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## Note

# Rapid analysis of serum lactate dehydrogenase isoenzymes by high-performance ion-exchange chromatography

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Isoenzymes of lactate dehydrogenase (L-lactate; nicotinamide-adenine dinucleotide oxidoreductase, EC 1.1.1.27, LDH) are tetramers composed of H and M subunits, i.e. H4, H3M1, H2M2, H1M3 and M4. At present, in clinical laboratories, the most common technique for the separation and measurement of serum LDH isoenzyme activity is electrophoresis. However, this method involves laborious and time-consuming procedures such as enzyme staining and drying of the support. The intensity of the stain is either estimated by absorbance or fluorescence densitometric scanning, and the results are usually semiquantitative. Moreover, the activities of unstable components (LDH-3, LDH-4 and LDH-5) can be lost, especially during the separation [1- 3].

The development of high-performance ion-exchange chromatography allowed protein separations in less than 30 min, while preserving their activities. However, silica-based ion exchangers could not be used with mobile phases of high pH. Furthermore, proteins or lipids were frequently irreversibly adsorbed at the surface of the packing. In our previous report [4], we investigated the use of a three-step linear gradient and TSK gel DEAE-5PW (75 × 7.5 mm I.D.), hydrophilic-polymer-based supports [5], for the analysis of LDH isoenzymes in serum.

The purpose of this work was to shorten the analysis time while maintaining the required resolution. By using shorter columns and a five-step linear gradient of sodium chloride, the analysis time was reduced to 10 min per sample. More than a thousand samples of human serum could be analysed using a single column. Futhermore, the quantity of reagents required was reduced concomitantly with the separation time.

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## EXPERIMENTAL

### *Apparatus*

The high-performance liquid chromatographic (HPLC) equipment, CCP8000, consisted of a CCPM computer multipump, AS-48 automatic sample injector, CO-8000 column oven, FS-8000 fluorophotometric detector, DG-3 degasser and CP-8000 chromatographic processor. The following columns were used: TSK gel DEAE-5PW (10 μm particle diameter, 50 × 7.5, 50 × 6.0, 75 × 4.6, 35 × 4.6 mm I.D.). The columns contained a filter element which served as a guard column. All instruments, as well as the columns, were from Toyo Soda (Tokyo, Japan). The reagent pump was a Technicon Auto Analyzer proportioning pump (Technicon Instruments, Tarrytown, NY, U.S.A.). The pH meter was an Autocal pH meter (Radiometer A/S, Copenhagen, Denmark).

### *Materials*

Tris(hydroxymethyl)aminomethane and human albumin were purchased from Sigma (St. Louis, MO, U.S.A.), diethanolamine, D,L-lactic acid, sodium hydroxide and hydrochloric acid were from Katayama (Osaka, Japan), and Brij-35 (30% solution) from Technicon International (Saint Denis, France). Solvents A and B were prepared as follows: A, tris(hydroxymethyl)aminomethane (1.211 g) was dissolved in ca. 900 ml of distilled water, adjusted to pH 8.0 at 37°C by adding 3.9 ml of 1 *M* hydrochloric acid and the solution was made up to 1 l with distilled water to obtain a 10 m*M* Tris—HCl buffer (pH 8.0); B, sodium chloride (29.225 g) was dissolved in 1 l of solvent A. The substrate buffer was prepared as follows: diethanolamine (42 g) was dissolved in ca. 500 ml of distilled water, mixed with 14.33 g of D,L-lactic acid and 7 ml of 10 *M* sodium hydroxide and the mixture was made up to ca. 950 ml with distilled water, and allowed to stand overnight. Finally, the pH was adjusted to 8.7 at 37°C, and made up to 1 l with distilled water. Solvents A and B and the substrate buffer were filtered through 0.45-μm membrane filters. Coenzyme reagent was prepared as follows: tris(hydroxymethyl)aminomethane (2.42 g) and β-$NAD^+$ (95.6 g) were dissolved in ca. 900 ml of distilled water and adjusted to pH 3.5—4.0 with 2.5 *M* sodium hydroxide. The solution was diluted to 1 l with distilled water and filtered through a glass filter. Aliquots of 15 ml were stored in 30-ml vials and dry-freezed. Solvents A and B and the substrate buffer and coenzyme reagent are stable for at least one year at 2—8°C. Before use as a reaction reagent, a vial of coenzyme reagent was dissolved in 500 ml of substrate buffer and 1.7 ml of Brij-35 (30% solution) [6].

