Journal of Chromatography, 378 (1986) 450—455
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3107

Note

Measurement of lactate dehydrogenase isoenzyme 1/isoenzyme 2 ratio by a batch separation method

MANCERHY P. MENON*, SHERRYL MILLER and BRYAN S. TAYLOR

Department of Chemistry, Savannah State College, Savannah, GA 31404 (U.S.A.)

(First received November 12th, 1985; revised manuscript received January 29th, 1986)

It has been well documented that the measurement of the lactate dehydrogenase (LDH) isoenzyme 1/isoenzyme 2 ratio in serum samples of patients suffering from heart problems is useful in the diagnosis of myocardial infarction [1—5]. The diagnosis is, however, based on the LDH-1/LDH-2 ratio obtained mostly from electrophoretic data [3, 6] or the results from miniature column chromatography [5, 7]. Agarose gel electrophoresis produces good separation, but the method is relatively expensive and often semi-quantitative [8]. Even improved column chromatographic techniques [9, 10] are tedious and suffer from carry-over of one fraction to the other, especially among LDH-1, LDH-2 and LDH-3 fractions.

We have developed radioimmunoassays for the individual LDH isoenzymes 1 and 2 [11], but found that both isoenzymes cross-reacted with the antisera to their counterparts if their concentration exceeded ca. 40 ng. Our ultimate objective in this work was to develop a rapid and simple procedure for the separation of LDH-1 and LDH-2 isoenzymes present in serum samples and then to quantify them using radioimmunoassays.

Morin [12] has reported a batch fractionation method for the separation of creatine kinase MM and MB isoenzymes using a macroporous strong anion exchanger (AG MP-1). We attempted to use a similar fractionation technique coupled with heat treatment of the serum samples to destroy the heat-labile LDH-5, LDH-4 and LDH-3 isoenzymes prior to the spectrophotometric measurement of LDH-1/LDH-2 ratios. This report presents the procedure we have developed and the values for LDH-1/LDH-2 ratios in ten randomly selected serum samples.

0378-4347/86/$03.50 © 1986 Elsevier Science Publishers B.V.

EXPERIMENTAL

Materials

Analytical-grade macroporous anion-exchange resin, AG MP-1, was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.), β-nicotinamide-adenine dinucleotide (NAD^+) and other chemicals from Sigma (St. Louis, MO, U.S.A.). The Titan gel agarose electrophoresis system was procured from Helena Labs. (Beaumont, TX, U.S.A.) and filter samplers from American Scientific Products (Stone Mountain, GA, U.S.A.). Human sera used for the study were obtained from Memorial Medical Center (Savannah, GA, U.S.A.).

Methods

About 25 g of the macroporous anion-exchange resin, AG MP-1, chloride form, 200—400 mesh, were mixed with 100 ml of 1 *M* sodium hydroxide to convert it into the basic form. After allowing it to sit for 10 min, the slurry was poured into a Büchner funnel and the excess of sodium hydroxide solution was drained off. The resin was then washed with 400 ml of 1 *M* sodium hydroxide and rinsed with deionized water until the effluent was slightly acidic. The filter cake was removed and suspended in 100 ml of ten-fold concentrated primary buffer (0.20 *M* Tris—HCl, pH 8) and allowed to equilibrate for 30 min. After filtration, the cake was washed several times with the primary buffer (0.02 *M* Tris—HCl, pH 8) until the pH and conductivity of the supernatant were the same as those of the primary buffer. The mixture was filtered through a Büchner funnel and the cake allowed to dry. The cake was then powdered and spread on a watch glass to be dried overnight in an air oven at 40°C. The equilibrated and dry resin was stored in a bottle. About 15 g of the powdered resin were stirred with enough volume of the primary buffer to give a suspension, 0.5 ml of which contained 90 ± 5 mg of dry resin.

The following buffers were used for the measurement of the percentage recovery of individual LDH isoenzymes 1, 2 and 3 and also for the separation of LDH-1 and LDH-2 from serum samples: buffer A (0.02 *M* Tris—HCl, pH 8.0), buffer B (0.02 *M* Tris—HCl + 0.075 *M* sodium chloride, pH 7.8), buffer C (0.02 *M* Tris—HCl + 0.15 *M* sodium chloride, pH 7.4) and buffer D (0.02 *M* Tris—HCl + 0.25 *M* sodium chloride, pH 7.0).

