Journal of Chromatography, 134 (1977) 83–90

CHROM. 9747

ENZYME-SELECTIVE DETECTOR SYSTEMS FOR HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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(Received October 15th, 1976)

SUMMARY

This paper describes the performance of absorbance and fluorescence detectors in two configurations of post-column reactors for selectively detecting and quantitating isoenzymes eluted from a high-pressure liquid chromatography column. Superb resolution of the isoenzymes of lactic dehydrogenase is illustrated by the use of new ion-exchange materials.

INTRODUCTION

In earlier papers[1,2] we have described separation of isoenzymes by high-pressure liquid chromatography (HPLC). These papers describe the conditions and materials for separation. Chromatograms were obtained by fraction collection with subsequent manual or automated determinations of the enzyme activity in each fraction. Separations were made in about 30 min, however, many hours were required to produce a chromatogram. Therefore, it was necessary to develop an on-stream detector system to realize the benefits of the small separation times. In this paper, several detection schemes will be discussed and evaluated. Regnier and co-workers[3–6] have also described detector systems, separation materials, and separations of high molecular weight proteins and enzymes.

Isoenzymes are different proteins which catalyze the same reaction. These separate proteins may differ in molecular weight, size, and/or charge. Isoenzymes have classically been separated and measured by electrophoresis and column techniques using compressible materials such as Sephadex, BioGel, etc. Electrophoresis suffers primarily by being at best semi-quantitative and the compressible column supports require long times for separations. With the advent of incompressible ion-exchange supports such as Corning DEAE-Glycophase and Vydac (The Separations

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Group, Hesperia, Calif., U.S.A.), it has become possible to separate isoenzymes with high-pressure techniques. The remaining problem, that of detection, has only been recently approached[5].

Since isoenzymes are proteins, they can be detected at 280 nm, but this is general for any protein and as such cannot be used for selectively detecting enzymes. Since enzymes are generally assayed by measuring the rate of the reaction which they catalyze, a post-column reactor system must be used to quantitate the amount (activity) of the enzyme; specificity is obtained because enzymes are generally highly specific to a given substrate (reactant). The detection system described herein, is specific to the nicotinamide adenine dinucleotide (NAD) substrate (co-factor). This system was chosen since it provides for a general detection system and because most enzyme-catalyzed reactions can be coupled to this redox reagent. In this paper, we have chosen the lactate dehydrogenase (LD) (E.C. 1.1.1.27) isoenzyme system because it has been extremely well characterized[7,8]. LD catalyzes the following reaction:

$$\text{lactate} + \text{NAD} \overset{\text{LD}}{\rightleftarrows} \text{pyruvate} + \text{NADH}$$

NADH is the reduced form of NAD and it absorbs strongly and is excited strongly at 340 nm. (The possibility of electrochemically oxidizing NADH is also present).

The detection strategy is to mix the reagents (lactate, NAD, buffer) with the analytical column effluent. As will be discussed, the problem of correcting for blanks is serious when serum, urine, or tissue samples are used. Since a rate must be measured in-stream, the absorbance or fluorescence must be measured at two points in time or at two different temperatures[9]. The latter is illustrated in Fig. 1 and has the advantage of simplicity since blank correction is automatic and the difference in absorbance or fluorescence readings is directly proportional to the instantaneous activity. Serious problems, however, arise in maintaining identical flow-rates in the split streams owing to differential viscosity problems as result of the NaCl gradient. Therefore, it will be necessary to use the in-line scheme of Fig. 2.

In this approach a blank is read at detector 1 and is subsequently subtracted from the readings at detector 2. Since the pumps operate at constant flow-rate, the delay coil produces a constant delay time, Δt. The difference in absorbance or fluorescence, ΔA and ΔF, respectively then provides a measure of the activity, Act (measured in international units per liter). The relationship is:

$$Act = k\Delta A/\Delta t = k'\Delta F/\Delta t$$

The system is calibrated by injecting a standard into the detector system by the loop valve situated at the end of the column (Fig. 1).

This approach, however, also presents problems which arise from the delay time, Δt, and from band spreading. It is therefore necessary to convolute a matrix of detector 1 readings before subtraction from detector 2 readings. A computer program to perform this operation is currently being developed and will be reported later. Therefore, the work described herein was done with a single detector and with "pure" samples which did not require large blank corrections.

This paper describes the experimental results of the separation, resolution, HETP, etc., and compares the various detector types described above.

