradient was begun automatically 2 min after injection. It was advanced at a rate of 3%/min for 4 min, 6%/min for 2 min and 3%/min until the end of the gradient. For each run 100 μl of sample was injected. The column was washed with buffer A for at least 15 min before each injection.

The solution for the reagent pump was 6.5 m*M* NAD (nicotinamide adenine dinucleotide, Grade AA; Sigma, St. Louis, Mo., U.S.A.), 86 m*M* L-lactate (Sigma) in 1.5 *M* 2-amino-2-methyl-1-propanol (AMP), pH 9.0, which was also obtained from Sigma. The flow-rate from the reagent pump was 1.00 ml/min while the gradient pumping system delivered solvent at a rate of 2.00 ml/min, producing a combined flow-rate of 3.00 ml/min through the post-column reaction coil. At this flow-rate, the residence (or reaction) time in the coil was 79 sec.

Serum samples were obtained from the Clinical Laboratory at the University of South Alabama Medical Center. They were frozen after collection and stored in the freezer. On thawing, the serum samples were directly injected into the chromatographic system. Samples that were obviously hemolyzed were discarded. The LD isoenzyme controls were obtained from Helena Laboratories (Beaumont, Texas, U.S.A.; Lot Nos. 1785131 and 585131). This material was reconstituted with 1.75 ml of buffer A to yield about 1000 U/l of LD activity. The most strongly retained

isoenzyme, LD-1, was obtained in a purified form, more than 95% homogeneous, from Sigma. The stock LD-1 solution was prepared by diluting the ammonium sulfate suspension with buffer A. Human tissues were obtained at autopsy from the University of South Alabama Medical Center and stored at −20°. A combined homogenate of heart, spleen and liver was prepared in buffer A as described earlier[4].

The assay of the stock LD-1 solution was done by adding a 100-μl aliquot of the solution to 3.0 ml of the assay solution containing 2.2 m*M* NAD, 29 m*M* L-lactate and 0.5 *M* AMP, pH 9.0. The initial rate of change in absorbance at 340 nm was determined with a Beckman Model 25 spectrophotometer (Beckman Instruments, Fullerton, Calif., U.S.A.). The LD-1 activity recovered from the chromatographic column was assayed by adding 1 ml of the collected solution to 2.0 ml of the assay solution. The resulting solution contained 4.3 m*M* NAD, 57 m*M* L-lactate and 1.0 *M* AMP (pH 9.0). The initial rate of change in absorbance at 340 nm was then determined.

The column fractions for subsequent electrophoresis were collected manually by following the activity profile recorded at detector 2. The fractions were collected and immediately stored in ice. The presence of the assay reagents did not interfere with subsequent electrophoresis and staining for LD activity. The electrophoresis of these fractions was done at the Clinical Laboratories (University of South Alabama Medical Center) with the Helena "Zip Zone LDH Isoenzyme" procedure. The identity of the LD isoenzyme(s) in the column fractions was determined by comparison with heart and liver controls purchased from Helena.

Serum LD isoenzymes were separated by anion-exchange chromatography in 30 min or less, and the column effluent was continuously combined with the LD assay reagent. Before the enzymatic reaction commenced, the background absorbance was measured at detector 1. After the reaction mixture exited the post-column reactor, the reaction product (NADH) was measured at detector 2. This latter response reflected both the NADH profile and the background absorbance.

RESULTS AND DISCUSSION

The distribution of LD isoenzymes in an abnormal serum sample with a total LD activity* of 502 U/l is shown in Fig. 1. The upper trace shows the chromatographic profile measured at detector 2, and the lower trace shows the background absorbance measured at detector 1. It is apparent that the profile due to the activity of the LD isoenzymes has been distorted by changes in the background absorbance, particularly in the region of the LD-1 and LD-2 peaks. With the previously described computer program[6], it is possible to deconvolute the response of detector 2 and produce the response attributable to the isoenzyme activity alone. The removal of the background absorbance produces the chromatogram shown in Fig. 2. The isoenzyme peaks are now resolved to baseline, and the baseline drift apparent in Fig. 1 has now been removed leaving a nearly flat baseline. The percentages of the total area of the LD-5, LD-4, LD-3 (sum of LD-3′ and LD-3), LD-2, and LD-1 peaks were found to be 2.5, 1.2, 10.9, 28.4 and 56.9%, respectively. The preponderance of LD-1 and LD-2 activity (85.3% of the total) is indicative of a significant contribution

* For the procedure in our clinical laboratory, the normal limits are 100–225 U/l.

