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REVIEW

SEPARATION AND MEASUREMENT OF ISOENZYMES AND OTHER PROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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1. INTRODUCTION

Isoenzymes are different proteins that catalyze the same reaction. They vary in chemical and physical properties, several of which are the basis for analyses, e.g., net charge, conformation, size, heat lability, substrate and inhibitor affinity, and immunospecificity.

Isoenzymes are synthesized in vivo under genetic control [1]; hence different isoenzymes are more optimized for different metabolic functions. For example, lactate dehydrogenase found in myocardial tissue is geared toward aerobic oxidation whereas the isoenzymes in muscle and liver tissue are optimized for anaerobic reduction. This provides for some degree of organ and/or organelle specificity which explains the clinical interest in determining isoenzyme content as opposed to total enzyme activity. Although isoenzymes

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are measured in tissues and fluids other than serum, most measurements are made on the latter specimen. There are two basic groups of serum enzymes [2]: serum-specific enzymes that have a function in serum, e.g., ceruloplasmin, pseudocholinesterase, lipoprotein lipase, and coagulation enzymes; serum-nonspecific enzymes have no function in serum and are present as the result of excretion, e.g., acid and alkaline phosphatase (ACP and AP and α-amylase), or cellular damage, e.g., lactate dehydrogenase (LD), creatine kinase (CK), and aspartate and alanine aminotransferases (AST and ALT). The serum-nonspecific isoenzymes are of principal interest because their organ specificity aids in ascertaining excretory function or cellular damage.

There are two isoenzyme families that are routinely profiled in the clinical laboratory: LD (EC 1.1.1.27) and CK (EC 2.7.3.2). Therefore, most research on high-performance liquid chromatographic (HPLC) separations has been devoted to these. Fractionation of LD and CK plays a major role in the diagnosis and monitoring of myocardial infarction (MI) because both enzymes have molecular forms selective to cardiac tissue. LD-1 and LD-2 are characteristically elevated in cardiac tissue damage while LD-4 and LD-5 are predominant in muscular or hepatic damage. LD-1 and LD-2 elevation occurs 12 to 24 hours after the onset of severe chest pain.

CK is expressed in three dimeric isoenzymes composed of the M and B subunits; MM, predominant in skeletal muscle; BB, in brain; and MB, in heart. MB and BB are detectable in serum of certain disease states producing tissue damage. CK-BB appears in the serum of patients with brain tissue injury [3, 4], renal damage [5], and certain carcinomas (e.g. gastrointestinal and prostatic [6, 7]. The MB isoenzyme appears in the serum of diagnosed Duchenne's muscular dystrophy [8]; its activity rises sharply within 4—6 h after MI, peaks within 12—24 h, and generally returns to normal levels within 48 h [9]. Wagner et al. [10] report that in a comparison of four clinical measurements for sensitivity and specificity in MI detection, CK-MB assay is 99% specific with 0% false negatives. Thus, the specificity and sensitivity and the rapidity of CK-MB elevation coupled with the prolonged LD-1:LD-2 elevation provide a two-part analysis that confirms MI with almost 100% predictive value. Therefore, the high interest in these isoenzymes is readily apparent.

The most common technique for the separation and measurement of isoenzyme activity is electrophoresis. A small sample, ca. 10 μl, is streaked on the medium and separation is made in an electrical field by the differential migration of the fractions. This separation is based principally on the net charge of the isoenzymes. After the separation step (ca. 1—3 h) the medium is incubated with substrate (e.g. lactate and NAD for LD) to develop products to stain the location of the isoenzyme. The intensity of the stain is proportional to the activity of the isoenzyme. The intensity is either estimated by visualization or by absorbance or fluorescent densitometric scanning. The method is semiquantative, labor intensive, slow (ca. 2—4 h), but offers the advantage of high throughput because multiple samples (about 20) can be processed simultaneously.

Differential techniques have been based on the different physical properties of isoenzymes. Differential heat lability [11] has been used to estimate the

heart isoenzymes of LD which are stable after heating serum at 65°C for 30 min. Differential substrate specificity has been used [12] to estimate the heart fraction of LD by use of α-hydroxybutyrate rather than L-lactate as the substrate. Differential inhibition has been used to estimate the LD heart isoenzyme by substrate inhibition with pyruvate [13], prostatic acid phosphatase by inhibition with tartrate, and alkaline phosphatase fractionation by inhibition with urea and L-phenylalanine. These differential methods are generally characterized by poor accuracy and poor specificity and have largely been replaced by other techniques. In recent years, immunoassays have become popular for the assay of specific isoenzymes, notably CK-MB [14] and prostatic acid phosphatase [15]. These methods can be automated, but one cannot always be certain that the antibodies are specific, e.g., that the antibody to CK-MB will not crossreact with CK-BB. The methods also measure mass as opposed to activity; therefore, both active and inactive enzyme proteins are measured. The merits of this technique are still under debate.

Recently, disposable minicolumn ion-exchange techniques have been developed mainly to measure the heart fractions of CK and LD. Mercer [16] originally described a small anion-exchange column (DEAE-Sephadex) made with a Pasteur pipet. The column was eluted with a series of buffers to obtain the MM, MB, and BB fractions of CK. Later, Mercer and Varrat [17] described a similar technique for the simultaneous separation of CK-MB and LD-1 and LD-2. The activity of the eluates was determined by subsequent manual assay. Similar techniques have been automated on the duPont aca, an automated clinical analyzer that discretely determines many clinical analytes with individual reagent packs. The liver fraction of LD was determined by passing the sample through a small anion-exchange column in the head of the analysis pack. The LD-5 (liver) fraction was not retained because it is cationic under the conditions used. Recently, a similar ion-exchange technique was implemented for CK-MB on the aca.

These chromatographic methods are generally more sensitive, quantitative, and faster than the other methods discussed above with the exception of the very high sensitivity of radioimmunoassay. However, manual methods are extremely labor intensive and because only a given eluate fraction is subsequently measured, one cannot be sure of the resolution of one fraction from another. Many of these methods are commercially available in the form of kits.

Classical liquid chromatographic (LC) separations are in routine use in research laboratories for purification and preparative-scale separation of enzymes; however, the slow flow-rates involved have generally made these techniques unacceptable for routine analytical work.

Modern HPLC separations, although not currently in routine use, offer the advantages of speed (5—20 min depending on the isoenzyme system and desired resolution), excellent recovery (ca. 90%) and capability of automation (e.g. automatic sample injection and data processing). Continuous post-column detection of isoenzyme activity offers the advantages of immediate visual display of the profile, extreme sensitivity (< 1 I.U./l), and precise assay of activity (1—2% relative standard deviation). Accuracy is difficult to estimate owing to the lack of suitable standards for this or any other enzymatic assay. This is the current state-of-the-art; however, many technological develop-

ments were needed to achieve these goals.

We shall identify some of the key developments that were necessary to achieve separation and measurement of isoenzymes by HPLC. In 1973, Hetano [18] separated the isoenzymes of alcohol dehydrogenase (EC 1.1.1.1) by steric exclusion chromatography. Later Eltikov et al. [19] modified the surface of glass beads with γ-aminopropyltriethoxysilane to yield hydrophilic media that did not denature proteins. Without surface modification [20] using HPLC ion-exchange separation of CK isoenzymes, the most significant component, CK-MB, was denatured on the Vydac column (The Separations Group, Hesperia, CA, U.S.A.). This demonstrated in a negative fashion that column packings would be a major key in isoenzyme separation by HPLC.

Another major problem at this time plaguing HPLC separations and analyses of isoenzymes was speed. Although a separation could be completed within 20 min, many hours were required for the collection of fractions and subsequent assay of activity; thus, the need for continuous detection in the form of post-column reactors (PCR) became apparent.

In 1958, Spackman et al. [21] described one of the earliest PCRs: a reagent stream of ninhydrin was mixed with the effluent of a classical ion-exchange column for the specific detection and measurement of amino acids. In 1965, Hicks and Nalevac [22] introduced a split-stream, self-blanking, dual-detector PCR for the continuous kinetic detection and measurement of LD isoenzymes as they emerged from a DEAE-Sephadex column. This was a logical extension of earlier work generally regarded as the first reports for flow injection analysis of glucose [23] and total LD [24].

Early recognition that these two factors, appropriate column materials and PCRs, were vital in the development of rapid separation, detection, and measurements of isoenzymes by HPLC prompted intensive research in these areas. Although many recent advances in HPLC pumps and accessories have improved mechanics, we shall limit discussion to detailing the specifics of column packings, PCR development, and specific applications.

Aspects of the topics to be presented in this work have recently been reviewed by Regnier and Gooding [25] and by Bowers and Bostick [26].

Table 1 qualitatively summarizes (our judgement) the relative advantages of some existing techniques for the separation and measurement of isoenzymes. Because HPLC with PCR is relatively new and not clinically proven, certain of the advantages are projected. Because immunoassay methods measure mass rather than activity, resolution and recovery cannot be compared. Note only HPLC + PCR and scanning electrophoresis present complete isoenzyme activity patterns.

2. POST-COLUMN REACTORS

Hicks and Nalevac [22] reported the first enzymatic post-column reactor system. They used relatively large-bore plastic tubing and low-pressure peristaltic pumps to monitor the LD activity eluted from a gravity-feed ion-exchange column using large sample volumes. Such a reactor was not directly adaptable to HPLC.

In their initial work, Kudirka and co-workers [20, 27] found that collec-

TABLE 1

RELATIVE ADVANTAGES OF SELECTED ISOENZYME SEPARATION AND MEASUREMENT TECHNIQUES

Advantage	HPLC + PCR	Electrophoresis	Immunoassay	Minicolumn
Speed	++	— —	+	— —
Labor intensity	—	— —	+	— —
Automation	++	—	++	— —
Precision	++	— —	++	+
Sensitivity	++	—	++	+
Resolution	++	+	NA*	+
Recovery	+	+	NA	+
Throughput	— —	++	++	— —
Complete presentation	++	+	—	—

*NA = not applicable.

tion with discrete manual assays of fractions for enzyme activity was unacceptable and that a continuous detection system was required. Chang and co-workers [28, 29] first introduced a PCR based on HPLC pumps and associated plumbing and demonstrated the continuous detection of LD and CK isoenzymes. Later, Schroeder et al. [30] and Schlabach et al. [31] simultaneously reported continuous detection apparatus for monitoring the activity of isoenzyme eluates. The principles of these systems are shown in Fig. 1.

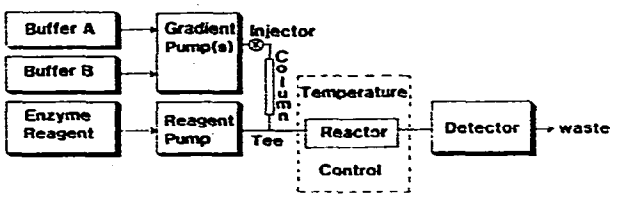


Fig. 1. Single-detector post-column reactor. See text for description.