### *Chromatographic conditions*

The conditions for the five-step linear gradient elution were as follows: starting eluent, 7% B; 0.7-min linear gradient from 7 to 9% B; 0.5-min linear gradient from 9 to 20% B; 1.8-min linear gradient from 20 to 26% B; 3.3-min linear gradient from 26 to 33% B and then 70% B, followed by 0.5-min isocratic elution, and return to starting conditions. The anion exchanger was equilibrated for 3.2 min prior to the following injection. Samples (100 μl) of human serum diluted four-fold with solvent A were injected at a flow-rate of

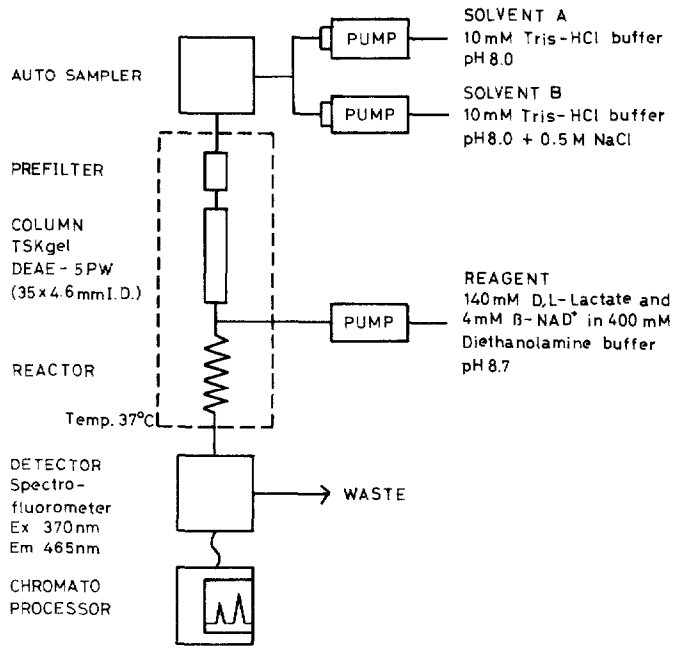


Fig. 1. HPLC flow diagram for the determination of serum LDH isoenzymes.

1.6 ml/min. The effluent was mixed with the reaction reagent in a 1:1 ratio and incubated in the reaction coil for 3 min at 37°C. Reduced nicotinamide-adenine dinucleotide (NADH) formed in this reaction was quantified fluorimetrically using an excitation wavelength of 370 nm and emission wavelength of 465 nm (Fig. 1). LDH activity was calculated from the NADH peak area.

## RESULTS AND DISCUSSION

Linear gradients of sodium chloride have been used frequently for the analysis of LDH isoenzymes [9—12]. Using continuous gradients, it was impossible to completely separate all isoenzymes owing to disparate intervals in the isoelectric points (p*I*). The elution pattern shown in Fig. 2a and b was obtained using 5-min and 10-min continuous gradient elutions of sodium chloride, respectively. Three peaks were obtained for LDH-3, i.e. LDH-3″, LDH-3′ and LDH-3 [7]. While the continuous gradients provided good resolution of LDH-3 compared with the five-step linear gradient method, LDH-2, LDH-1 and albumin could not be separated in less than 10 min.

The best resolution and the shortest analysis time were obtained using a 35 × 4.6 mm I.D. TSK gel column and a five-step linear gradient. A chromatogram of a control adult serum (total activity 230 I.U./l) diluted four-fold with solvent A is shown in Fig. 3. The elution order was: LDH-5, LDH-4, LDH-3, LDH-2 and LDH-1. LDH-3 gave two peaks, in agreement with the results of previous reports [4, 7, 8]. The elution time of LDH-3 was taken as that of the second peak, and the peak area as the sum of the two peaks.

Albumin exhibited strong fluorescence intensity under the conditions of the experiment. In order to avoid interferences, its separation from the LDH peaks was mandatory.

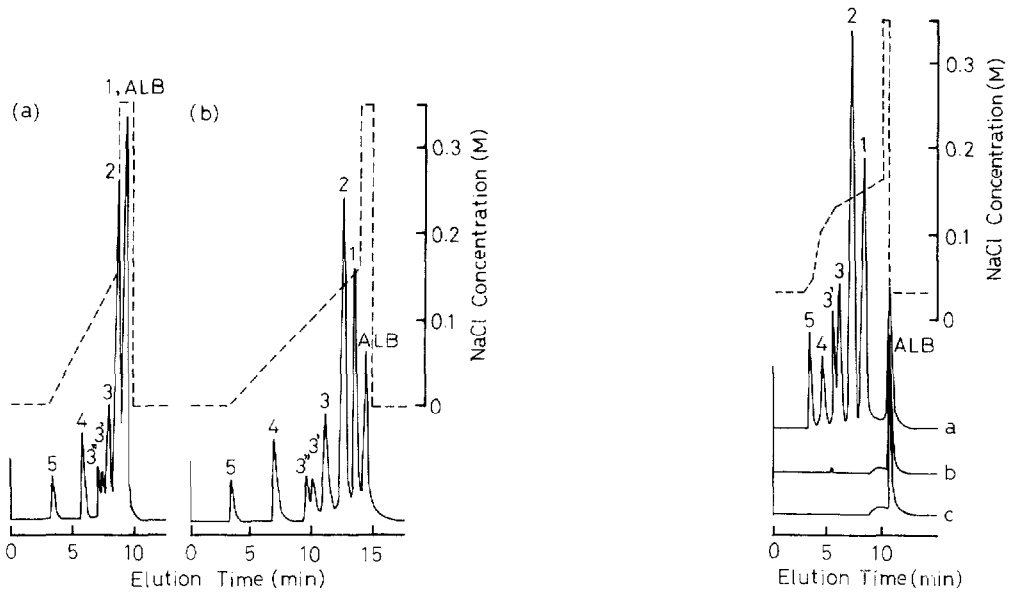


Fig. 2. Chromatograms of LDH isoenzymes (1—5) and albumin obtained by high-performance ion-exchange chromatography on a TSK gel DEAE-5PW column (35 × 4.6 mm I.D.). Linear gradient of sodium chloride from 0 to 0.15 *M* in 5 min (a) and in 10 min (b). Other conditions were the same as in Fig. 3.