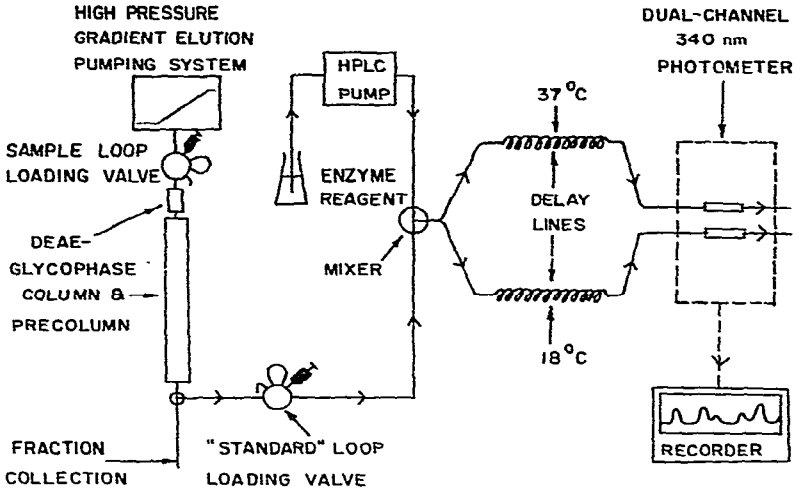

Fig. 1. Flow diagram of the HPLC system with a parallel stream reaction detector. This system affords a directly measurable signal, $A_{upper} - A_{lower}$, related to the amount of enzyme eluting from the column.

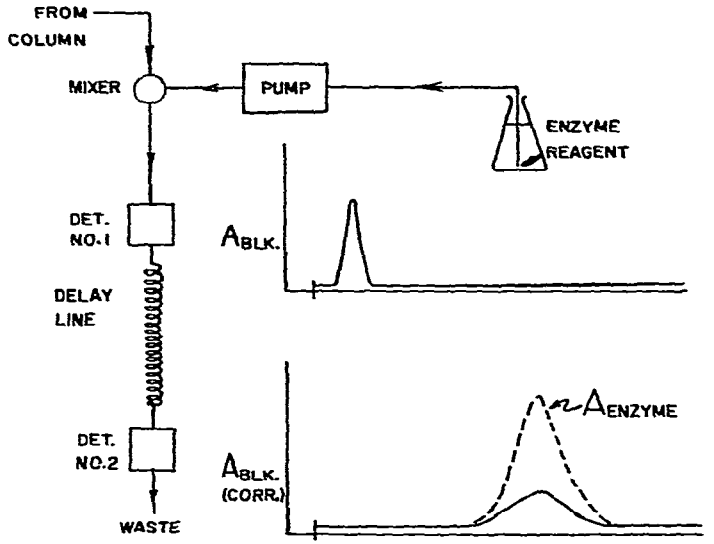

Fig. 2. Enzyme reaction detector using single stream sequential arrangement. The blank (A_{BLK}) is obtained at detector no. 1 and must be mathematically shifted in time and broadened to reflect the band spreading in the coil. The resultant blank (A_{BLK}(corr.)) is subtracted from the detector no. 2 response to obtain the response due to the enzyme catalyzed reaction. Detector 1 is one side of a dual 340-nm absorbance detector. Detector 2 is in two sections, the first of which is the second side of the dual 340-nm absorbance detector. The second section of detector 2 is an Aminco flow fluorometer set up for NADH fluorescence (340 nm excitation, 457 nm emission).

EXPERIMENTAL

The high-pressure, gradient-elution pumping system shown in Fig. 1 was obtained from Spectra-Physics (Santa Clara, Calif., U.S.A.; two Model 740 pumps and controllers, a Model 744 solvent programmer, and a Model 714 pressure monitor). Sample injection was made using a Model LIB syringe-injection port (Whatman, Clifton, N.J., U.S.A.) and, later, by a Model 70-10 six-port, sample-loop valve (Rheodyne, Berkeley, Calif., U.S.A.).

Column tubing, end fittings, frits, and connection tubing were obtained from Whatman (Cat. Nos. LA 316, LP 315, LA 225, and LA 301, respectively). The packing was a 5–10-μm particle size ion-exchanger (Corning Medical, Medfield, Mass., U.S.A.; DEAE-Glycophase/CPG-250). Columns were packed with a Model 705 Stirred-Slurry Apparatus (Micromeritics, Norcross, Ga., U.S.A.) using isopropanol as the solvent. Following packing, the isopropanol was displaced by purging with several hundred milliliters of distilled water. Pre-columns, 5 cm in length, were used also on occasion. These were assembled in the same manner as the analytical columns, but dry packed with 37–74-μm particle size Corning DEAE-Glycophase/CPG-550.