Schlabach et al. [31] used a column (600 × 4.1 mm I.D.) for LD packed with inert glass beads for the reactor to reduce band-spreading; however, Schroeder et al. [30] obtained satisfactory resolution by using tightly wound coils of stainless-steeel tubing (1830 × 0.05 cm). Schlabach et al. [31] compared the theoretical plate behavior of capillary and packed-bed reactors (Fig. 2) and found packed-bed reactors more efficient. Huber et al. [32] also compared and discussed the theory of tubular and packed-bed reactors. They concluded that to optimize reactor design to acceptable band broadening, the efficiency of the reactor must exceed the column efficiency; therefore, for long reaction times packed-bed reactors are superior. They also observed that the dispersion of coiled tubular reactors exhibited local minima in the h (plate height) vs. u (linear flow velocity). Toren et al. [33], Hoffman and Halász [34], and earlier Horvath et al. [35] observed similar phenomena. To date, these observations have not been explained; however, this beneficial

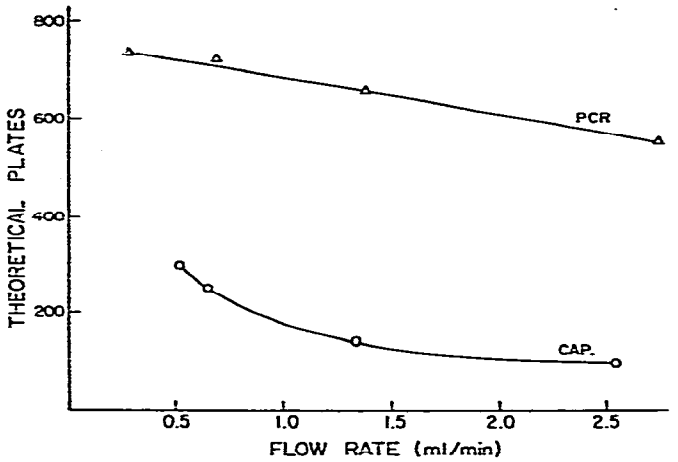


Fig. 2. Comparison of band-spreading between a capillary tubing (CAP) and a packed-column reaction vessel (PCR). The capillary tubing was a 15.25 m coil of 0.5 mm I.D. while the packed column was a 600 × 4.1 mm precision bore tube. The column was packed with Whatman glass spheres. The solute was 0.5 m*M* *p*-nitrophenol and the mobile phase was 0.01 *M* NaH_2PO_4 buffer (pH 8). Both the main pump and reagent pump were filled with 0.01 *M* NaH_2PO_4 buffer and driven at the same rate. Reprinted from ref. 31, p. 96, courtesy of the authors.

effect can be used to good advantage in PCRs. Frei et al. [36] also discuss the optimization of PCRs with respect to reaction time. The most detailed discussion and theoretical analysis of PCRs has been presented by Deelder et al. [37] who also studied segmented-flow reactors. They conclude that tubular reactors are acceptable for fast reactions ($\leqslant$ 30 sec), packed reactors should be used for intermediate reactions, and the segmented-stream reactors should be used for slow reactions.

Whereas these considerations are very important, especially for non-kinetic post-column assays, relatively little work has been done to characterize PCRs used for enzyme assays, for which the apparent resolution does not suffer severely because the detectable product increases (generally linearly) with time. Also, open tubular, helically coiled reactors in these applications do not greatly affect resolution because (1) Aris—Taylor dispersion [38] does not apply because of the beneficial effects of secondary flow [39], (2) large sample volumes, $>$ 50 μl, are used to achieve sensitivity, and (3) detection of typically less than eight active components permits good resolution. Open tubular coils offer significant advantages over packed reactors: they are easier to prepare; they are volumetrically stable, i.e., they do not fracture and clog with usage [40]; and they have superior heat transfer properties, facilitating the good temperature control required for the extremely temperature-dependent, enzymatic kinetics [41].

A gradient pumping system is generally required to separate isoenzymes by ion exchange. Buffer B is usually Buffer A to which either sodium acetate or sodium chloride has been added. Sodium chloride is more efficient for the ionic gradient than sodium acetate, but it corrodes the stainless-steel tubing, necessitating thorough flushing after each use [42]. The reagent stream is

mixed with eluate, and the ensuing reaction is allowed to proceeed in the reactor.

Because LD catalyzes the following reaction:

$$\text{L-lactate} + \text{NAD} \xrightarrow{\text{LD}} \text{pyruvate} + \text{NADH}\ (\lambda_{max} = 340\ \text{nm}, \lambda_{ex} = 340\ \text{nm}, \lambda_{em} = 455\ \text{nm})$$

the activity can be measured by monitoring either the absorbance or fluorescence of NADH (the reduced form of NAD, nicotinamide adenine dinucleotide). If the reagent concentrations (in this case NAD and L-lactate) are optimized, zero-order kinetics apply, hence:

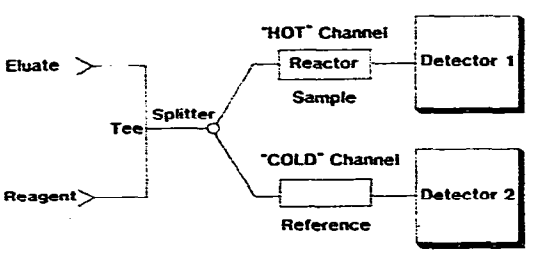
$$\text{Activity} = k\frac{\Delta\text{NADH}}{\Delta t} = k'\frac{\Delta A}{\Delta t} = k''\frac{\Delta F}{\Delta t}$$

where Δt is the residence time of the reactor and ΔA and ΔF are the changes in absorbance and fluorescence, respectively. The residence time is constant for a given total flow-rate (gradient + reagent) and is given by V_r/Q where V_r is the reactor volume and Q is the volumetric flow-rate.

With relatively pure samples few problems with this simple PCR were noted [31]; however, with human sera and tissue extracts [30] significant interferences, high reagent blanks and drifting baselines were observed. For other enzymatic reactions, notably CK, a significant lag-phase (time to reach steady state, especially for coupled reactions) is observed; therefore, additional plumbing and an additional detector are required.

Fig. 3 illustrates the general configuration of a dual-detector PCR operating in the parallel mode. This is the original design of Hicks and Nalevac [22]. Fig. 4 graphically illustrates how this design circumvents the effects of interferences, reagent blanks and drifts. Two identical reactors with identical flow-rates and nearly identical detectors are required because the residence time, Δt, must be identical to subtract the instantaneous detector responses. The same stringency is not required for the detectors which can be calibrated or compensated to obtain equal response. Interfering substances, blanks and detector drifts can be distinguished from reacting or enzymatic peaks because their difference between channels does not change with time.

The reference or cold channel is maintained at a lower temperature (conveniently ambient or 0°C); the greater the temperature difference the greater the sensitivity, i.e. Δ response. Observe (Fig. 4) that the reference starting



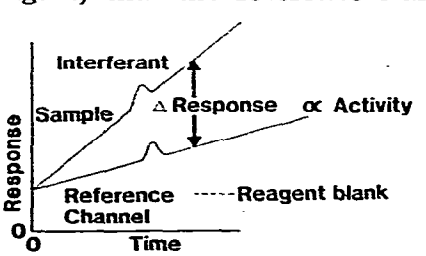


Fig. 3. Dual-detector post-column reactor, parallel mode. See text for description.

Fig. 4. Mode of operation for parallel PCR. See text for description.

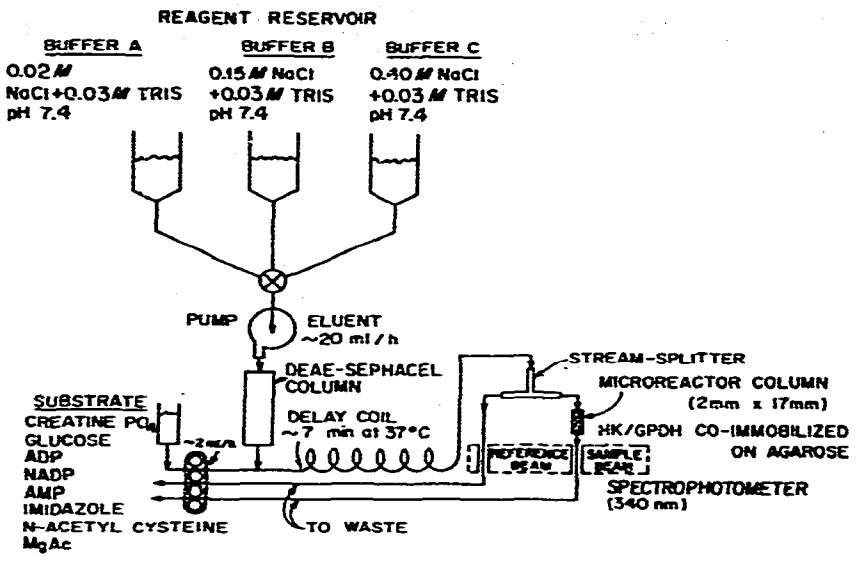


Fig. 5. Schematic for the chromatographic separation and continuously referenced monitoring of CK isoenzymes with a microreactor containing immobilized enzymes. Reprinted from ref. 43, p. 1409, courtesy of the authors and Clinical Chemistry.

point, $t = 0$, corresponds to the reagent blank and that the detector drifts (due to gradient effects, etc.) will be constant for both channels.

The Oak Ridge group [43—45] has described some very innovative modifications of the parallel stream reactor by incorporating immobilized enzyme microreactors in the sample channel instead of elevating the temperature in conjunction with novel electrochemical and bioluminescence detectors. The basic design was first reported by Denton et al. [43]. Fig. 5 illustrates the principles of this approach for monitoring CK isoenzymes. Although they used classical LC columns in this work, Denton et al. [44] later adapted this reactor to HPLC separations. Later, Bostick et al. [45] extended the system for the simultaneous detection of CK and LD by monitoring the bioluminescence of ATP and the amperometric current from a redox couple, coupled to the LD-catalyzed reaction. To illustrate the principles in more detail, Denton et al. [43] incorporated an immobilized enzyme microreactor in the sample channel for the determination of CK isoenzymes by the Rosalki [46] method:

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} + \text{NAD(P)} \xrightarrow{\text{G-6-PDH}} \text{gluconolactone-6-phosphate} + \text{NAD(P)H (spectral properties similar to NADH)}$$

The reagent consists of creatine phosphate, ADP, and D-glucose; HK and G-6-PDH are immobilized on agarose. ADP is adenosine diphosphate, ATP

is adenosine triphosphate, HK is hexokinase (EC 2.7.1.1), and G-6-PDH is glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Again, reagent blanks and interferences tend to cancel because their responses are identical. The immobilized microreactor conserves coupling enzymes by reuse, thus reducing operating costs.