Fig. 3. Chromatograms of LDH isoenzymes and albumin obtained by high-performance ion-exchange chromatography on a TSK gel DEAE-5PW column (35 × 4.6 mm I.D.). Chromatographic conditions: solvent A, 0.01 *M* Tris—HCl buffer (pH 8.0); solvent B, 0.5 *M* sodium chloride in solvent A; flow-rate, 1.6 ml/min; injection volume, 100 μl; gradients: starting eluent, 7% B; 0.7-min linear gradient from 7 to 9% B; 0.5-min linear gradient from 9 to 20% B; 1.8-min linear gradient from 20 to 26% B; 3.3-min linear gradient from 26 to 33% B and then 70% B, followed by 0.5-min isocratic elution, and return to starting conditions. Samples: (a) normal human serum (total activity, 230 I.U./l); (b) the same as a (sample blank); (c) 40 mg/ml standard solution of human albumin dissolved in solvent A.

Reproducibilities of elution times and peak areas of LDH-1—5 were determined from ten injections of adult human serum (230 I.U./l). The coefficients of variation of elution times were 0.32, 0.28, 0.23, 0.38 and 0.48% for LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5, respectively, and those of peak areas were 1.09, 0.80, 1.73, 2.71 and 1.50%, respectively (Table I). The linear dynamic range determined using samples of patient serum (1300 I.U./l) diluted with solvent A was 10—1100 I.U./l.

The life-time of a TSK gel DEAE-5PW column (35 × 4.6 mm I.D.) was investigated by making repetitive injections of human serum with periodic injections of 100 μl of 0.1 *M* sodium hydroxide every 30 samples (Fig. 4) [13]. More than a 1000 injections could be made without significant loss of resolution.

The separation of LDH isoenzymes is based on differences in electrostatic interactions which largely govern the ion-exchange process. Only a partial area of the surface of isoenzymes comes into contact with the stationary phase, and the retention is governed by the number of charges associated with the adsorption—desorption process. Since there are both acidic and basic residues within

TABLE I

WITHIN-ANALYSIS REPRODUCIBILITY OF ELUTION TIMES AND PEAK AREAS OF LDH ISOENZYMES

Total activity = 230 I.U./l; $n$ = 10.

| LDH isoenzyme | Elution time (mean ± S.D.) (min) | Coefficient of variation (%) | Peak area (mean ± S.D.) (I.U./l) | Coefficient of variation (%) |
|---|---|---|---|---|
| LDH-1 | 8.33 ± 0.027 | 0.32 | 60.7 ± 0.66 | 1.09 |
| LDH-2 | 7.30 ± 0.021 | 0.28 | 91.8 ± 0.73 | 0.80 |
| LDH-3 | 6.27 ± 0.015 | 0.23 | 45.1 ± 0.78 | 1.73 |
| LDH-4 | 4.63 ± 0.017 | 0.37 | 17.7 ± 0.48 | 2.71 |
| LDH-5 | 3.52 ± 0.017 | 0.48 | 14.7 ± 0.22 | 1.50 |

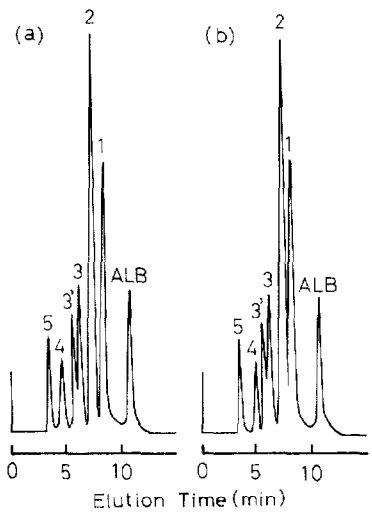


Fig. 4. Chromatograms of LDH isoenzymes and albumin from the 1st (a) and the 1000th (b) run of TSK gel DEAE-5PW (35 × 4.6 mm I.D.).

the M or H subunits of the molecule, its net charge is pH-dependent. The exact p$I$ values of LDH-1—5 depend both on the type of amino acids and molecular structure. LDH isoenzymes 1—4 were retained on anion-exchange columns at pH 8.0 (above their p$I$) because they have a net negative charge, while LDH-5 eluted in the void volume since it is cationic at pH 8.0 (below its p$I$).

The separation of the LDH-3 isoenzyme into several peaks might be due to molecular multiplicities resulting from different steric arrangements formed by random association of H and M subunits. However, the details have not yet been clarified.

We are currently using this assay for routine testing of serum LDH isoenzymes because of its high sensitivity, reproducibility, speed of analysis and low cost per sample compared with the electrophoretic method.

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