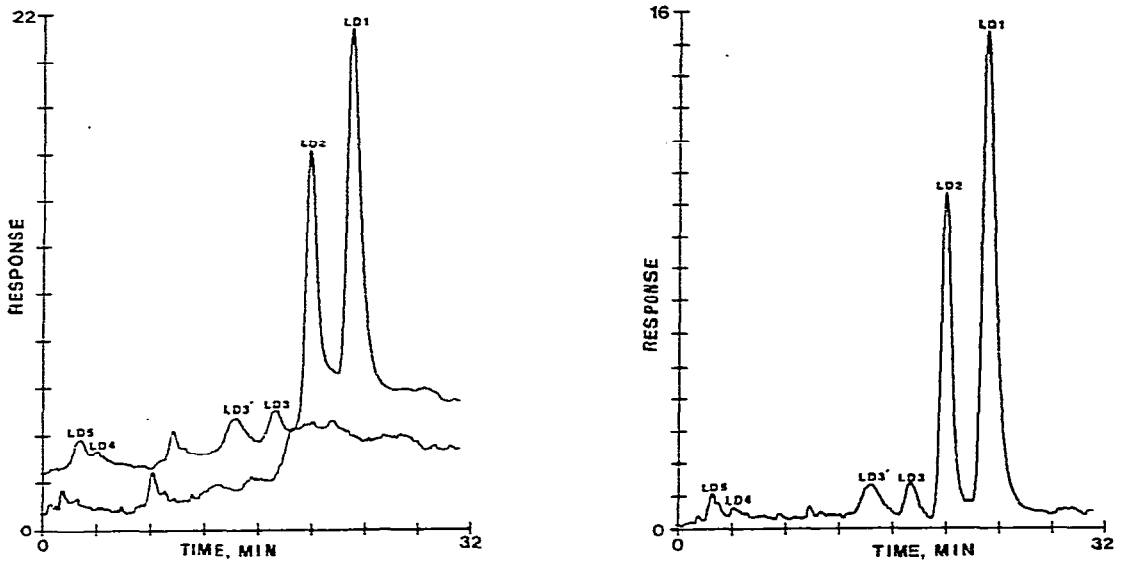


Fig. 1. Chromatogram of serum LD isoenzymes from a patient who had a myocardial infarct. The upper trace was recorded at the downstream detector (detector 2). The lower trace shows the background absorbance, as it was observed at the upstream detector (detector 1). Total serum LD activity was 502 U/l.

Fig. 2. Profile of serum LD isoenzymes resulting from the correction for background absorbance (in Fig. 1). The dispersion coefficient used in the computer program was 0.45 sec. The percentages of total area are: LD-5, 2.5; LD-4, 1.2; LD-3', 6.5; LD-3, 4.4; LD-2, 28.4 and LD-1, 56.9%.

of LD isoenzymes from heart tissue to the serum isoenzyme profile. The elevation of LD-1 over LD-2 activity is called the "LD-flip"[7]. Since LD-2 is predominant in normal sera, the "LD-flip" is a good indication of myocardial infarction[7], which was the clinical diagnosis.

The distribution of LD isoenzymes in this sample was also determined electrophoretically. This distribution is shown in Fig. 3. The percentages of total area of the LD-5, LD-4, LD-3, LD-2 and LD-1 peaks were determined by densitometry and found to be 1.2, 2.8, 19.6, 33.3 and 43.1%, respectively. This result is in qualitative agreement with our finding but reveals a lesser amount of the clinically important LD-1 isoenzyme. This same observation has been made by Schlabach *et al.*[8].