Schlabach and Regnier [47] also reported the immobilization of HK and G-6-PDH on the glass beads of their packed-bed reactor. They found difficulty in reaching steady state when both coupling enzymes were immobilized and obtained best results when only the G-6-PDH was immobilized.

The PCRs previously discussed were successful because in the first case Hicks and Nalevac [22] obtained identical flow-rates as a result of the relatively large diameter tubing used which greatly reduced back pressure, while in the second case Denton et al. [43] had both channels at the same temperature and pulled the reference stream through the detector. An initial attempt by Schroeder et al. [30] to employ similar techniques failed because identical flow-rates could not be maintained as a result of the temperature-dependent viscosity difference between the hot and cold channels, in addition to the increasing viscosity of the eluate as a result of the gradient. However, this problem was surmounted by using a pneumatically activated, stream-switching valve to divert the stream alternately between channels [48] (Fig. 6). Because linearity and sensitivity were sacrificed, Fulton et al. [49, 50] used a dual-detector, serial mode PCR as shown in Fig. 7. In this system a post-column injector is provided for calibration standards and for determining the total sample activity. The predelay coil allows complete mixing and the lag-phase of the reaction to pass before measurement.

Several difficulties arise from this design. As seen in Fig. 8, to obtain a point-by-point corrected chromatogram, peaks appearing at detector 1 must be time- and shape-transformed. This requires a computer. Simple, total area corrections will be discussed later. The algorithm used for these transformations has been described [49] and tested [50]. The mass balance equation, $\partial c/\partial t = D\partial^2 c/\partial x^2 - u\partial c/\partial x$, where c is the concentration, t is time, D is the

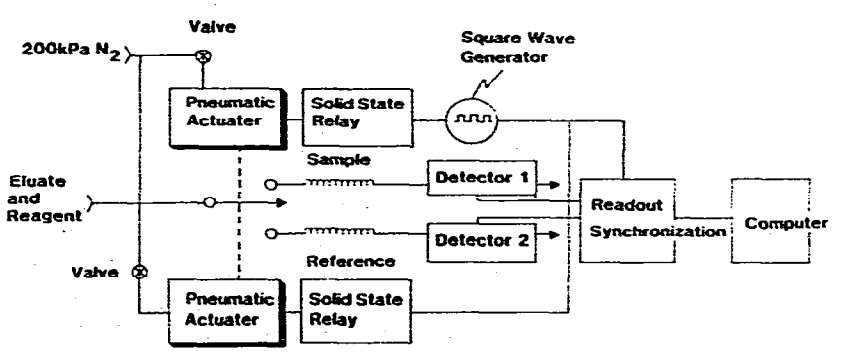


Fig. 6. Parallel stream dual-detector PCR with stream switching. A precision square wave generator controls the opening and closing of the valves that alternately divert the stream for equal periods between the sample and reference channels and also synchronizes the readings of the two detectors after switching noise has subsided. A computer acquires the data and performs a direct subtraction of the detector readings.

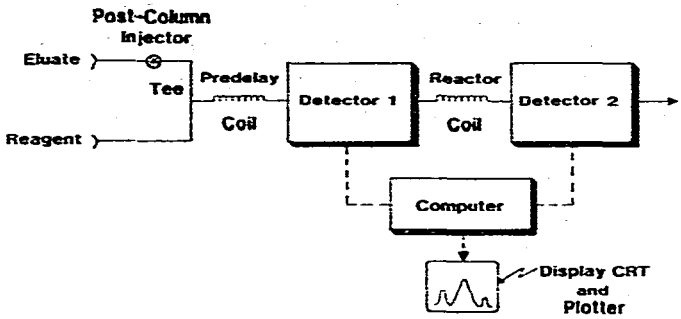


Fig. 7. Dual-detector post-column reactor, serial mode. See text for description.

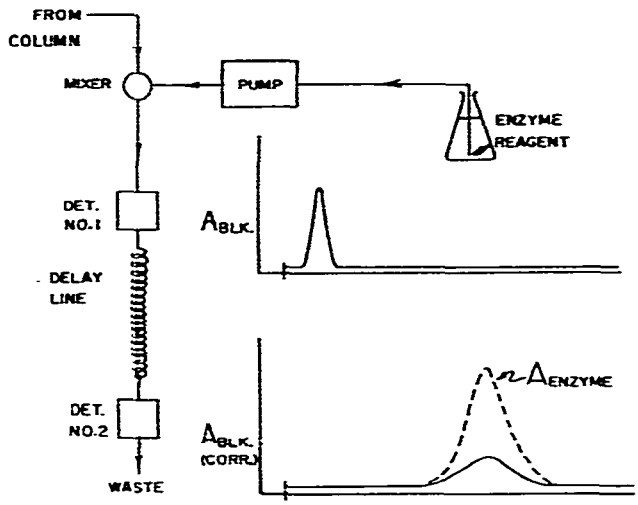


Fig. 8. Enzyme reaction detector using single stream sequential arrangement. The blank ($A_{BLK.}$) is obtained at detector No. 1 and must be mathematically shifted in time and broadened to reflect the band-spreading in the coil. The resultant blank, $A_{BLK.}$ (corr.) is subtracted from the detector No. 2 response to obtain the response due to the enzyme-catalyzed reaction. Detector 1 is one side and detector 2 is the second side of the dual 340-nm absorbance detector.

dynamic diffusion coefficient [39], x is the linear distance and u is the linear flow-rate, is solved [49] by the Savitsky—Golay method [51].

The solution requires only one empirical parameter, K, that depends on tubing dimensions, flow-rate, viscosity, and diffusion coefficient. K values are determined iteratively by subtracting the transformed detector 1 response from detector 2 response until no area remains [49], as illustrated in Fig. 9. A more dramatic illustration of the power of this correction is evidenced in the chromatograms of serum from a myocardial infarction patient [50] (Figs. 10 and 11).

Schlabach et al. [48] have described a simple scheme of data reduction for the dual-detector PCR operating in the serial mode. The computer merely obtains peak areas after time-transformation and subtracts the areas to obtain a net activity. Although a computer was used in this application, it should

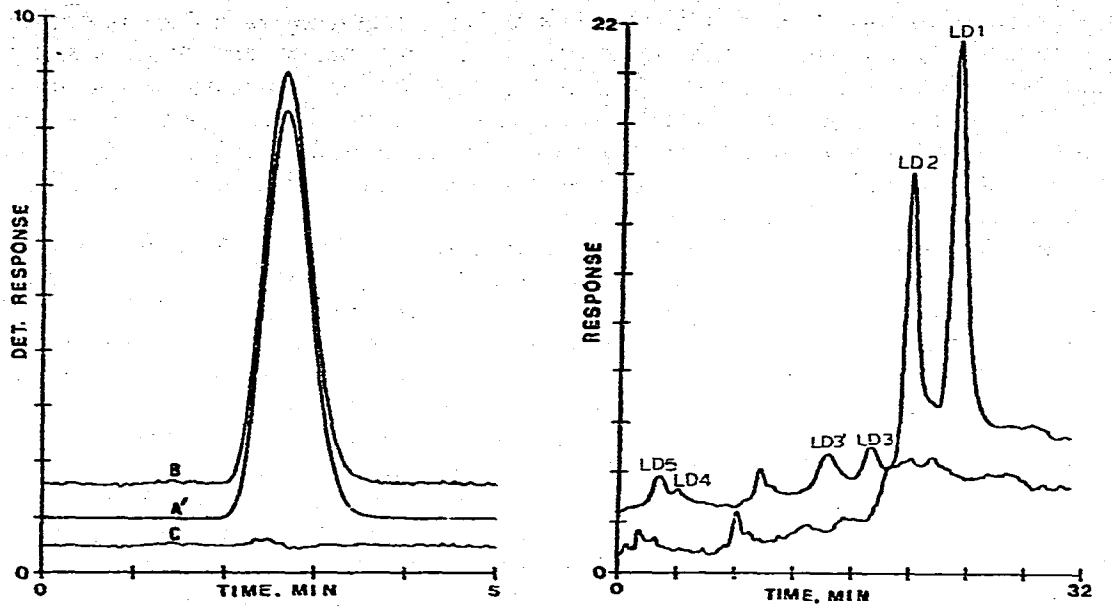


Fig. 9. Comparison of the predicted and observed response profiles at detector 2. Trace A' is the dispersed (K = 0.303 sec) and time-transformed profile of the response from detector 1 using a 79-sec delay time. Trace B is the response profile observed at detector 2. Trace C is the result of the point-by-point subtraction of trace A' from trace B.

Fig. 10. Chromatogram of serum LD isoenzymes from a patient who had a myocardial infarct. The upper trace was recorded at the downstream detector (detector 2). The lower trace shows the background absorbance, as it was observed at the upstream detector (detector 1). Total serum LD activity was 502 I.U./l.

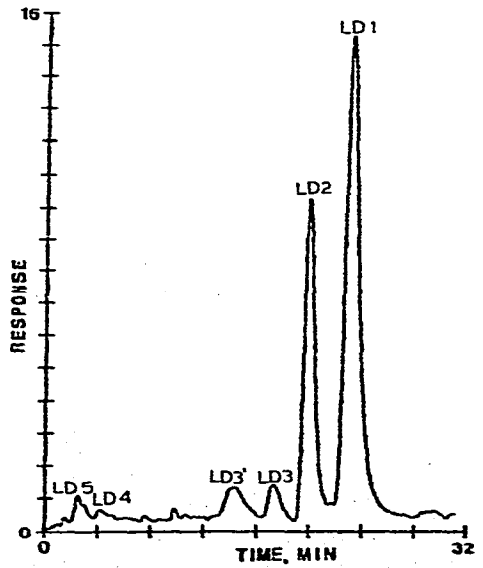


Fig. 11. Profile of serum LD isoenzymes resulting from the correction for background absorbance in Fig. 10. The dispersion coefficient used in the computer program was 0.45 sec. The percentages of total area are: LD-5, 2.5%; LD-4, 1.2%; LD-3', 6.5%; LD-3, 4.4%; LD-2, 28.4%; and LD-1, 56.9%.

be possible to monitor each detector with an integrating recorder to obtain net areas. Calibration of the detectors (both absorbance and fluorescence) and the calculations used to obtain activities from the measured peak areas are also discussed. Schlabach et al. [52] also described PCR apparatus using four detectors (two absorbance and two fluorescence) simultaneously. The system was later used to identify serum interferences that have grossly different absorbance and fluorescence spectra.

