SEPARATION OF SERUM LD ISOENZYMES BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Separation and quantitative estimation of the isoenzymes of lactate dehydrogenase(LD) in serum were accomplished with capillary electrophoresis system. An uncoated fused silica capillary column 50 cm long, 75 μ m I.D. and substrate containing running buffer including L-lactic acid and NAD⁺ were used for the separation of serum LD isoenzymes. The resulting product of "NADH" was detected at 340 nm. Injection of 10 nL of five fold diluted serum sample were performed by pressure injection within 2 seconds. The isoenzymes were separated at 10 kV of voltage for 5 min, by turning off the voltage applied for 30 min incubation at 24° C for reaction between substrate and isoenzymes, and applying voltage of 30 min. Under these conditions, the isoenzymes of LD were detected by a NADH generated as isoenzyme of LD-5 emerged at 20 min, LD-1 peak at 23.5 min with close to baseline separation of the other isoenzymes which emerge between LD-5 to LD-1, after the emergence to LD-1 peak, followed another peak, termed "sample shock". The results obtained by the proposed method correlated well with those by gel electrophoresis systemes($r=0.92\sim0.98$) for each five LD isoenzymes, respectively. Within -run precision CVs for 5 replicate analysis were 3.01 (LD-3, mean 14.6%) % $\sim$ 7.82 % (LD-4, mean 4.22 %.), respectively.

KEY WORDS: Capillary electrophoresis, LD isoenzymes, analytical method

INTRODUCTION

Lactate dehydrogenase (LD) is one of the important enzyme markers for liver and cardiac diseases. It is composed of four chains of two types, H(heart) and/or M(muscle) subunits which give rise to five isoenzymes. Each enzyme has a different net charge and can be separated electrophoretically into five fractions, LD1(H₄) ~LD5(M₄). The current method for LD isoenzyme measurement is by electrophoresis. However, whole process of electrophoresis is very cumbersome, time consuming and skill- dependent. Capillary electrophoresis is a new tool for macromolecular separation. It can be accommodated with high electrical fields and is well suited for

automation. In 1992, Hsieh et. al. (1) developed LD isoenzymes determination by capillary electrophoresis system. However, this system is insufficient for serum LD isoenzymes determination for the sake of poor detection limits and poor separation (2). Therefore, in this study, we have devised an improved capillary electrophoresis method for fractionating LD isoenzymes, and have evaluated its utility in resolving the isoenzymes in serum.

MATERIALS AND METHODS

We used Beckman P/ACE system 2100 (Beckman Coulter Inc., Brea, CA, USA) for performing capillary electrophoresis using column and reagents supplied by the manufacturer (Beckman Coulter instrument). Analysis and capillary column preparation were as follows: Uncoated fused silica capillary was used in cartridge (75 μ m I.D. x 57 cm) format. Daily, before starting an electrophoresis run, the capillary

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was washed with 1 mol/L of NaOH for 1 min followed by distilled water wash for an additional 5 min and then rinsing with running buffer for 15 min to equilibrate the column. Tris (hydroxymethyl) aminomethane buffer (25 mmol/L, pH 8.7) containing 51.6 mmol/L of L-lactate and 8.26 mmol/L of NAD^+ was used as the running buffer. Capillary was rinsed with running buffer for 15 min and equilibrated with running buffer for an additional 30 sec prior to injection of sample.

Serum samples from patients and human LD standard (Sigma, St. Louis, MO, USA) were diluted 5 times with Tris buffer, prior to injection. Samples were introduced by pressure injection for 2 sec (approximately 10 nL). Capillary electrophoresis was performed at 24° C, 10 kV for 5 min. The power was turned off for 30 min to allow the reaction between isoenzyme and LD substrate to be completed (parking time). Separation was resumed by applying a voltage 10 kV 30 min. LD isoenzyme activity was monitored by following the absorbance of reduced NADH at 340 nm (Figure 1).

Fig. 1. Principle of LD isoenzymes measurement by capillary electrophoresis.

RESULTS AND DISCUSSION

Migration of NADH

Figure 2 (A) shows NADH from purified LD2 and NADH solution (B) by proposed method. First Sample Shock (SS) appeared from 16 min to 24 min (A) (due to sample movement into the capillary column with substrate containing running buffer). Only NADH

solution also showed sharp peak at 24 min (B).

Applied voltage

We initially employed 10 kV for separation of LD isoenzymes for 5 min. Applied voltage and initial application time affected both the migration time of NADH and separation of individual isoenzymes. With an applied voltage of 5 kV, migration time of NADH was about 40 min; if the initial application time was 2 min, LD isoenzymes peak very close, and did not resolve well. If the initial application time was 10 min, isoenzymes were washed out of the column (data not shown).