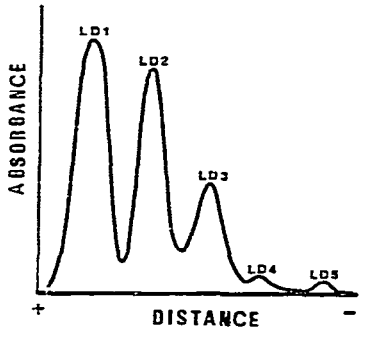
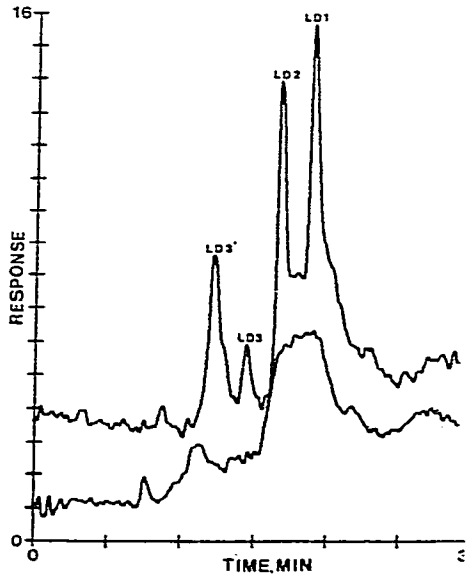


Fig. 3. Electrophoretic scan of the sample chromatographed in Fig. 1. Peak assignments and percentages of the total peak area are: LD-1, 43.1; LD-2, 33.3; LD-3, 19.6; LD-4, 2.8 and LD-5, 1.2%.

The distortion in the LD activity profile due to changes in background absorbance is usually more pronounced when the resulting NADH concentrations are low, and higher detector sensitivities (lower attenuation) must be used. Higher sensitivity increases the magnitude of the background peaks attributable to sample interferences. The chromatogram of serum LD isoenzymes in a normal sample is shown in Fig. 4 and illustrates the effect of increased sensitivity on the magnitude of the background peaks. While the change in background absorbance (lower trace) in the LD-2 to LD-1 region was observed to be about 15% of the maximum peak (upper trace) in Fig. 1, this change was found to be about 40% in Fig. 4. This distortion is so bad that it makes even a qualitative assessment nearly impossible. It would appear that the LD-1 peak is the dominant peak (upper trace) in Fig. 4, but when the background absorbance is removed (Fig. 5), LD-2 is seen to be the dominant peak. Although LD-1 and LD-2 are again the major peaks, they only constitute about 69% of the total activity which is typical for a normal serum LD isoenzyme distribution. The noisy baseline seen in Fig. 5 is also a direct result of increased sensitivity (decreased attenuation) and indicates that further increases in sensitivity are not possible.



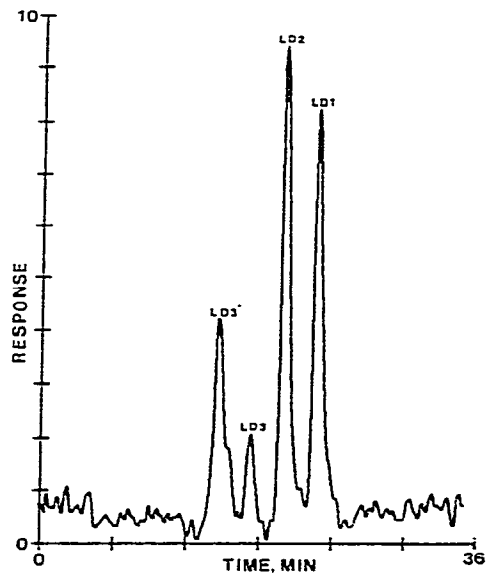


Fig. 4. Chromatogram of serum LD isoenzymes from a patient with a normal level of serum LD activity. Total serum LD activity was 140 U/l. The upper and lower traces were observed at detector 2 (downstream) and detector 1 (upstream), respectively.