Rauschbaum and Everse [53] have adapted a stopped-flow instrument [54] to monitor automatically the activity of enzymatic eluates from classical LC columns, i.e., cellulose and Sephadex. A small aliquot of the eluate is withdrawn and mixed with reagent. The reaction is monitored kinetically by absorbance detection for 15 sec. The principal application would appear to be in location activity during routine purifications; however, no information is available on application for HPLC detection and the continuous monitoring required for the accurate measurement of peak activity.

A unique reaction detector has been described by Zare et al. [55] and is illustrated in Fig. 12. HPLC is used to separate an antibody—antigen complex from the sample matrix. The fluorescent antibody tag (fluorescein) is activated by increasing the pH with sodium hydroxide. The tag is detected in droplets emerging from the reactor by excitation at 325 nm with a highly focused laser beam.

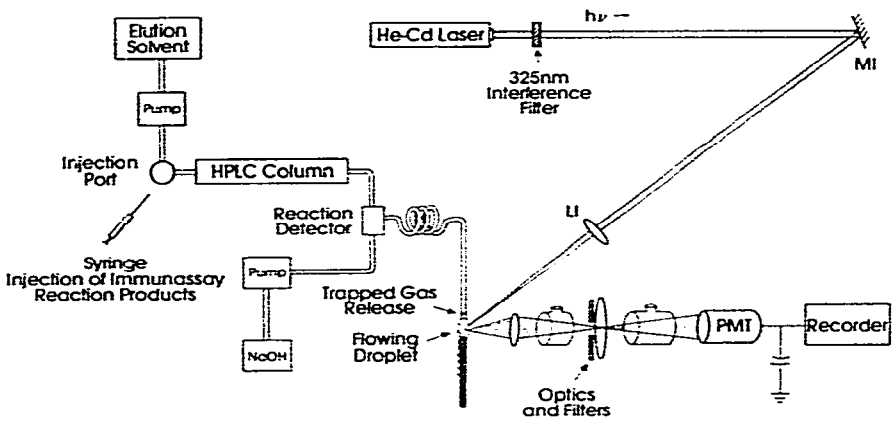


Fig. 12. LC and fluorescence immunoassay combined for separation and specificity. Reprinted courtesy of E.M. Chait and R.C. Ebersole, Anal. Chem., 53 (1981) 682, copyright 1981 American Chemical Society.

In summary, for practical use it was necessary to develop PCRs to monitor the column eluates continuously. Single detector systems proved inadequate because of endogenous interferences and blanks in biological samples. Parallel mode, dual detectors are inherently mechanically complex, but offer advantages in data processing because a simple, differential input, integrating recorder might be used rather than an expensive computer system. The serial mode system offers the advantage of a mechanically simple and trouble-free system, but with complex and expensive data reduction.

Packed-bed reactors demonstrate lower pressure drops and reduced band-spreading over open tubular designs for a given residence time; however, they are mechanically unstable, they do not have good heat transfer properties, and they are even difficult to put in a water bath.

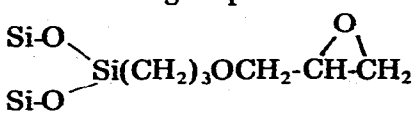
3. COLUMN PACKINGS

The key to the separation of proteins is the column packing. Although we will discuss some steric exclusion chromatography, the bulk of isoenzyme separations has been effected by ion-exchange chromatography, because isoenzymes typically have very similar molecular weights and differ only in net charge, especially the subunit-type isomers, e.g. LD and CK.

3.1. Ion-exchange supports

The early HPLC ion exchangers were coated phase materials that had a very low capacity. High-capacity columns are essential because of the large amounts of total protein present in the matrix of biological samples. For our initial work, Kudirka et al. [20] originally chose Vydac because of its relatively high capacity of ca. 100 μequiv./g.

The most significant development has been the synthesis of DEAE Glycophase-CPG™ (Corning Medical, Medfield, MA, U.S.A.) by Chang and coworkers [28, 29], subsequently patented by the investigators [56]. Glycophase is a hydrophilic coating with glycidyl groups covalently attached to the surface Si-OH groups to form



to which DEAE (diethylaminoethyl), a weak anion-exchange group, or CM (carboxymethyl), a cation-exchange group, may be added at the epoxy moiety. CPG is controlled-pore glass with practical pore size ranging from 100—530 Å for ion-exchange applications. The average particle sizes ranged from 5—10 μm to 37—74 μm. The latter provides adequate resolution for the five LD isoenzymes [28, 30]; however, the superior resolution achieved with 5—10 μm particle size packings reveals greater multiplicity of individually separated isoenzymes (see Applications). The capacity of these packings is ca. 1—2 mequiv./g [28].

Because these supports are not widely available, research by Schlabach [40] demonstrated that other chemistries can be used for the synthesis of anion exchangers from porous inorganic particles. A simple three-step route [40] to these materials consists of (1) silylation to introduce the covalently bound glycidoxy group with an oxirane monomer, Si-O-CH(O)-CH_2; (2) introduction of a polyfunctional amine, e.g., tetraethylenepentamine, to the epoxy group to which may also be added a specific ion-exchange group, -NR_2, where R is an alkyl group, via the appropriate amine epoxide, (3) crosslinking to stabilize the coating with bifunctional epoxides.

Cation-exchange supports may be synthesized by reacting the covalently bound amine groups with the appropriate anhydride to form a carboxylic

acid group bound through an amide linkage. These materials were available through Pierce (Rockford, IL, U.S.A.), but may not be available now.

Chang et al. [29] later reported on the synthesis of a variety of Glycophase supports using five different oxirane monomers in the first silylation step. In addition to the DEAE support they prepared a QAE (quaternary amine exchanger) by the addition of a methyl group to covalently bound DEAE support. CM and sulfonyl propyl (SP) Glycophases were prepared by copolymerizing an allyl oxirane with triglycidyl glycerol in the first step with subsequent oxidation to the carboxylic acid, and in the presence of bisulfite to the sulfonic acid derivative, respectively.

Recently, newer and even simpler chemistries [57] have been reported and patented [58] in which polyfunctional amines are first adsorbed onto an inorganic porous support (silica, alumina, titania) to form a monolayer subsequently crosslinked to form a permanent coating. These materials have capacities ranging from 0.3—2.6 mequiv./g and are more uniform from batch to batch because the particles do not tend to aggregate. The supports were prepared by soaking the support materials in a solution of amine in methanol. The slurry was evacuated partially to remove air from the pores and then completely to remove the methanol. This resultant material was covered with a solution of crosslinking epoxide resin in dioxane. After the reaction was completed, the product was washed with acetone, water, acetone and dried under vacuum. Best results seemingly were achieved with polyethyleneimine-6, with pentaerythritol tetraglycidyl ether as the tetrafunctional crosslinking agent. These materials are known as PEI supports.

Synchropak AX-300 is a commercially available anion-exchange support (Synchrom, Linden, IN, U.S.A.). It is a 10-μm spherical pellicular PEI support with 300 Å pores and a capacity of about 40 mg hemoglobin per g support. Hydrophilic, HPLC steric exclusion supports are also available from SynChrom, as Synchropak GPC with 100, 300, 500, and 1000 Å pore sizes.

For describing the ion-exchange capacity of supports used to separate macromolecules, more appropriate figures-of-merit may be obtained expressing the absorption capacity in terms of grams of protein per gram support. Chang et al. [59] described an assay based on the absorption of hemoglobin. Later, Schlabach [40] described a more sensitive assay based on the absorption of cytochrome C. Hancock et al. [60] reported a more classical assay based on picric acid absorption.

Toren [33] has used both DEAE-Glycophase (received as gift from Corning Glassworks, Corning, NY, U.S.A.) and Synchropak AX-300 in recent studies. Both produce well-resolved chromatograms of LD isoenzymes after direct injection of human serum. There are, however, differences in these supports. Glycophase offers somewhat better resolution, a possible requirement for the study of multiple forms of a particular isoenzyme; however, it has a lower capacity. The higher capacity of the Synchropak AX-300 makes it ideal for clinical applications where large sample volumes are required, but longer equilibration times between runs are required and a steep convex gradient aids in improving the resolution of the first peaks. We have also observed that when chromatographic purified isoenzymes in which less total protein is present, dilution with 5% albumin is required with the Synchropak AX-300

and to a lesser extent with Glycophase to obtain reproducible retention volumes when compared to lyophilized control and human sera that contain endogenous albumin.

3.2. Steric exclusion supports

Steric exclusion chromatography of proteins is primarily used for purification and isolation in clinical applications in which size differences exist, e.g., serum proteins, and acid and alkaline phosphatase isoenzymes. Regnier and Noel [61] described a preparation of Glycophase-bonded supports that is similar to the synthesis of the DEAE-Glycophase discussed above. Hydrophilic, incompressible HPLC supports were prepared by silylation of controlled porosity glass with γ-glycidoxypropyltrimethoxysilane to form Glycophase G, $Si\text{-}(CH_2)_3OCH_2\text{-}\underset{\displaystyle OH}{\underset{|}{CH}}\text{-}CH_2OH$, covalently bound to the glass surface. Since the coating will change the pore size of the particle, pore diameters were determined by measuring the steric exclusion characteristics with standard markers. The average thickness of the coating was determined to be about 18 Å.

Gooding et al. [42] and Vanecek and Regnier [62] describe the Synchropak GPC steric exclusion chromatography supports in a general way. Presumably the materials are similar to Glycophase prepared by silylation of inorganic supports with various organosilanes. They also discuss optimal chromatographic conditions for the HPLC of proteins by steric exclusion and anion-exchange chromatography as well as presenting several applications.

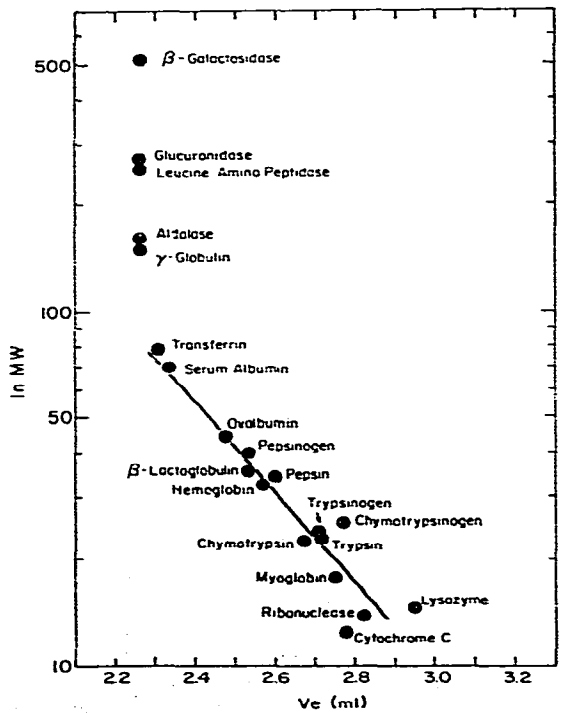


Fig. 13. Correlation of elution volume and ln MW in 0.1 *M* sodium acetate, 0.1 *M* sodium sulfate, pH 5.0. Reprinted courtesy of D.E. Schmidt, Jr., R.W. Giese, D. Contron and B.L. Karger, Anal. Chem., 52 (1980) 177, copyright 1980 American Chemical Society.