Recovery measurement

Human LDH-1, LDH-2 and LDH-3 isoenzymes were isolated from myocardial tissue obtained from autopsy material and purified by DEAE-cellulose anion-exchange column chromatography [11]. The purity of the isolated isoenzymes was ascertained by agarose gel electrophoresis [13]. The percentage recovery and percentage contamination of each of the isoenzymes in the LDH-2 and LDH-1 fractions were measured as follows: 0.5 ml of the resin suspension was introduced into a 10 cm × 1.5 cm tube to which 0.1 ml of the isoenzyme (activity 500 U/l) was added, followed by 0.4 ml of buffer A. The filter sampler (a one-side fritted plastic tube which exactly fitted into the test tube) was introduced and depressed to within 1 cm of the surface. The mixture was shaken for 1.5 min for the absorption of the enzyme. The sampler

was then filled with 4 ml of the primary buffer A and raised until the buffer entered the tube below the sampler. The mixture was vortexed for 15 s, the sampler was depressed until it squeezed the resin and the clear filtrate that entered into the sampler was discarded. Buffer B (2 ml) was introduced into the sampler and the plunger was raised. The mixture was vortexed for 15 s, the sampler was depressed and the filtrate was discarded as before. These two steps removed all of LDH-5 and LDH-4 and most of LDH-3 isoenzymes in the serum sample, when analyzed. The process was repeated two times with 2 ml of buffer C and the combined filtrate was collected as LDH-2 fraction. The process was repeated two more times with 2 ml of buffer D and the combined filtrate was collected as LDH-1 fraction. The activities of LDH-2 and LDH-1 fractions and those of the stock solutions of the isoenzymes were measured at 37°C (conversion of lactate to pyruvate). The sample (2 ml) and the concentrated substrate (1 ml) were used to increase the sensitivity and the precision of the measurement [10]. In the case of more concentrated stock solution of LDH isoenzymes, 0.1 ml of LDH-2 or LDH-1 was diluted to 2 ml with buffer C before the absorbance measurement. From the total activity added and that recovered from each fraction, the percentage recovery was computed.

Procedure for the measurement of LDH-1/LDH-2 ratio in sera

From our experiments it was quite clear that there was some cross-contamination between fractions LDH-3 and LDH-2 and LDH-1 and LDH-2. Similar cross-contamination has also been reported by others [9, 14] among LDH-3, LDH-2 and LDH-1 during the separation of isoenzymes by column chromatography. In order to eliminate the cross-contamination of the heat-labile isoenzyme fractions including LDH-3, Hunter et al. [14] suggested a heat treatment procedure in which the plasma samples are heated at 55°C for 20 min prior to the separation. This technique was incorporated in our batch separation method. The procedure finally adopted was the following: 1 ml of serum taken in a small test tube was heated in an incubator at 55°C for 20 min. The tube was allowed to cool to room temperature. The pre-heated serum (0.5 ml) was added to 0.5 ml of the resin suspension (90 ± 5 mg of dry resin) that was kept in the separation tube (10 cm × 1.5 cm). The filter sampler was inserted into the tube and depressed to within 1 cm of the surface. The mixture was shaken for 1.5 min. The sampler was then filled with 4 ml of the primary buffer A and it was raised until all the buffer was dispensed to the tube. The mixture was vortexed for 15 s and then allowed to settle for 5—10 min or until the supernatant was clear. If the supernatant is cloudy one may have problems with the filtration. One filter sampler should be used only once. To reduce the separation time, one may process several samples in a sequential manner. The rest of the procedure was the same as was described in the previous section. We have analyzed ten randomly chosen serum samples to measure the LDH-1/LDH-2 ratio by the batch separation method. On the average it takes only 6 min for the separation of LDH-2 and LDH-1 per sample. We have also measured the same ratios in the same sera using the electrophoretic technique for comparison with our results.