Fig. 5. Profile of serum LD isoenzymes resulting from the correction for background absorbance (in Fig. 4). The dispersion coefficient used in the computer program was 0.40 sec. The percentages of total area are: LD-3', 21.3; LD-3, 9.2; LD-2, 40.4 and LD-1, 28.6%.

Although the interference present in the LD-2 to LD-1 region has not been definitely characterized, preliminary data indicate that it is likely due to serum protein(s). Since serum contains many low molecular weight compounds that also absorb to some extent at 340 nm and since many drugs also absorb at that wavelength, the background profile of a serum sample can be complex and unpredictable. An example

of such a complex background profile is shown in Fig. 6 (lower trace). The removal of the background absorbance produced the chromatographic profile shown in Fig. 7. The background peaks appearing between LD-4 and LD-3′ in Fig. 6 (upper trace) were completely eliminated by deconvolution algorithm. The shoulder on the front of LD-2 in Fig. 6 was also removed; the resulting baseline was nearly flat, and a a substantial improvement in resolution was achieved between LD-3 and LD-2.

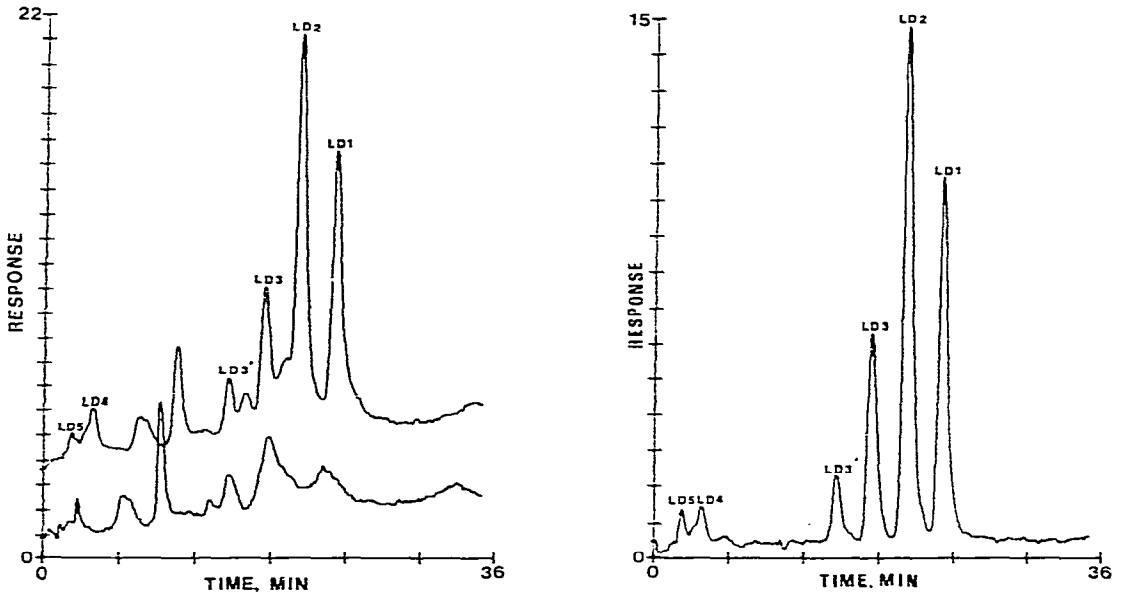


Fig. 6. Chromatogram of serum LD isoenzymes from a patient with an elevated level of LD activity. Total serum LD activity was 444 U/l. The upper and lower traces were observed at detector 2 (downstream) and detector 1 (upstream), respectively.

Fig. 7. Profile of serum LD isoenzymes resulting from the correction for background absorbance (in Fig. 6). The dispersion coefficient used in the computer program was 0.46 sec. The peak assignments are the same as in Fig. 6. The percentages of total area are: LD-5, 1.1; LD-4, 2.2; LD-3′, 4.2; LD-3, 17.4; LD-2, 44.0 and LD-1, 31.0%.

