Kawasaki Med. J. Vol. 3: No. 4, 287-294, 1977

SERUM LDH_5 ISOZYME DETERMINATION BY A COLUMN METHOD (" CHEMIPHAR " TEST)

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Accepted for Publication on Aug. 2, 1977

Abstract

A LDH isozyme assay by column chromatography (" Chemiphar " kit) was compared with the conventional electrophoretic method, and its usefulness as a routine procedure for estimation of LDH_5 isozyme was recognized. It was simple in manipulation, requires short time for getting the result and gave satisfactorily precise estimations of LDH_5 (coefficient of variation was 7.3 %).

It showed a good correlation with the agar gel electrophoretic method (correlation coefficient, r=0.93), but gave values slightly higher than those obtained by electrophoresis on account of contamination of the LDH_5 effluent from the column with minute amount of LDH_4 isozyme. However, this is insignificant for exact interpretation of the estimations. In this method the coloration for estimation of LDH isozyme was successfully stabilized by use of Merudola Blue as hydrogen acceptor instead of the conventional Phenazine Methosulfate.

This column (" Chemiphar ") method is recommended for screening the sera with abnormal LDH_5 isozyme activity.

INTRODUCTION

Among various methods of isozyme assay, electrophoresis on agar gel, cellulose acetate membrane, and starch gel have been widely used for identification. Enzymes such as LDH, Alk-P, CPK, LAP, Amylase, and γ-GTP have been examined for their isozymes electrophoretically. Electrophoresis has an advantage of easy recognition of isozymes with the naked eyes, but suffers from disadvantages of considerably complicated manipulation and long time required for assay. Recently, a new method for LDH isozyme assay using column chromatography was developed to eliminate the disadvantage inherent in electrophoresis in the laboratory of the " Nippon Chemiphar ". This is said to be characterized

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by a small amount of sample and short time necessary for assay. Therefore, an investigation was undertaken to evaluate this column method as a LDH isozyme estimation in comparison with the conventional agar gel electrophoresis method.

MATERIALS AND METHODS

1. Principle

On the "Chemiphar" DEAE cellulose* an aliquot of 0.05 ml of fresh serum is gently placed with a Sanz's pipet, and allowed to stand for 30-60 seconds until it is absorbed completely. Myocardial LDH ($LDH_{1,2}$) is absorbed to DEAE cellulose, but hepatic LDH (LDH_5) remains free, and this is eluted easily by addition of 5 mM phosphate buffer (pH 8.0) to the column. Hepatic LDH can, therefore, be estimated by the LDH activity assay of the effluent. The activity of myocardial LDH is obtained by subtraction of myocardial LDH activity from the estimation of total LDH activity of the untreated serum which is carried out independently.

2. Measurement of LDH activity

The effluent from the column was assayed for LDH activity in accordance with the instructions described in the manual attached to the "Chemiphar" kit. The color reagent employs Merudola Blue as its main component in the place of Phenazine Methosulfate which is used as hydrogen acceptor in the conventional methods.[1~2)]

	Sample	Sample
Effluent	Total volume 0.35 ml	———
Buffer for elution	———	0.35 ml
Substrate reagent	0.25 ml	0.25 ml
Color reagent	0.25 ml	0.25 ml
Shaking		
Enzyme reaction at 37°C for 15 min.		
Reagent for stopping enzymatic reaction	3.0 ml	3.0 ml
At the end of incubation at 37°C for 15 min, absorbance of the sample solution was measured at 500 nm, adjusting zero point with the blank solution.		

* Obtainable from the Nippon Chemiphar Co., LTD, 2-2-3 Iwamoto-cho, Chiyodaku, Tokyo.

3. Column procedure

A cap attached to the top of the "Chemiphar" column is removed. The column is mounted on a column stand equipped with an adaptor funnel for introducing buffer solution, and allowed to stand till excess buffer in the column disappears (About 5 min. are required). The column thus prepared is ready for measurement.