Schmidt et al. [63] have demonstrated good separation and recovery of proteins with commercial steric exclusion chromatography material (LiChrosorb DIOL, Merck, Darmstadt, G.F.R.) that is similar to that of Regnier and Noel [61]. The correlation of molecular weight and elution volume is shown in Fig. 13.

3.3. Other columns

Readily available reversed- and normal-phase columns have also been used for protein separations [64, 65]. A very intriguing type of support has recently been reported by Ohlson et al. [66] who prepared HPLC affinity packings on macroporous inorganic supports. The procedure is similar to the preparation of Glycophase except that, after silylation of the glass with γ-glycidylpropyltrimethoxysilane, the coating is converted to the aldehydic form by periodate oxidation: $Si\text{-}(CH_2)_3OCH_2CHO$. The aldehydic form is then coupled to anti-human serum albumin or N^6-(6-aminohexyl)-AMP by reaction with alkaline aqueous sodium borohydride. Mixtures of bovine serum albumin, human serum albumin, pig heart and rabbit muscle LD, and liver alcohol dehydrogenase were successfully resolved using these supports. Recently Sportsman and Wilson [67] have reported on the chromatographic properties of antibodies immobilized on silica particles and coined the term high-performance immunoaffinity chromatography (HPIC). They successfully immobilized anti-human-IgG and monoclonal anti-insulin on LiChrosphere Si 1000. Typical chromatograms for IgG and insulin are shown in Figs. 14 and 15. We envision

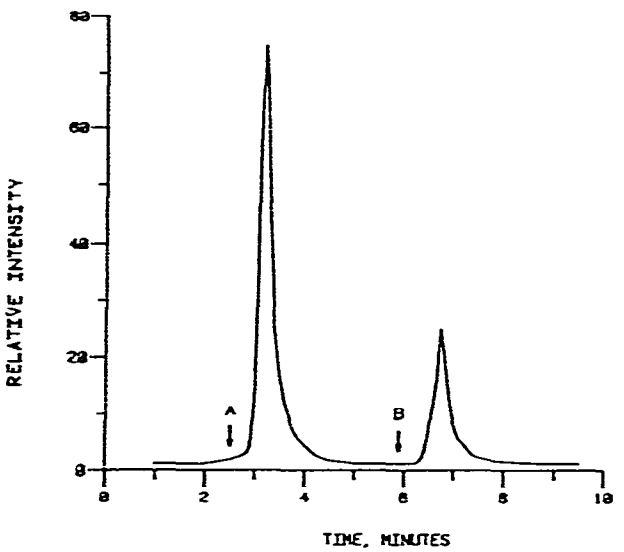


Fig. 14. HPIC chromatogram for IgG system: immunosorbent antihuman-IgG attached to LiChrospher Si 1000; column, 4 cm × 2 mm I.D.; flow-rate, 0.5 ml/min; mobile phase "A" (reservoir 1), 1% phosphate buffered saline, pH 7.4; mobile phase "B" (reservoir 2), 0.01 *M* phosphate buffer, pH 2.2; antigen (IgG) concentration, 1.4 mg/ml; volume injected, 10.0 μl; fluorimetric detection, excitation 283 nm, emission 335 nm. Reprinted courtesy of J.R. Sportsman and G.S. Wilson, Anal. Chem., 52 (1980) 2013, copyright 1980 American Chemical Society.

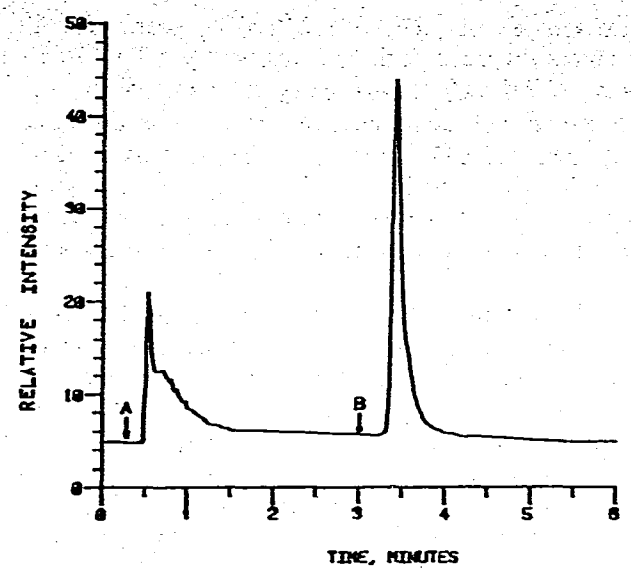


Fig. 15. HPIC chromatogram for insulin: immunosorbent, BE CB6 monoclonal attached to LiChrospher Si 1000; column, 4 cm × 2 mm I.D.; flow-rate, 0.30 ml/min; mobile phase "A" (reservoir 1), 1% phosphate buffered saline, pH 7.4; mobile phase "B" (reservoir 2), acetonitrile in 1% phosphate buffered saline; antigen (insulin) concentration, 4.0 μg/ml; volume injected, 50 μl; fluorimetric detection, excitation 390 nm, emission 470 nm. Reprinted courtesy of J.R. Sportsman and G.S. Wilson, Anal. Chem., 52 (1980) 2013, copyright 1980 American Chemical Society.

great potential for this type of support in the determination of clinically significant proteins if specific supports can be designed using the all but one technique reported by Anderson et al. [68] for classical affinity chromatography (Sepharose) in which antibodies to all species except the sought-for species are bound to the support phase.

Significant applications of the supports and reactors will be discussed in the following sections.

4. APPLICATIONS

The preponderance of the literature has been devoted to the subunit isoenzymes separated by anion-exchange chromatography; especially the cardiac enzymes LD and CK. These will be discussed in detail followed by other applications. Because these techniques are not in routine use, most of the papers are research oriented; however, we are prepared to commence a clinical trial shortly.

4.1. Lactate dehydrogenase

Lactate dehydrogenase (LD) is a tetramer of two subunits, H and M, that has a molecular weight of 140,000 daltons. All five possible combinations have been observed by a wide variety of separation techniques. The five combinations are H_4, H_3M, H_2M_2, HM_3, and M_4, commonly denoted in electrophoretic literature as LD-1, LD-2, LD-3, LD-4, and LD-5, respectively. In ad-

dition further multiplicity has been observed [49, 50, 52, 69] primarily in LD-3 (3 peaks) and LD-4 (2 peaks) when separations are made by HPLC; this demonstrates the superior resolution of HPLC. There may be multiplicity in the other peaks; however, LD-5 elutes in the void volume since it is cationic at the typical pH values used for separation; LD-1 and LD-2 are not as well resolved owing to decreasing column efficiency with increasing retention time. No clinical significance has been attached to these multiplicities, nor have they been fully explained. A third subunit, C, is found in testicular tissue and spermatozoa [70].

Typically, LD is separated at a pH of about 7.8 at flow-rates of 1—2 ml/min (to avoid degrading the column) with sodium chloride or sodium acetate gradients of 0 to ca. 0.3 *M*. Schlabach et al. [52] have recently reported the separation of LD isoenzymes in human serum with a recovery of 90% of total activity. The resulting isoenzyme activity profiles (chromatograms) were in excellent agreement with clinical findings. The isoenzyme profiles were acquired and analyzed by the computer system and programs described by Fulton et al. [49] with both absorbance and fluorescence detection. The limit of sensitivity was about 1 I.U./l, the linear dynamic range was 1.5—1500 I.U./l, precision approached 1% relative standard deviation (C.V.), and recovery of applied activity was approximately 92%. Fig. 16 compares the

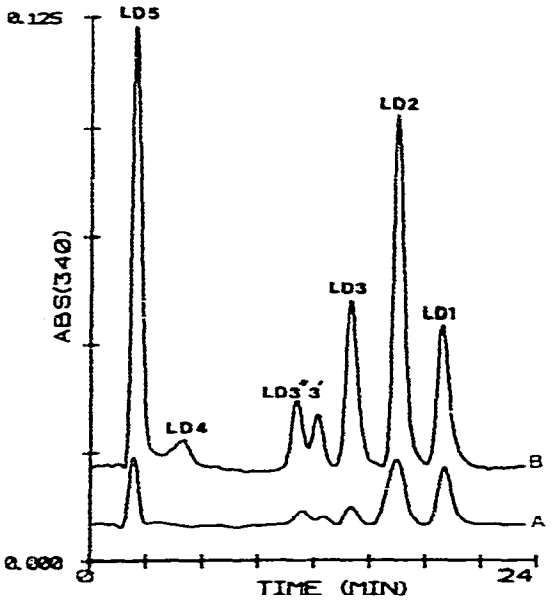


Fig. 16. Comparison of LD isoenzyme profiles from the sera of a normal (A) and an abnormal (B) individual. The isoenzyme activities in (A) were 19.3, 0, 6.1, 2.6, 7.2, 37.0, and 26.3 I.U./l for LD-5, LD-4, LD-3'', LD-3', LD-3, LD-2, and LD-1, respectively. The isoenzyme activities in (B) were similarly: 131.9, 15.3, 22.3, 17.9, 61.7, 121.8, and 57.1 I.U./l. The weak buffer was 0.02 *M* Tris HCl adjusted to pH 7.8. The strong buffer was weak buffer to which was added 0.15 *M* hydrochloric acid. The column was 18 × 0.5 cm I.D. packed with 5—10 μm DEAE-Glycophase. The flow-rate was 2 ml/min, the sample size was 102.8 μl, and the gradient was begun at 2 min after injection and was advanced at 5%/min. Reprinted courtesy of T.D. Schlabach, J.A. Fulton, P.B. Mockridge and E.C. Toren, Jr., Anal. Chem., 52 (1980) 729, copyright 1980 American Chemical Society.

serum profiles of a normal and abnormal individual and clearly illustrates the multiplicity in LD-3, and describes the experimental conditions. Fig. 17 compares the LD profiles of a patient admitted to the cardiac care unit on admission and 18 h later. Observe the dramatic increase in LD-1 and LD-2 and the so-called LD flip; i.e., LD-1 activity > LD-2, indicative of MI.