RESULTS AND DISCUSSION

The results of the recovery studies are presented in Table I. In each extraction, 0.3 ml of the buffer remained within the resin. Both LDH-2 and LDH-1 activities were corrected for this residual volume. Absorbance measurements were made at 340 nm and the activities (A) computed using the following relationship [10]:

$$A\ (\mathrm{U/l}) = [(\Delta A/\mathrm{min})V_t]/(0.001/\mathrm{min})V_s \tag{1}$$

where V_t, the total volume of each fraction, is 4.6 ml and V_s, the sample volume, is 2 ml. Although the percentages recoveries for LDH-1 and LDH-2

TABLE I

PERCENTAGE RECOVERY AND PERCENTAGE CONTAMINATION OF LDH-1 AND LDH-2 ISOENZYMES USING THE BATCH SEPARATION TECHNIQUE

The LDH-1 and LDH-2 fractions were 4 ml each.

Standard	Contamination (%)		Recovery (%)	
	LDH-1 fraction	LDH-2 fraction	LDH-1 fraction	LDH-2 fraction
LDH-1		19.7 ± 1.1	41.2 ± 5.5	
LDH-2	21.2 ± 4.1			41.7 ± 1.4
LDH-3	14.8 ± 4.0	49.1 ± 3.2		

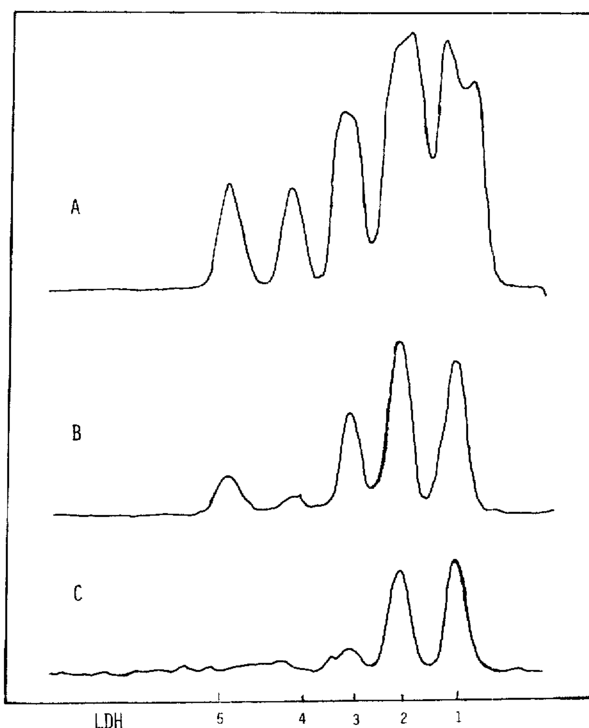

Fig. 1. Electropherograms of a typical serum sample taken before and after heat treatment procedure. (A) Myocardial standard; (B) serum before heating; (C) same serum after heating.

are almost identical, they are less than desirable. The lower percentage recovery may be attributed to the fact that the entire separation is a non-equilibrium and rapid process and the washing steps may remove some of the adsorbed enzyme. There is also evidence for the incomplete absorption of LDH-2 on the resin and a trailing trend for LDH-1 during the desorption step.

The electropherograms for a crude myocardial standard and those of a typical serum sample taken before and after the heat treatment procedure are depicted in Fig. 1. The LDH-3 in the combined fraction of LDH-3, LDH-2 and LDH-1 was found to be reduced from 22.4 to 12.2% by the heat treatment procedure. No trace of LDH-5 or LDH-4 was seen in the electropherogram of the sample after heating. However, there is a slight increase in the LDH-1/LDH-2 ratio after the heat treatment procedure. Since LDH-3 in the serum samples has been reduced considerably, the carry-over of LDH-3 to LDH-2 fraction is only minimal. The contamination between LDH-1 and LDH-2 fractions was corrected using the following simultaneous equations:

$$A_{t2} = 100(A_{m2} - A_{t1}I_1)/R_2 \quad (2)$$

$$A_{t1} = 100(A_{m1} - A_{t1}I_2)/R_1 \quad (3)$$

where A_{t2} and A_{t1} are the total and A_{m2} and A_{m1} the measured activities of LDH-2 and LDH-1 in 1 ml of serum, I_2 and I_1 (0.212 and 0.197) are the correction factors for contamination, and R_2 and R_1 (41.7 and 41.2) are the recoveries for LDH-2 and LDH-1, respectively. Using the measured values of A_{m2} and A_{m1} and the predetermined values of I_2, I_1, R_2 and R_1, the total LDH-2 and LDH-1 activities and LDH-1/LDH-2 ratio in each serum sample were computed. The LDH-1/LDH-2 ratios measured by the new batch separation procedure are presented in Table II along with those measured by the electrophoretic method. The table also includes the mean of the two measured values and also the total LDH for each serum sample measured under the same experimental conditions. It is to be pointed out that the LDH-1/LDH-2 ratios