The primary eluent was a 20-mmole/l tris(hydroxymethyl)aminomethane (Tris, "Trizma base", reagent grade; Sigma, St. Louis, Mo., U.S.A.) buffer adjusted to pH 7.80 with acetic acid. The secondary eluent was a 20-mmole/l Tris buffer, containing also 150 mmole/l of NaCl, adjusted to pH 7.80 with acetic acid after addition of NaCl.

The "standard" loading valve, situated at the end of the column and used to inject known volumes of pre-assayed sample for calibration of the reaction detector, was a Model 204590 six-port, sample-loop valve (DuPont, Wilmington, Del., U.S.A.).

The mixer was a combination of a Swagelok union tee (Crawford Fitting, Cleveland, Ohio, U.S.A.; Cat. No. ss-100-3) followed by a 3-cm tube (about 3/32 in. I.D.) filled with 3/32-in. ball bearings. The delay line(s) was a 60-ft. length(s) of 0.020 in. I.D., 1/16 in. O.D., stainless-steel tube.

The enzyme reagent was made by mixing 135 g of 2-amino-2-methyl-1-propanol, 22.5 ml of a 60% sodium lactate solution (Pfanstiehl, Waukegan, Ill., U.S.A.), and 4.5 g of β-nicotinamide adenine dinucleotide (β-NAD; Sigma, Cat. No. N-7524), adjusting to pH 9.0 with HCl and diluting to 1.0 l. This reagent was delivered to the mixer under constant flow by a Model 712-31 Solvent Delivery System (Laboratory Data Control, Riviera Beach, Fla., U.S.A.). A nominal 7-μm pore size filter (Nupro, Cleveland, Ohio, U.S.A.; Cat. No. ss-2F-7) was placed between this pump and the reagent-containing flask.

A Waters Model 440 dual-channel absorbance detector, fitted with two 340-nm conversion kits (Waters Assoc., Milford, Mass., U.S.A.), was used for monitoring the absorbance as shown in Fig. 2. A third detector, a filter fluorometer, was placed in series immediately after the second absorbance detector shown in Fig. 2 so as to enable some comparisons of on-line fluorescence *versus* absorbance to be made. This detector was an Aminco Model J4-7461 Fluorometric Analyzer System (American Instrument, Silver Spring, Md., U.S.A.) containing a General Electric No. F4TF/BL ultraviolet (UV) source, a Corning 7-60 primary filter, a Wratten 2A secondary filter, and an 18-μl sample-volume flow cell (Aminco; Cat. No. J4-7476).

LD isoenzymes (A_4, 450 U/mg, Cat. No. 15382; A_3B, 100 U/mg, Cat. No.

15381; A_2B_2, 200 U/mg, Cat. No. 15380; AB_3, 200 U/mg, Cat. No. 15379; B_4, 360 U/mg, Cat. No. 15378) were obtained commercially as suspensions in ammonium sulfate solutions (Boehringer Mannheim, New York, U.S.A.). These suspensions were diluted with primary eluent (above) and refrigerated at 4°. These solutions and mixtures thereof were used to establish optimum chromatographic conditions for LD isoenzyme separations.

RESULTS AND DISCUSSION

Most of the samples were mixtures of commercially available LD isoenzymes. In the different mixtures the activity of the individual isoenzymes and the total activity were varied over a reasonably broad range and included values typical of patient sera. Samples also included selected patient sera obtained from the U.W. Hospital Clinical Chemistry Laboratory. These sera were chosen for their high values as assayed by normal laboratory procedures. Various sample volumes and detector sensitivity settings were employed. Typical results for synthetic mixtures are shown in Figs. 3, 4 and 5. Fig. 6 is a manually corrected patient serum.

The differences between the results shown are explained by differences in the analytical columns used during this work (compare Figs. 3 and 4) by the inclusion or deletion of a pre-column*, by the impending failure of one of the pumps in the