4. Agar gel electrophoresis for LDH isozymes

To prepare an agar gel plate a sufficient amount of purified agar is dissolved in veronal buffer solution (pH 8.4, $\mu=0.04$) by heating to get a final concentration of 0.8 g/dl. Electrophoresis is conducted at a constant current of 5 mA/cm for about 60 min. by cooling in the layer of chilled petroleum ether. After electrophoresis, the agar gel plate is treated with the color reagent containing substrates for enzyme reaction at 37°C for 90 min. The reaction is stopped and the agar plate is subjected to automatic densitography after washing with distilled water and drying.

POINTS TO BE INVESTIGATED

1. Precision

Thirty-six samples of normal and abnormal sera were assayed in duplicate.

Examination of a deca-plicated measurements was made with a serum sample obtained from a patient with chronic hepatitis by caliculation of the average, mean value, the standard deviation, and the coefficient of variation.

2. Dependence of column adsorption on the sample volume.

Degree of LDH adsorption to the DEAE cellulose column was examined with various volumes of serum (50 μl, 150 μl, 200 μl, and 300 μl) to estimate the adsorption limit.

3. Change in absorbance of the colored solution with elapse of time.

Absorbance of the colored solutions at 500 nm was measured in a Shimadzu Model UV-200 spectrophotometer with intervals after making color reaction with serum samples obtained from the patients with various diseases as well as with the standard solutions.

4. Absorption curve

The absorption curves of the colored solutions were traced by a Shimadzu Model UV-200 spectrophotometer.

5. Electrophoretic isozyme patterns of the effluent

Ten serum samples from the patients with various diseases which

had been examined for their isozyme patterns of the effluents from the column were studied by agar gel electrophoresis.

6. Comparison of the column method with the agar gel electrophoretic procedure.

The column and the agar gel electrophoretic methods were compared with each other with respect to LDH_5 activity. The enzyme activity obtained by the agar gel electrophoretic method was expressed in terms of the international unit (mIU/ml) by dividing the Wroblewski units by 2.07.

RESULTS AND DISCUSSION

The column method for LDH_5 isozyme estimation is easy in procedure. The disagreement between the duplicate estimations of the same samples was within the range of 0.5–14.6 mIU/ml.

The deca-plicated estimations of a sample of mean LDH_5 activity of 27.6 mIU/ml gave a variance with standard deviation of 2.02 and coefficient of variation of 7.3 %. The precision is therefore fairly satisfactory, although this is 2.5 times as large as the CV for ordinary determination of serum total LDH activity.

The adsorption strength of DEAE cellulose which retains LDH_1–LDH_4 isozymes to the column was examined with increasing amounts (starting from 50 μl) of sample serum applied to the column and use of correspondingly increased volumes of elution buffer. Application of serum in amount beyond 200 μl caused the appearance of LDH isozymes other than LDH_5 in the effluents (Figure 1). When serum sample is loaded as much as 300 μl, the major portion of LDH isozymes passed through the column without being adsorbed to DEAE cellulose. It is accordingly thought that less than 150 μl of serum is recommended as an adequate amount of specimen. Volume of 50–150 μl will be the most appropriate.

Absorbance of the colored solution rose by about 2.5 % in 1 hr after coloration, whereas by less than 1.1 % in 1 hr when the solution was colored to full extent by incubating at 37°C for 15 min. The latter procedure (incubation) is satisfactorily precise as a routine quantitative analysis.

The absorption spectrum of the colored solution showed an absorption peak at 500 nm (Figure 2).

The result of comparison of the column method with the agar gel electrophoresis method with respect to isozyme and the isozyme patterns seen in the effluent from the column are worthy of special mention.