TABLE II

MEASURED LDH-1/LDH-2 RATIOS IN TEN RANDOMLY CHOSEN SERUM SAMPLES

Sample	Total LDH activity (U/l)	LDH-1/LDH-2 ratio		$[(B-E)/B]100$*
		Batch separation	Electrophoresis	
1	320	0.959	0.856	10.7
2	465	0.958	0.709	26.0
3	440	0.645	0.795	(23.0)**
4	420	0.959	1.047	(9.2)
5	405	0.674	0.675	(0.1)
6	355	0.957	1.056	(10.3)
7	235	0.675	0.567	16.0
8	210	0.645	0.752	(16.6)
9	245	0.644	0.799	(24.0)
10	470	0.701	0.916	(30.6)

*B = LDH-1/LDH-2 ratio measured by batch separation method; E = LDH-1/LDH-2 ratio measured by electrophoresis method.

**Parentheses indicate that the percentage difference is negative.

obtained by peak integration from the electropherogram taken on different days differ from each other by more than 5%. It is, therefore, not surprising that our values for the LDH-1/LDH-2 ratios differ slightly, in most cases, from the electrophoretic values for the same samples. Previous authors have also reported that LDH-1/LDH-2 ratios based on electrophoretic data were only semiquantitative [7, 14]. The standard deviation computed from each mean value between samples was 0.076 and the coefficient of variation was 9.7%. Although the accuracy is somewhat limited owing to a slightly higher percentage of error, the method holds promise for measurement of the LDH-1/LDH-2 ratio rapidly in serum samples of patients suspected of heart ailment.

ACKNOWLEDGEMENTS

This research was supported by the Division of Research Resources of the National Institute of Health (MBRS Grant No. 5 S06 RR08144-08, Project No. 5). We are grateful to Dr. Jane Jennings for providing us with the test serum samples.

REFERENCES

1 J.A. Lott and J.M. Stang, Clin. Chem. (Winston-Salem, NC), 26 (1980) 1241–1250.
2 G.S. Wagner, C.R. Roe, L.E. Limberd, R.A. Rosaii and A.C. Wallace, Circulation, 47 (1973) 263–269.
3 C.R. Ro, Ann. Clin. Lab. Sci., 7 (1977) 201–209.
4 M.D. Cohen, J. Djordjevich and S. Jacobsen, Med. Clin. North Am., 50 (1966) 193–209.
5 G. Vasudevan, D.W. Mercer and M.A. Varat, Circulation, 57 (1978) 1055–1057.
6 F.Y. Leung and A.R. Henderson, Clin. Chem. (Winston-Salem, N.C.), 25 (1979) 209–211.
7 D.W. Mercer, Clin. Chem. (Winston-Salem, NC), 21 (1975) 1102–1106.
8 P.H. Springell and T.A. Lynch, Anal. Biochem., 74 (1976) 251–253.
9 Mei-Yung Hsu, M.M. Kohler, L. Barolla and R.J.L. Bondar, Clin. Chem. (Winston-Salem, NC), 25 (1979) 1453–1457.
10 M.P. Menon, G. Anderson and G.K. Nambiar, Anal. Chem., 55 (1983) 1385–1390.
11 M.P. Menon, G.K. Nambiar and R.M.G. Nair, J. Radioanal. Nucl. Chem., 92 (1985) 123–132.
12 L.G. Morin, Clin. Chem., 22 (1976) 92–97.
13 T.L. Gollas (Editor), Electrophoresis and Laboratory Procedures, Proc. No. 6, Helena Labs., Beaumont, TX, 1984, p. 4-1.
14 I. Hunter, B. Attock and T. Palmer, Clin. Chim. Acta, 135 (1983) 73–82.