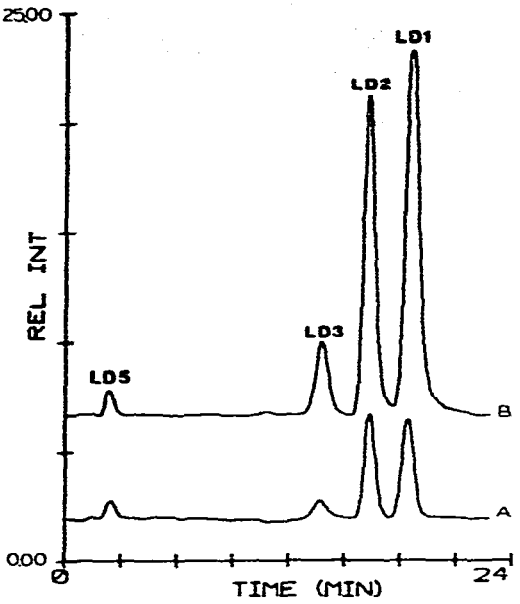


Fig. 17. Comparison of sequential, LD isoenzyme profiles from the sera of another patient in the cardiac care unit. (A) Admission profile. The isoenzyme activities in (A) were 6.3, 9.8, 40.8, and 43.2 I.U./l for LD-5, LD-3, LD-2, and LD-1, respectively. (B) Profile 18 h after admission. The isoenzyme activities in (B) were similarly 7.5, 33.5, 130.0, and 183.0 U.I./.l, respectively. The experimental conditions are described in Fig. 16. Reprinted courtesy of T.D. Schlabach, J.A. Fulton, P.B. Mockridge and E.C. Toren, Jr., Anal. Chem., 52 (1980) 729, copyright 1980 American Chemical Society.

Earlier, Schlabach et al. [48] described a similar determination of LD isoenzyme activity profiles, but with decreased sensitivity and linear dynamic range, 17—1700 I.U./l because only absorbance detection of the NADH produced was used. Therefore, it may be concluded that fluorescence detection improved sensitivity ca. ten-fold. This paper also described a parallel-stream reactor that did not perform as well as the serial-stream reactor; the linear dynamic range was 20—1000 I.U./l.

Fulton et al. [50] obtained LD profiles to evaluate the serial reactor and computer programs used to analyze the data [49]. This paper also compares HPLC separations with classical electrophoresis using lyophilized control sera.

Schlabach et al. [69] obtained LD isoenzyme activity profiles with a packed-bed, single-detector PCR. They evaluated column materials prepared by different procedures [40, 57]; however, most of the work was with the PEI material [57]. Schlabach et al. [69] used human serum samples and demonstrated elevated LD-1 and LD-2 with the associated flip pattern for a patient with MI.

The profile (Fig. 18) of an accident victim who suffered a broken hip reveals a greatly elevated LD-5 peak normally associated with muscular or hepatic damage. Schlabach et al. [69] also illustrated that the sensitivity of the reactor may be increased by elevating the temperature for all isoenzymes except LD-5 which is known to be temperature-labile.

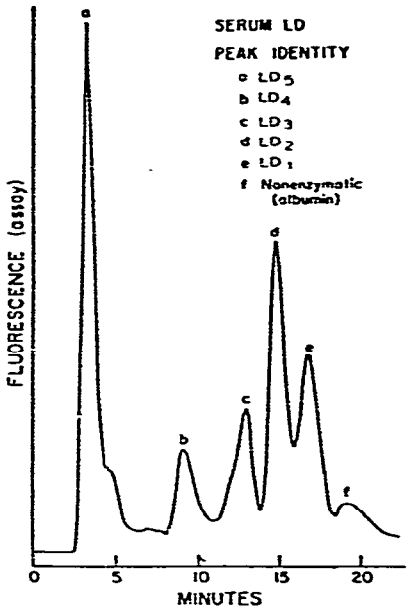


Fig. 18. Serum LD isoenzyme profile for a patient after a severe accident. The serum sample was directly chromatographed on a diethyl-2,3-epoxypropylamine column with a 20-min linear gradient at a flow-rate of 1.25 ml/min. The LD assay reagent was added at 0.8 ml/min. The post-column temperature was 40°C and the resulting NADH was detected by its fluorescence. Reprinted from ref. 69, p. 1358, courtesy of the authors and Clinical Chemistry.

Schlabach et al. [31] compared packed-bed reactors with open capillary reactors using a DEAE-Glycophase column and relatively pure LD samples. Fig. 2 clearly demonstrates the superior band-spreading behavior of the packed-bed variety. However, Toren [71] has observed that the large sample volumes required for serum samples (to achieve sensitivity) primarily determine the resolution. Schlabach et al. [31] also described and derived optimal particle sizes for use in packed-bed reactors. Simultaneously, Schroeder et al. [30] also reported on a capillary reactor with a single detector. The limiting factors were drifts and interferences with autopsy and serum specimens, but continuous recording of the LD activity profile was feasible.

Earlier, Kudirka et al. [27] demonstrated the excellent resolution that could be obtained on DEAE-Glycophase columns as is indicated in Fig. 19. Observe the baseline resolution of most peaks and the multiplicity in LD-4 and LD-3. In spite of the obvious improvement in resolution and freedom from drifts and interferences by using fraction collection (small volumes) with subsequent kinetic assay of activity, the tedium of determining 140

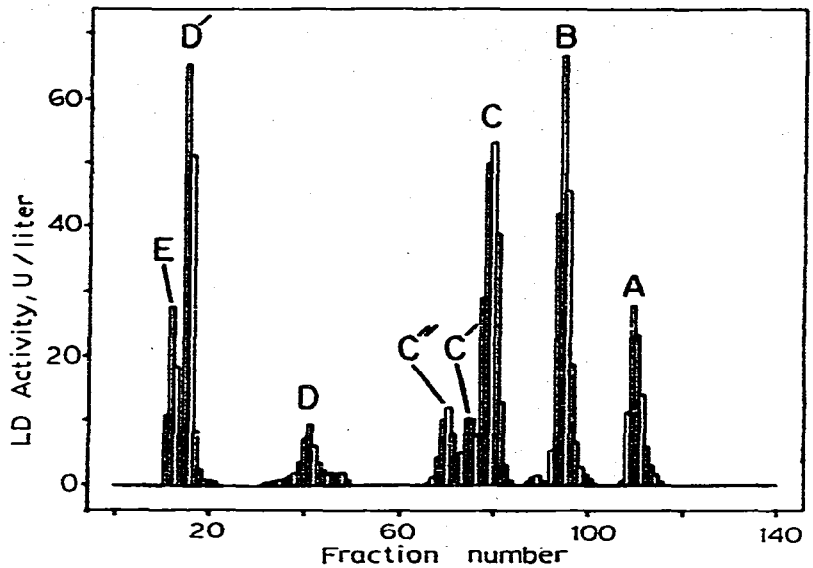


Fig. 19. Chromatographic separation of LD isoenzymes in a spleen extract. Peaks: E = LD-5; D = LD-4; C = LD-3; B = LD-2; A = LD-1. Undetermined LD peaks are designated by letters marked with primes. Reprinted from ref. 27, p. 473, courtesy of Clinical Chemistry.

fractions mitigates against this procedure. With the introduction of the first PCR by Chang et al. [28] such tedium was obviated. After examining a series of support materials, they determined that non-porous spherical sodium silicate glass beads were the most satisfactory material available for their packed-bed reactor to minimize band-spreading and adsorption. Enzyme-dependent variables such as temperature, pH, substrate concentration, and time were also studied and adjusted in the PCR to assure detection linearity.

Alpert and Regnier [70] described the preparation of the PEI column packings and demonstrated the efficacy of the material by resolving the C-subunit isoenzymes of LD (LD-X) in murine testicular tissue and human semen. The three isoenzymes observed appear between LD-3 and LD-4 as shown in Fig. 20. See Fig. 20 for experimental details.

Denton et al. [44] described a unique PCR system for producing LD and CK isoenzymes profiles. They used the parallel-stream approach discussed earlier with an immobilized enzyme reactor: diaphorase (EC 1.6.4.3) for LD and HK and G-6-PDH co-immobilized for CK.

The diaphorase is used to couple the NADH produced to a redox-indicator dye coupled as follows:

NADH + diaphorase—FAD → diaphorase—FADH + NAD

diaphorase—FADH + 2,6-dichlorophenolindophenol (2,6 D) → diaphorase—FAD + 2,6 D (reduced, λ_{max} = 600 nm)

FAD and FADH are the oxidized and reduced forms of flavin adenine dinucleotide, respectively. This reactor system greatly reduces reagent cost

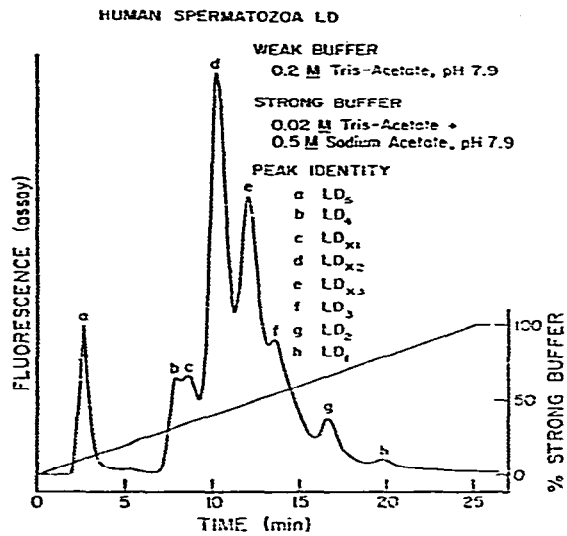


Fig. 20. Activity profile of spermatozoa LD isoenzymes from a 27-year-old human. The column was a 25 × 0.41 cm PEI 6—LiChrospher. The sample was 100 μl of 105,000 g supernatant, diluted two-fold. Reprinted from ref. 70, courtesy of the authors.

especially for CK; unfortunately, it also reduces resolution. However, Bostick et al. [45] later improved the system and were able to measure LD and CK simultaneously by monitoring the LD-catalyzed production of NADH electrochemically with a vitreous carbon electrode and the CK-catalyzed production of ATP by bioluminescence with the luciferin—luciferase reaction to achieve reactor specificity for both isoenzyme systems.

4.2. Creatine kinase

Creatine kinase (CK) isoenzymes are also subunit types that consist of dimers of M (muscle) and B (brain) subunits. The molecular weight of the dimer is about 80,000 daltons. All three possible isoenzymes have been observed, and they are generally denoted as CK-MM (the major constituent of normal and abnormal sera), CK-MB (the cardiac specific isoenzyme), and CK-BB (of brain origin and usually not present in human serum). In addition, several reports describe variant CK types, e.g., a mitochondrial CK distinctive from any cytoplasmic isoenzymes [72—75] and active—inactive forms of CK-BB [76].