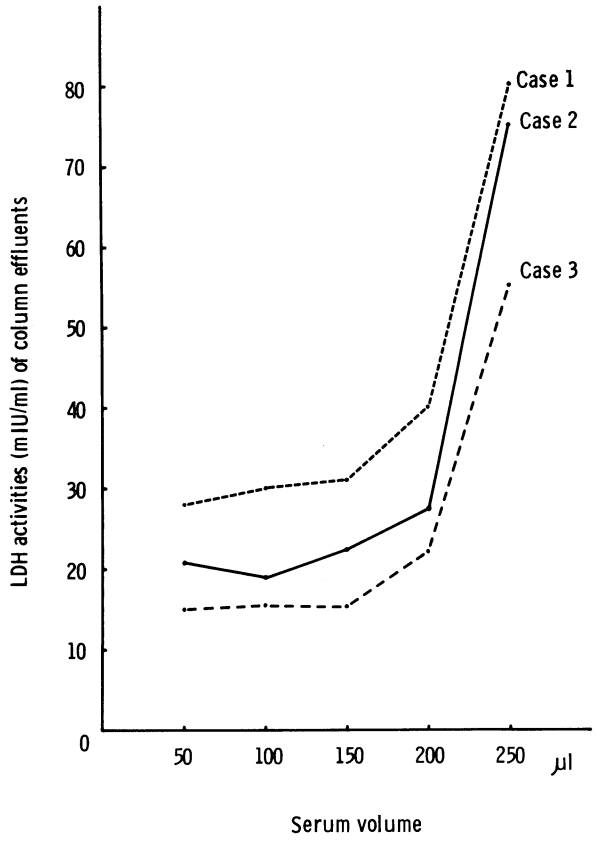

Fig. 1. Relationship between serum volume applied on the column and LDH activities passed through the column.

The correlation between these two methods was excellent: the coefficient of correlation was 0.93 but the regression line was $y=1.09x+2.05$, indicating that the column method tended to give estimations of DDH_5 activity slightly higher than those obtained by the agar gel electrophoresis method.

Examination of the isozymes contained in the effluents by agar gel electrophoresis disclosed the presence of faint band of LDH_4 in addition to the distinct isozyme band of LDH_5 (Figure 3). Tendency toward higher estimations of LDH_5 activity by the column method in comparison with those obtained by electrophoretic procedure will, therefore, be accounted for by the slightest contamination of the effluents with the trace amount of LDH_4.

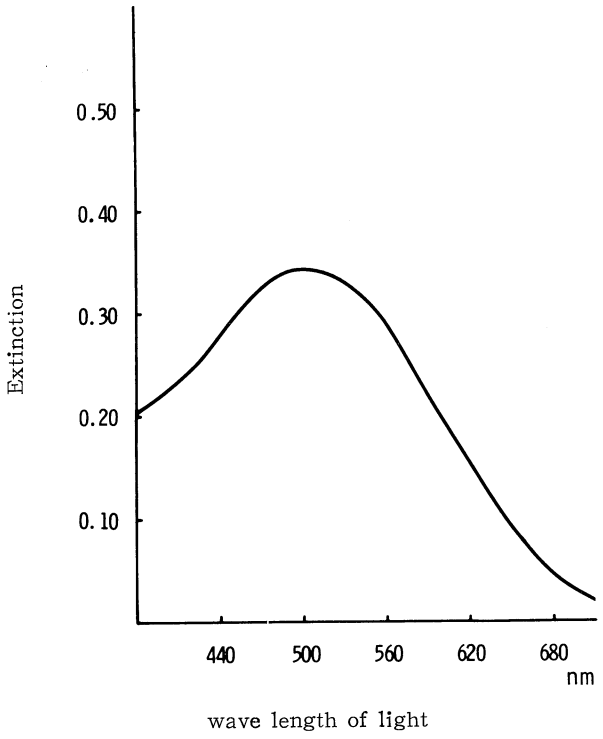

Fig. 2. Absorption curve of colored solution

The most important point to which attention should be paid lies in the manipulation for the application of the sample serum to the top of the column. Application of 50 μl of serum is recommened in the standard procedure, but as much as 150 μl may be applied without invalidation of the estimation.