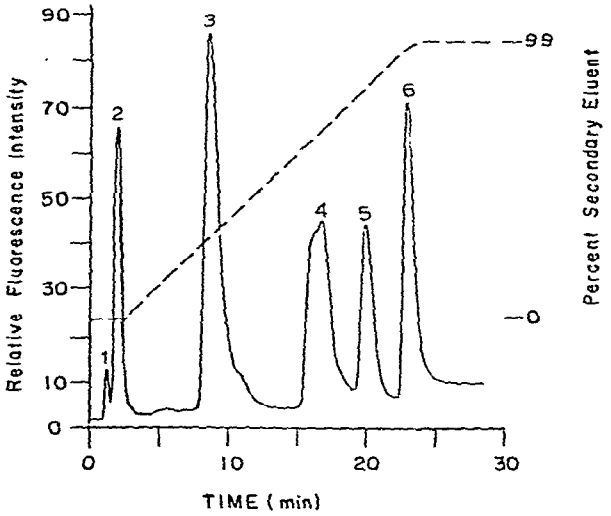

Fig. 3. Chromatographic separation of LD isoenzyme mixture with on-line reaction and detection, output of fluorescence detection section of detector 2 of Fig. 2. Syringe Injection. 6 cm × 4.6 mm I.D. pre-column. 10 cm × 4.6 mm I.D. analytical column. Eluent flow-rate, 2.0 ml/min; enzyme reagent flow-rate, 1.0 ml/min. Aminco Multiplier setting of 3. The 10-μl sample contained: 1.4×10^3 U/l A_4, 2.5×10^3 U/l A_3B, 6.2×10^2 U/l A_2B_2, 1.2×10^3 U/l AB_3, and 1.1×10^3 U/l B_4. Peak 1: artifact due to stoppage of column flow during sample injection; peak 2 = A_4; peak 3 = A_3B; peak 4 = A_2B_2; peak 5 = AB_3; peak 6 = B_4.

* The inclusion of a pre-column, packed with larger diameter particles than the analytical column causes some extra band spreading and prohibited the resolution of the A_2B_2 band into its three separate peaks (compare Figs. 3 and 4).

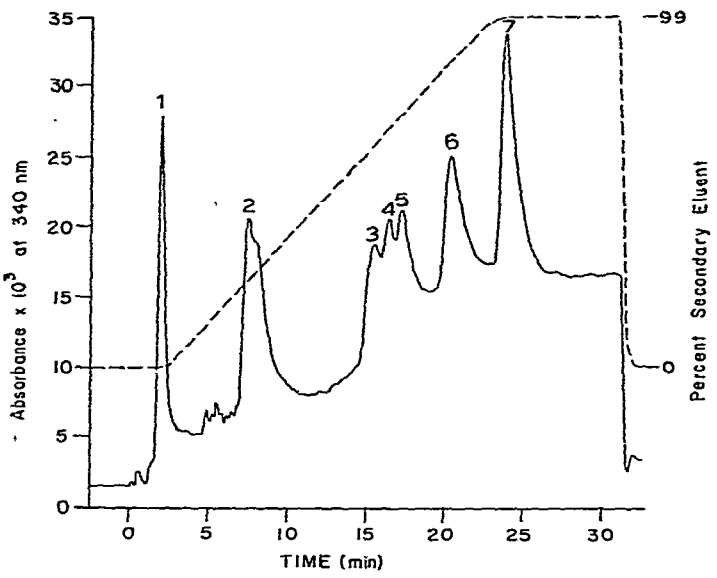

Fig. 4. Chromatographic separation of LD isoenzyme mixture with on-line reaction and detection, output of absorbance detection section of detector 2 of Fig. 2. Loop-valve injection, no pre-column. Analytical column, 20 cm × 4.6 mm I.D.; eluent flow-rate, 2.0 ml/min; enzyme reagent flow-rate, 1.0 ml/min. The 20-μl sample contained: 3.6×10^2 U/l A_4, 4.0×10^2 U/l A_3B, 4.0×10^2 U/l A_2B_2, 4.0×10^2 U/l AB_3, and 4.2×10^2 U/l B_4. Peak 1 = A_4; peak 2 = A_3B; peaks 3–5 = A_2B_2; peak 6 = AB_3; peak 7 = B_4.

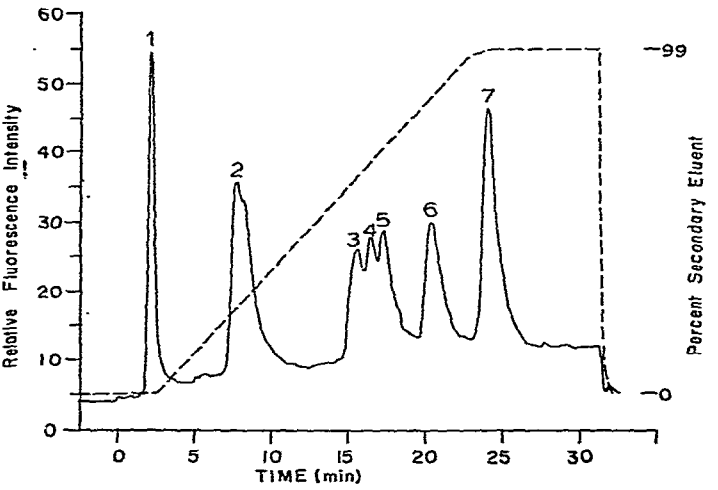

Fig. 5. Chromatographic separation of LD isoenzyme mixture with on-line reaction and detection. Same chromatographic run as shown in Fig. 4, except this is output of fluorescence detection section of detector 2 of Fig. 2. Aminco multiplier setting of 3. Peak numbers as in Fig. 4.