The accuracy of our peak assignments was evaluated by comparing our results for samples prepared from human tissue extracts with the results obtained from electrophoresis.

A chromatogram of LD-isoenzymes in a sample prepared from combined human heart, liver and spleen tissue is shown in Fig. 8. With the gradient and pH conditions used, LD-5 was incompletely resolved from LD-4. However, it is seen that LD-3 was clearly resolved into three separate peaks. Repeated injections of this sample yielded the same result.

To confirm the identity of our peak assignments, especially the three peaks in the LD-3 region, column fractions were collected and analyzed by electrophoresis. The electrophoretic results confirmed our peak assignments and indicated that the three LD-3 peaks migrated like the LD-3 isoenzyme in the control sample. The presence of multiple bands for a single LD isoenzyme has been ascribed to the presence of allelic variations[9,10] and the noncovalent association of LD-isoenzymes

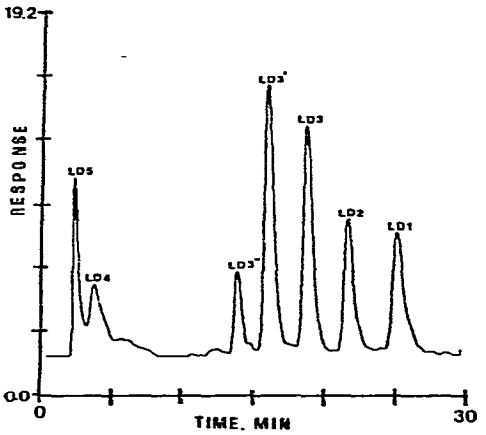


Fig. 8. Profile of LD isoenzymes in an extract prepared from human tissues after correction for background absorbance observed at detector 1. The extract was prepared from combined heart, liver and lung tissue. The dispersion coefficient used in the computer program was 0.36 sec. The percentages of total peak area are: LD-5, 11.4; LD-4, 7.3; LD-3'', 5.9; LD-3', 26.0; LD-3, 22.1; LD-2, 13.2 and LD-1, 14.1%.

with other serum proteins[11]. Multiple LD isoenzymes were also observed by Schlabach *et al.*[8] who separated LD isoenzymes on a similar column.

We determined the column recovery by introducing a sample of LD-1 at the head of the column. Later an identical sample was introduced below the column and just ahead of the PCR. The resulting peak areas were compared. The experiment was run in quadruplicate with an average recovery of 89.5 $\pm$ 1%. In a second experiment, the same LD-1 samples were introduced as before, but 10-ml fractions of the effluent at detector 2 were collected and assayed. This experiment produced an average recovery of 90.1 $\pm$ 1% for quadruplicate samples. This indicates good column recovery and excellent agreement between the activities determined by the PCR system and the manual procedure.

The dynamic range of the PCR was evaluated by making triplicate injections of six solutions of purified LD-1, with activities ranging from 3 to 1500 U/l. The isoenzyme was chromatographed with the conditions described in Materials and methods. The peak height was determined and converted to absorbance units. When these absorbance data were correlated with enzyme activity, the regression line was found to have a slope of 1.005 and a zero intercept. The linear correlation coefficient ($n = 18$) was found to be 0.999.

The repeatability of the LD isoenzyme profile was determined with five replicate injections of the LD isoenzyme control. The LD-1 and LD-2 isoenzymes were found to be the dominant peaks and LD-5 was present at a very low level and was nonretained. The retention times for the LD-4, LD-3 (largest peak), LD-2 and LD-1 isoenzymes were found to have the following coefficients of variation (C.V.); 2.62, 0.67, 0.52 and 0.43% ($n = 5$), respectively. The C.V. values for the corresponding peak areas were substantially larger and were found to be 5.62, 4.56, 1.79 and 1.43%, respectively. The variation in the clinically important ratio of the LD-1 to LD-2 peak areas was found to be very small with a C.V. of only 0.62%. This primarily represents the random error associated with the determination of the

individual peak areas and likely represents the lower limit of variation since errors due to differences in injection volumes, lag phases and delay times have been eliminated. The injection valve in these experiments was found to have a C.V. of 0.55% ($n = 8$).