As previously discussed Denton et al. [44] described the simultaneous separation and quantitation of CK and LD. The immobilization of the coupling reagents for this assay is very important because Toren [71] has observed that the cost of CK assay reagents approaches $200 per day. The slight loss in resolution is immaterial in this case because only three already well-resolved peaks need to be detected. The principles of this approach were described earlier [43]; however, these investigators did not use HPLC techniques. Bostick et al. [45], as discussed earlier, were able to determine LD and CK isoenzymes simultaneously; the extremely sensitive bioluminescence detection system was further studied [77]. A trace activity of CK-MB was readily detected from a 50-μl sample.

A major problem in CK assays is the interference by adenylate kinase (AK, EC 2.7.4.3), also referred to as myokinase. AK interferes by its production of ATP from endogenous ADP. The effect is usually compensated by addition of selective inhibitors for AK in the assay reagent. Denton et al. [44] clearly illustrate this interference in Fig. 21. AK coelutes with CK-MM.

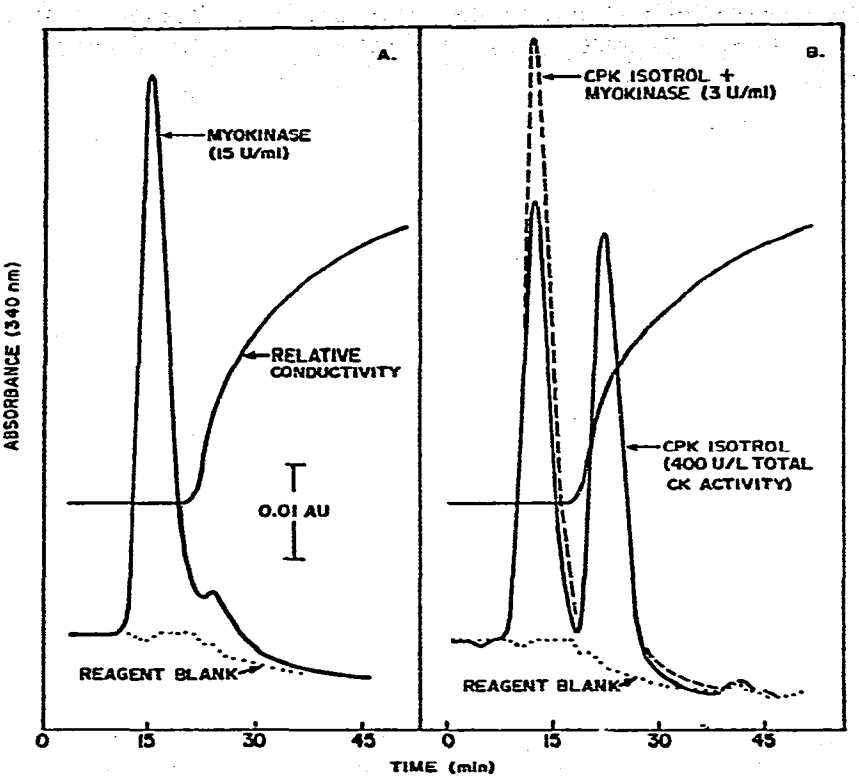


Fig. 21. Effect of myokinase (MK) in CK monitoring. (A) 72 μl MK (15 U.I./ml) in dilute buffer. Chromatographic conditions: column, 25 cm DEAE-Glycophase CPG 250; total flow-rate, 46 ml/h; 4-min hold at initial conditions (0.02 *M* sodium chloride + 0.03 *M* Tris, pH 7.4) followed by a 4% gradient, 0—100% B (B = 0.40 *M* sodium chloride + 0.03 *M* Tris, pH 7.4) in 32 min. Monitored at 340 nm, 0.1 a.u.f.s. (B) CPK Isotrol with 10 m*M* dithiothreitol (400 I.U./l total CK activity), solid line, and same CPK Isotrol + MK (3 I.U./ml), also with 10 m*M* dithiothreitol (430 I.U./l total activity), dashed line. Activities determined by kinetic assay. Reprinted from ref. 44, p. 182, courtesy of the authors and Marcel Dekker.

Because AK is a much smaller molecule than CK, Toren [33] attempted to alter the elution order by using a small pre-column, 5 × 0.4 cm I.D. of Glycophase CPG 100, a steric exclusion chromatography packing. Although they did not recover even added AK activity, the pre-column did, however, eliminate the AK interference without reducing the CK recovery.

Recently, Schlabach et al. [52] reported on the separation and assay of CK isoenzyme activity (experimental conditions identical to Fig. 16 with the exception that the sample volumes were 216.7 μl and the gradient was begun immediately at 15% weak buffer and advanced thereafter at 5%/min).

The limit of sensitivity was about 0.5 I.U./l; linear dynamic range was 0.5—500 I.U./l; precision approached 1% relative standard deviation (C.V.) and recovery of applied activity was approx. 97%. Fig. 22 illustrates the dramatic increase in CK-MB activity for the same patient described in Fig. 17. Later, Schlabach et al. [78] demonstrated that the serial-stream reactor could be used to identify the nonenzymatic interferences that appear in human serum. The major interferent in the CK-MB region was albumin and the interferent in the CK-BB region was pre-albumin.

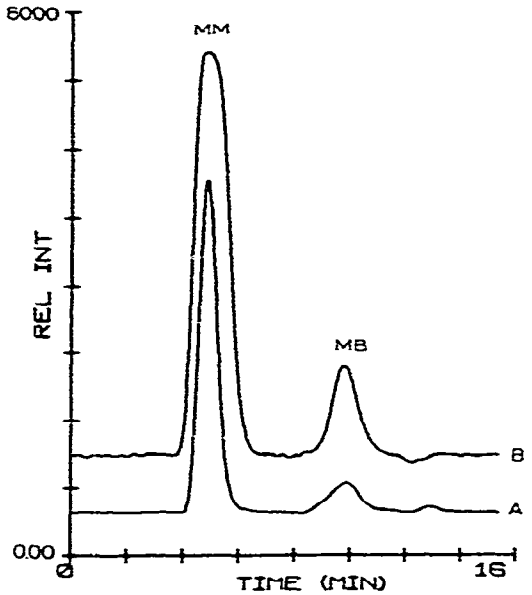


Fig. 22. Comparison of sequential, CK isoenzyme profiles from the same serum used in Fig. 17. (A) Admission profile. The CK-MM and CK-MB activities in (A) were 106.5 and 12.8 I.U./l, respectively. (B) Profile 18 h after admission. The CK-MM and CK-MB activities in (B) were 168.1 and 30.1 I.U./l, respectively. Reprinted courtesy of T.D. Schlabach, J.A. Fulton, P.B. Mockridge and E.C. Toren, Jr., Anal. Chem., 52 (1980) 729, copyright 1980 American Chemical Society.

The first HPLC separation of CK isoenzymes was reported by Kudirka et al. [20]. Unfortunately, the CK-MB activity was destroyed on the hydrophobic Vydac anion exchanger. Schlabach and Regnier [47], however, successfully resolved CK-MB on DEAE-Glycophase. They also demonstrated the efficacy of immobilizing one of the coupling enzymes, G-6-PDH as did Denton et al. [43]. Earlier Schlabach et al. [31] reported the detection and separation of CK and LD using soluble reagents.

4.3. Other enzymes

Schlabach and Regnier [47] have also demonstrated the separation and detection of alkaline phosphatase (AP, EC 3.1.3.1) and hexokinase (HK) from murine tissue extracts with DEAE-Glycophase columns. AP is a non-specific enzyme that cleaves phosphate groups from a wide variety of natural and artificial substrates. Total AP and its isoenzyme activity assays are pri-

marily used for the diagnosis of hepatic, bone, intestinal, and metastatic diseases. HK activity assays and separations are not common in clinical practice.

AP is most commonly assayed using the following reaction:

$$p\text{-nitrophenylphosphate} \xrightarrow{AP} \text{phosphate} + p\text{-nitrophenol} \quad (\lambda_{max} = 404 \text{ nm})$$

Fig. 23 illustrates the separation and post-column detection of AP employing the above reaction.

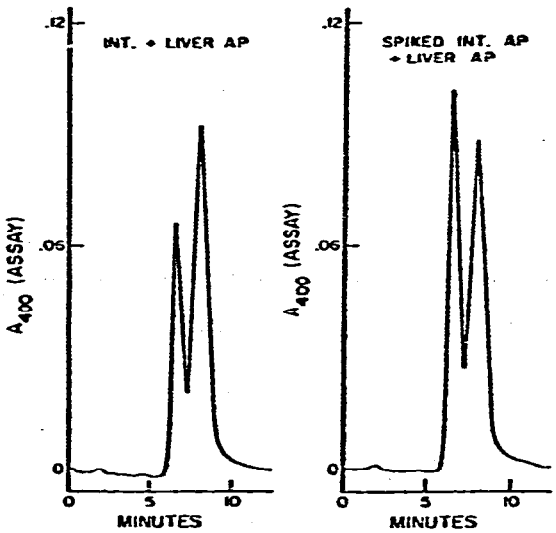


Fig. 23. Separation of a combined solution of liver and intestinal AP, and the separation of the same solution spiked with intestinal AP, eluted with a 15-min linear gradient reaching 0.15 *M* sodium chloride at a flow-rate of 1.5 ml/min. Both the weak and strong buffer contained 1 *M* urea. The AP assay solution was introduced into the column effluent at a rate of 2.0 ml/min. The post-column temperature was 40°C, and the resulting *p*-nitrophenol was detected by absorbance at 400 nm. Reprinted from ref. 47, p. 357, courtesy of the authors.

Schlabach and Regnier [47] separated and detected HK isoenzymes employing the following reaction sequence:

$$\text{D-glucose} + \text{ATP} \xrightarrow{HK} \text{glucose-6-phosphate} + \text{ATP}$$

$$\text{glucose-6-phosphate} + \text{NAD} \xrightarrow{\text{G-6-PDH}} \text{gluconolactone-6-phosphate} + \text{NADH}$$

They used both soluble (see Fig. 24) and immobilized G-6-PDH with similar results. Finally, they employed the above reaction sequence for CK isoenzymes, immobilized G-6-PDH, and immobilized HK and G-6-PDH in the reactor.