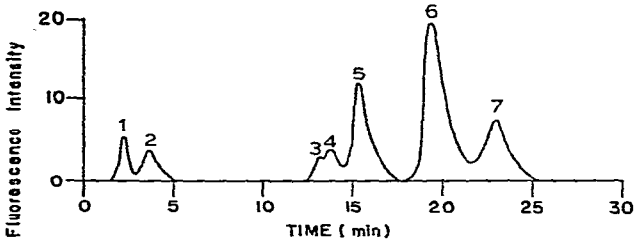

Fig. 6. Chromatographic separation of LD isoenzymes in a human serum sample. Output of fluorescence detection section of detector 2 of Fig. 2, roughly corrected for blank detector response. Loop-valve injection. No pre-column; analytical column, 20 cm × 4.6 mm I.D. Eluent flow-rate, 2.0 ml/min; Enzyme reagent flow-rate, 1.0 ml/min. Aminco multiplier Setting of 3. NaCl gradient same as shown in Figs. 3–5. The 20-μl sample contained a total serum LD level = 729 U/l. Peak 1 = A_4; peak 2 = unknown, not A_3B; peaks 3–5 = A_2B_2; peak 6 = AB_3; peak 7 = B_4.

solvent delivery system (note increased baseline drift in Fig. 4 *vs.* Fig. 3), and by differences in sample volume and activity.

In the synthetic mixtures LD3 is usually seen as three nearly equal peaks. The resolution of the major peaks is nearly to baseline in all cases.

The separation demonstrated by the elution patterns of Figs. 3–5 are comparable in resolution to earlier results from this laboratory[2], but show improved separation time. Since relatively small columns and low flow-rates have thus far been used and since the pressure required for these operating conditions (<2000 p.s.i.) has been well below the system maximum, there remains a considerable range of operating conditions in which to improve both resolution and separation time. Our calculation of HETP for the several peaks under various conditions indicates that flow-rate can be increased several fold with only a minor change in resolution. Thus, for the columns used here, separation times of about 5 min are accessible with resolution comparable to that shown.

Two factors seem most important in the evaluation of the detector system: its selectivity for the enzyme of interest and its sensitivity. Other factors of less importance involve band spreading, delay time, convenience, cost of operation, etc.

In the results shown in Figs. 3–5, an adequate response has been shown for two different detectors. The total LD activity in the injected samples was *ca.* 70 mU and 30 mU, respectively, indicating that the isoenzymes in serum samples with normal LD activity could be detected using injected sample volumes of about 100 μl.

The overall sensitivity is further determined by the reaction temperature and the delay time between detectors (or between reagent mixing and detection). The reaction time is determined by the coil volume and total flow-rate and was about 1.3 min for the cases shown. Increased reaction time would increase the peak to background ratio. However, this improvement is limited by the reagent concentration since substrate depletion would eventually cause non-linear response. Furthermore, longer delay coils would cause sufficient spreading of the eluted band eventually to negate any improvement in sensitivity.

We would rate the sensitivity of the detector system as adequate. The specificity of the detector system is acceptable but seems to be the limiting factor in its current operation. This should be much improved when our computer-automated blanking

procedure is implemented. Without a blank correction, flow-rate changes, reagent impurities, and other factors cause a rather marked change in the detector baseline*. Such effects can be compensated by blank correction using the output of detector 1 and even a crude correction, done manually, shows the improvement to the system specificity (Fig. 6).

ACKNOWLEDGEMENTS

The authors wish to thank the Corning Glass Co. for the gift of their experimental column support materials. The many helpful discussions with Professor Regnier's group are gratefully acknowledged. This work was funded by grants GM 21752 from the National Institutes of Health and GP-43624X from the National Science Foundation.

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* During the course of these experiments it became increasingly difficult to maintain the calibration of the dual pumps in the solvent delivery system. This caused a variation in the total column flow-rate during a gradient run and is evidenced as an apparent drift in the detector response. In the experiment shown in Fig. 6, this "apparent drift" has been compensated by a crude subtraction technique. In the experiments shown in Figs. 3–5, no compensation has been made and the "apparent drift" is evident.