Since Merudola Blue was used for the assay of LDH activity as an intermediate hydrogen acceptor instead of Phenzine Methosulfate (PMS), the absorbance of the thus colored solution did not undergo significant change by exposure to light. In addition, the color reagents can be stored for a long time. The blue color of Merudola Blue, however, may cause higher values in activity measurements.

It should be noted that a markedly higher value of isozyme assay may occasionally be obtained by the column method unless non-jaundiced and non-hemolyzed sera are used as samples. (Figure 4)

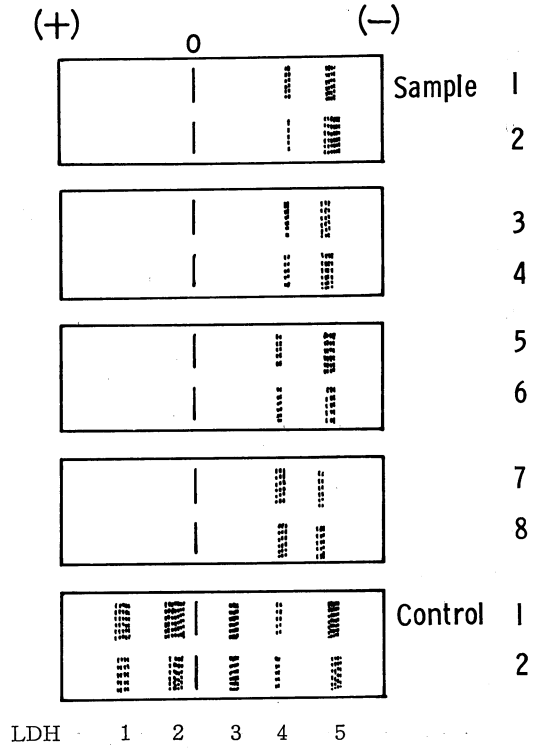

Fig. 3. LDH isozyme patterns of the column effluent (Sample 1 to 8) on agar gel electrophoresis

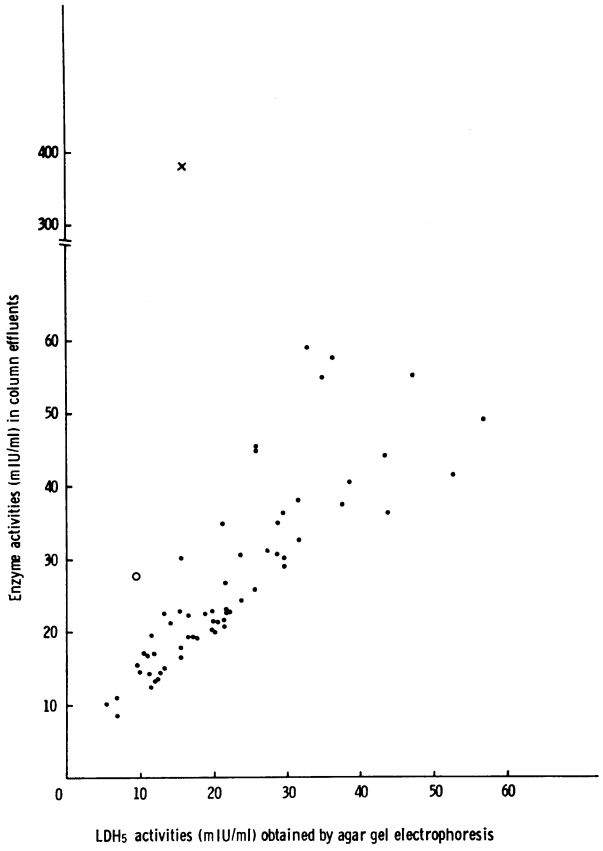

Fig. 4. Comparison of LDH_5 activities obtained on identical samples by column effluents and by agar gel electrophoresis.
×: indicating icteric serum
○: indicating hemolyzed serum

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