Bostick et al. [79] have separated and detected the two isoenzymes, A and B, of arylsulfatase (AS, EC 3.1.6.1) in samples of human body fluids. Like AP, AS is nonspecific and cleaves sulfate groups and may be assayed with synthetic substrates. In fact, they used *p*-nitrophenylcatechol sulfate

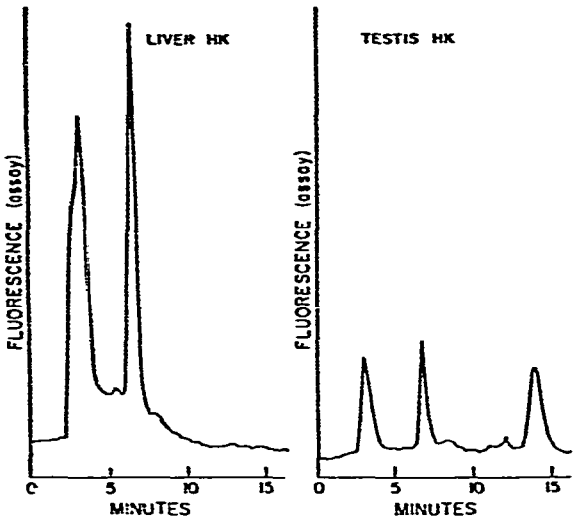


Fig. 24. Separation of HK isoenzymes in rat liver and testicular tissue extracts. The 105,000 *g* supernatant from each tissue extract was directly chromatographed with a 15-min linear gradient reaching 0.4 *M* sodium chloride at a flow-rate of 0.8 ml/min into the column effluent. The post-column temperature was 40°C, and the resulting NADH was detected by fluorescence. Reprinted from ref. 47, p. 360, courtesy of the authors.

(NCS) and 4-methylumbelliferone sulfate (MUS) in the PCR as substrates. The NC product is similar to *p*-nitrophenol in absorbance properties; however, the MU product may be detected fluorimetrically (λ_{ex} = 310 nm, and λ_{em} = 450 nm). AS isoenzyme fractionation is useful for the diagnosis of metastatic disease, especially malignant melanoma and colorectal cancer.

4.4. Serum proteins

Nonenzymatic serum proteins are routinely separated and measured in the clinical laboratory by electrophoresis. Such proteins include γ- and β-globulins, albumin, pre-albumin, hemoglobin and variants, lipoproteins, and albumin complexes. Because electrophoretic and ion-exchange techniques have similar separation mechanisms, HPLC with modern supports is a logical choice for future application because of its speed, sensitivity, and precision.

Alpert and Regnier [57] demonstrated the efficacy of serum protein separation on PEI anion-exchange supports. Fig. 25 is an excellent illustration of the resolution that may be achieved. Because serum proteins are present in large amounts (ca. 5 g/l), and because they are readily detected by absorbance methods at 280 nm, post-column reaction is not necessary. They were also able to resolve serum nucleotides by this technique. Regnier and Noel [61] likewise separated serum proteins, nucleic acids, and dextrans with steric exclusion chromatography with Glycophase CPG 100 (100 Å pore size) columns. Serum proteins were not as well resolved by this technique as with anion exchange [57].

Hemoglobin separations* are useful for the diagnosis and screening of

*See also the review by P. Basset, F. Braconnier and J. Rosa, J. Chromatogr., 227 (1982) 267.

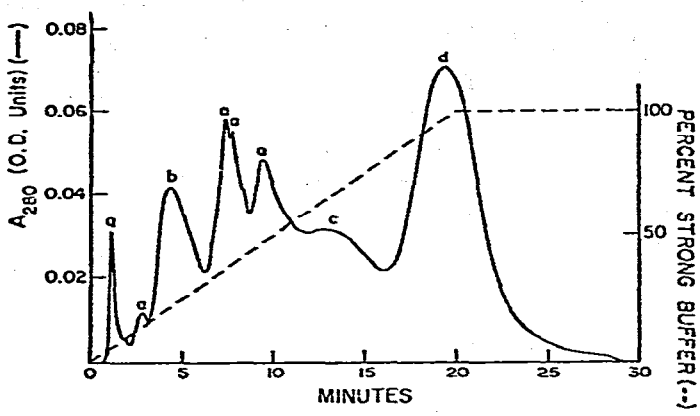


Fig. 25. Anion-exchange chromatography of human serum. Column, PEI 6—LiChrospher Si 500 (10 μm) crosslinked with pentaerythritol tetraglycidyl ether 25 × 0.41 cm; sample, 100 μl of serum, diluted 33%; temperature, 25°C; flow rate, 2.0 ml/min; inlet pressure, 9.66 MPa; detection, A_{280}, 0.195 a.u.f.s.; weak buffer, 0.02 *M* Tris acetate, pH 8.0; strong buffer, 0.5 *M* sodium acetate in weak buffer; peaks: a = γ-globulin; b = unidentified; c = β-globulin; d = albumin. Reprinted from ref. 57, courtesy of the authors.

genetic diseases such as sickle cell anemia and thalassemia (hemoglobinopathies with variant hemoglobins). Glycosylated hemoglobin separations have recently been used for the diagnosis and therapeutic monitoring of diabetes (fast hemoglobin or HbA_{1C}). Hemoglobin separations are typically made by electrophoresis and gravity-flow chromatography. Recently HPLC has been employed for hemoglobin separation. Chang et al. [28] and Gooding et al. [80] have separated the intact hemoglobin proteins by anion exchange. Others [64, 65] have separated and identified the chains or peptide fragments of hemoglobin by reversed-phase chromatography. HPLC on BioRex 70 cation exchanger has been used to determine HbA_{1C} [81—83]. Gooding et al. [80] achieved excellent resolution of hemoglobins on Synchropak AX 300 anion-exchange columns as evidenced in Fig. 26.

Bilirubin is the degradation product of hemoglobin and in the free unconjugated state is the major cause of jaundice, a common condition in neonates. Serum measurements of free bilirubin are difficult and erratic [84—86].

Lu et al. [87] determined free bilirubin, protein-bound bilirubin, total bilirubin, and percentage of high affinity binding sites by HPLC based upon prior separations on gel permeation supports. Fig. 27 is a typical neonatal serum profile with bilirubin bound to albumin. pH, ionic strength, interferences competing for binding sites and background absorbance were observed to be significant variables that must be controlled. However, the conclusion is that HPLC is much quicker, more quantitative, and requires less sample than the clinically utilized Sephadex gel method while achieving comparable results. Several additional articles on protein and polypeptide separations and detection by HPLC [88, 89], though not clinical, note the broadening horizons for HPLC in the areas of biochemical and physiological research and development.

While this review reveals that HPLC is still in its infancy as a routine clinical

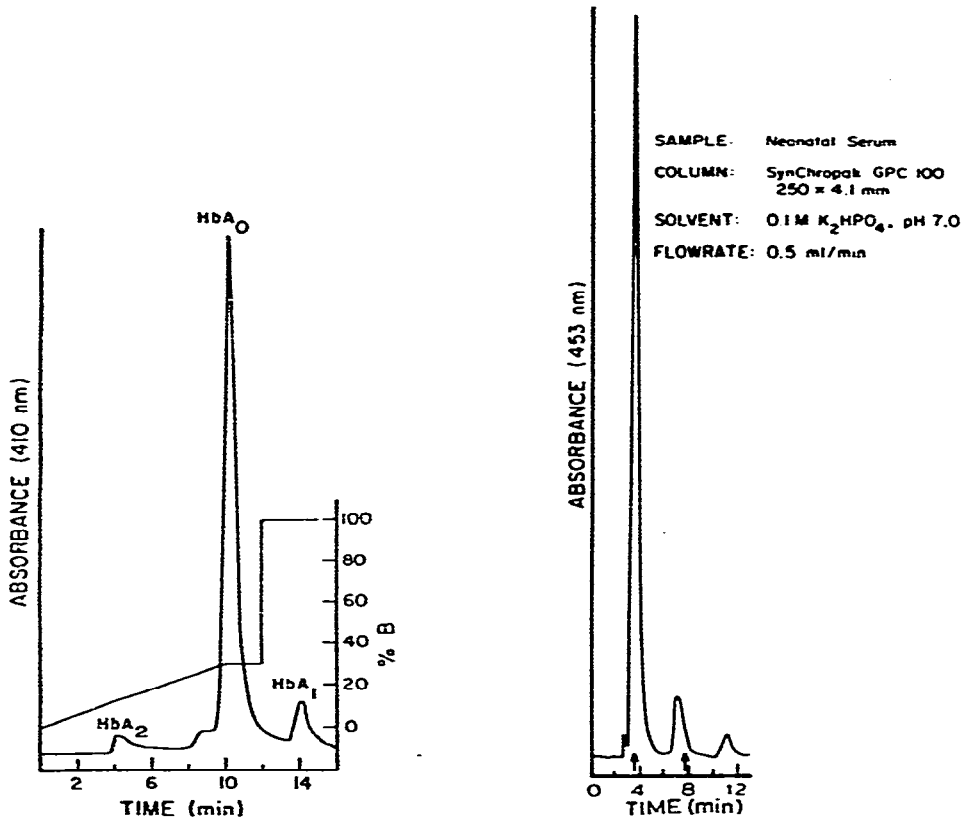


Fig. 26. Analysis of standard HbA_2 sample. Column: SynChropak AX300, 250 × 4.1 mm I.D. Solvents A and B were 0.02 *M* Tris (pH 8.0) and 0.02 *M* Tris + 0.1 *M* sodium acetate (pH 8.0) respectively, with a 10-min linear gradient from 0 to 30% then step to 100%; flow-rate, 2.5 ml/min; pressure, 8.28 MPa; detection at 410 nm Reprinted from ref. 80, p. 507, courtesy of the authors.

Fig. 27. Analysis of protein-bound bilirubin in neonatal serum. Arrows indicate injection of 20 μl of 0.1 mmol/l bovine serum albumin. Reprinted from ref. 87, p. 1609, courtesy of the authors and Clinical Chemistry.

tool for protein and enzyme separation, we have shown that its application in specific areas of enzyme research is well established and expanding. We foresee HPLC making a major impact in the clinical areas of enzyme separation and detection in the next few years as the necessity for rapidity and precision will have an increasingly high priority in achieving fast, accurate results for diagnosis and monitoring. The numerous advantages of HPLC over other analytical systems brightens its clinical usage. It is rapid, has a wide dynamic range and sensitivity, is easily automated, requires little sample preparation, and its reproducibility is free of most inherent human manipulative errors. While initial cost and sample throughput appear as disadvantages, increased technology, familiarity, and utilization in other areas will reduce these to minor factors to bring it into routine use.

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6. SUMMARY

We review the state-of-the-art of the separation of isoenzymes and other proteins by high-performance liquid chromatography (HPLC) and their subsequent, continuous detection and activity assay by post-column reaction (PCR). We describe the developments leading to current practice. These developments are categorized by column packings, post-column reaction detectors and applications in which specific analysis systems for isoenzymes, especially for lactate dehydrogenase (LD) and creatine kinase (CK) are discussed.

Although HPLC coupled with PCR detection is well developed and in many cases is analytically superior to other isoenzyme technologies, it is not at the present used for routine clinical applications. We expect this trend to be reversed with clinical demonstration and the addition of commercially available automation.

7. NOTE ADDED IN PROOF

Many new developments in the separation and detection of proteins, peptides and enzymes on all types of commercially available packings were recently presented at the International Symposium on HPLC of Proteins and Peptides, November 16 and 17, 1981, Washington, DC, U.S.A. Many of these papers will be published in 1982 issues of *Analytical Biochemistry*.

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