Parking Time

When parking time was increased, migration time of NADH (isoenzyme peak) was delayed slightly. Reaction temperature initially used was 24° C, with increasing temperature, peak area decreased (this temperature does not reflect capillary column temperature). We used a parking time of 30 min, which was dependent on total LD activity. If the total LD activity was below 500 U/L, 30 min parking time was optimal for resolving the isoenzymes.

Measurement of LD isoenzymes

Figure 3 shows electropherograms of mixture of pure LD isoenzymes (Sigma LD 1,2,3,5). The migration times for each LD isoenzymes were as follows: LD 5, 20.0 min, LD3, 21.8 min, LD 2, 22.6 min, LD1 23.6 min, second sample shock 24.0 min, respectively. Electropherogram obtained from a normal subject and a liver disease patient by capillary electrophoresis assay were shown in Figure 4 and Figure 5 respectively.

Linearity

We established the linearity of the method using NADH and pure human LD2. The response was linear up to NADH of 0~0.007 mg/mL and linear up to 20 U/L ~150 U/L of LD-2 activity.

Precision and Interferences

We analyzed patient sample (Total LD 360 U/L) 5 times for the within-run analysis and obtained a mean $\pm$ SD, CVs of 36.7 $\pm$ 1.26 %, 3.43 % for LD1,

Fig. 2. (A) NADH from LD2 [LD2 (Sigma) and running buffer incubated at 37°C 5 min, mixture was introduced to capillary column by pressure injection 2 sec and applied 10 kV (No Parking)]. (B) NADH solution (2mg/mL) was introduced to capillary column by pressure injection and applied 10 kV.

Fig. 3. Eletropherogram of LD isoenzymes standard (Sigma) mixture (LD1 230 IU/L, LD2 130 IU/L, LD3 240 IU/L, LD5 250 IU/L)

Fig. 4. Eletropherogram of normal subject (total LD 171 IU/L).

Fig. 5. Eletropherogram of liver disease patient (total LD 3880 IU/L).

33.28 ± 1.09 %, 3.28 % for LD2, 14.6 % ± 0.4 %, 3.01 % for LD3, 4.22 ± 0.33 %, 7.82 % for LD4, 11.2 ± 0.66 %, 5.89 % for LD5, respectively (Table 1). Normal concentration of various kinds of anticoagulants, glucose, ascorbic acid, hemoglobin and bilirubin had no effects on the results (data not shown)

Method comparisons

We compared the results of analyses of 20 serum samples by our capillary electrophoresis assay with those obtained using LD isoenzymes with Rep (Helena Labs, TX, USA). The results of the two assay methods

Fig. 6. Correlation of LD isoenzymes (%) between proposed capillary electrophoresis method and Helena electrophoresis method (agarose gel electrophoresis).

Table 1. Within-run precision

	LD1	LD2	LD3	LD4	LD5
1	37	33.2	14.3	4.5	11
2	36.5	33.2	14.5	4.6	11.2
3	34.8	34.5	15	4.2	11.2
4	37.8	33.9	14.1	3.9	10.3
5	37.3	31.6	15.1	3.9	12.1
N	5	5	5	5	5
Mean	36.76	33.28	14.6	4.22	11.2
SD	1.26	1.09	0.44	0.33	0.66
CV	3.43	3.28	3.01	7.82	5.89

showed good agreement giving the following correlation for each isoenzyme: (Y= our capillary electrophoresis, X = Helena Labs) LD1: $Y = 1.25x - 2.4$, $r = 0.92$, LD2: $Y = 1.08x + 2.8$, $r = 0.96$, LD3: $Y = 1.13x - 4.1$, $r = 0.92$, LD4: $Y = 0.91x - 2.0$, $r = 0.93$, LD5: $Y = 1.14x - 50$, $r = 0.98$, respectively (Figure 6).

Reference ranges

In our capillary electrophoresis method reference values for serum LD isoenzymes in 50 healthy adults were as follows : LD1 : 22.2~ 36.6%, LD2 : 35.5~

43.0%, LD3: 20.1~ 26.7 %, LD4 :3..2 ~ 15.0 %. LD5: 5..0 ~ 13.0 % ,respectively.

Conclusions

We have devised an improved capillary electrophoresis method for fractionating LD isoenzymes using parking time and have obtained its utility in resolving the isoenzymes in serum. In conclusion, proposed serum LD isoenzyme analysis by capillary electrophoresis system is sensitive, precise, easy for clinical use.

REFERENCES

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