In the above studies, mean peak areas for LD-5, LD-4, LD-3 (the sum of LD-3″, LD-3′ and LD-3), LD-2 and LD-1 were 0.2, 1.1, 14.6, 38.6 and 45.7%, respectively. The corresponding distribution of LD isoenzymes in this control sample was reported by the manufacturer to be 2, 9, 19, 33 and 37%, respectively[12].

To reduce analysis time, a steeper gradient was used. Another lot of the control sample was selected because it had more than twice as much LD-5 activity, which made LD-5 clearly visible (Fig. 9). In Fig. 9 the LD-1 isoenzyme is observed at about 17 min compared to about 24 min in the previous chromatograms. Although this sample was prepared from rat tissue, three peaks in the LD-3 region are clearly apparent in Fig. 9. The separation shown in Fig. 9 could be repeated at a rate of approximately three per hour. At a 50% usage rate, it would be possible to analyze 36 samples per day. We have found that column resolution is adequate for several hundred hours or more with periodic use and for at least a month with daily use.

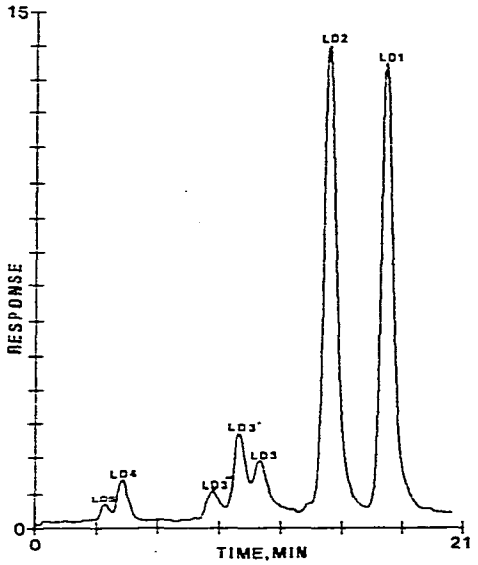


Fig. 9. Chromatogram of LD isoenzymes in a control sample. A "CK/LD Isoenzyme Control" from Helena (Lot No. 585131) was chromatographed with a gradient which began at 5% strong buffer and was increased linearly at a rate of 4%/min to 100% strong buffer. The percentages of total peak area are: LD-5, 0.8; LD-4, 2.8; LD-3″, 2.2; LD-3′, 6.6; LD-3, 5.7; LD-2, 43.1 and LD-1, 38.7%.

CONCLUSIONS

For enzyme assays, the weakness in most PCR systems has been the inability to correct for contributions to detector response arising from sample interferences, gradient effects, etc.[4,5,8]. Such deleterious contributions are superimposed on the

response resulting from the detection of the reaction product. The concentration of enzymatic product in a PCR is proportional to the amount of enzyme originally present in the sample. The chromatographic profile of the reaction product reveals the activity of each isoenzyme. Serum samples, however, contain interferences that distort this profile. By placing an identical detector ahead of the PCR coil and using a computer program[6], we have demonstrated a method that corrects for serum interferences. The corrected profile only reflects the distribution of isoenzyme activity.

The ability to correct for sample interferences and baseline drifts was shown to improve both the qualitative and quantitative evaluation of serum LD isoenzymes. Since sample interferences are ubiquitous, it is difficult to distinguish between activity peaks and interference peaks. Therefore, the ability to subtract out interferences permits the unambiguous identification of peaks having enzyme activity.

The excellent precision found in peak retention and peak area is attributable to the stability and resolution of the chromatographic column and the linear response of the PCR system. Combining the resolution and precision of this system with ability to correct for sample interferences will enable new discoveries to be made in the relationship between serum isoenzyme profiles and various disease etiologies.

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