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A CONTINUOUS-FLOW ENZYME DETECTOR FOR LIQUID CHROMATOGRAPHY*

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SUMMARY

A detection system has been developed for the selective and sensitive detection of enzymes eluting from a liquid chromatographic column. This system monitors a reaction that the enzyme catalyzes and provides a chemical amplification ranging from 10^4 to 10^5. The detection system consists of a reagent or substrate pump, a post-column reactor packed with non-porous spherical glass beads, and a photometric detector. A linear and selective response to a series of enzymes of clinical importance is demonstrated.

INTRODUCTION

With the advent of high-speed liquid chromatographic separations of proteins[1–3], enzymes of clinical significance can be resolved in minutes. Enzyme separations on chromatographic columns have classically been monitored by collecting and assaying column fractions for both protein and enzyme activity. Although this lengthy procedure is widely used in enzyme separation and purification on gel supports, it is totally inadequate for high-speed columns that generate fractions every 10 sec. This paper is the second in a series that reports the development of an enzyme-specific detector for high-performance liquid chromatography (HPLC)[3].

THEORETICAL

Quantitation of enzyme activity may be accomplished by incubating an enzyme (E) with an appropriate substrate (S) and monitoring the formation of product (P) after a fixed time. This obviously could be accomplished in a flow-through reactor in which the enzyme is mixed with substrate at the reactor inlet and the amount of product (P) monitored at the exit. Fig. 1 illustrates such a system.

There are two major problems in the design and construction of flow-through

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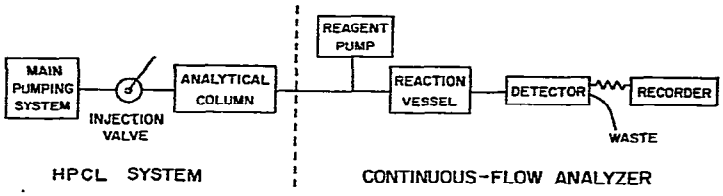


Fig. 1. Diagrammatic representation of the continuous-flow analyzer in union with a high-pressure liquid chromatography (HPLC) system.

reactor type detectors. One of these problems is distortion of the chromatographic profile by band spreading in the reactor and the other is enzyme–product demixing during passage through the system. These problems have been partially overcome in instruments such as the Technicon Auto-analyzers by segmenting the liquid reactant stream with air bubbles. The alternating gas–liquid segmentation diminishes intersegmental mixing while still allowing intrasegmental mixing of reactants. Snyder and Adler have presented an extensive analysis of sample dispersion in segmented flow analyzers[4,5].

It is tempting to suggest the use of an open tubular column as the reaction vessel in a flow-through reactor. Unfortunately, solutions flowing through open tubing without gas segmentation will experience significant band spreading due to the higher solution viscosity at the tubing walls. This is a well-known phenomenon that has been studied by Scott and Kucera[6] and described mathematically in an equation that predicts band broadening (V_i) in an open tube:

$$V_i^2 = \frac{d^4 FL}{24 D_m}$$

where d is internal tube diameter, L is the length of the tubing, F is the flow-rate, and D_m is the diffusion coefficient of the solute molecule. As can be seen in this equation, band spreading in an open tubular reactor is proportional to the square of the internal diameter.

Equations proposed by Giddings[7] to predict band spreading in chromatographic columns suggest that the use of tubular reactors packed with a particulate support will reduce band spreading. This is primarily due to the homogenization of flow across the tube by coupling phenomena of eddy and mobile phase diffusion. Through the efforts of many workers the effects of parameters such as particle size, mobile phase velocity, solute diffusion coefficient, and column dimensions on band spreading in a packed column are well understood. This knowledge allows one to choose proper supports and conditions to minimize band spreading in a packed flow-through detector. It should be noted, however, that chromatographic supports are designed to separate compounds, whereas separation of reactants in a continuous-flow reaction is intolerable. The enzyme, substrate(s) and product(s) must stay totally mixed while passing through the reactor just as they must remain mixed in a test tube assay.

The presence of pores in flow-through reactor packings has an important effect on band spreading. A column packing with pores as small as 40 Å in diameter

would separate many enzymes from their substrates and products by molecular sieving. On the other hand, very large pore diameter (1500 Å) supports would eliminate molecular sieving problems but introduce others. Although all enzymes, substrates, and products would totally penetrate the pores of the 1500 Å support, the enzyme band would spread more extensively than that of small molecules due to the smaller diffusion coefficient of the enzyme. Charged supports would also not be satisfactory because they would differentially retard molecules of opposite charge. Hydrophobic supports suffer the disadvantage that they selectively adsorb hydrophobic compounds. It is suggested from the above discussion that the ideal packing would be non-porous, neutral, and hydrophilic.

Kinetic considerations are also a major concern in chemically selective detectors. Linear response of the detector to different enzyme concentrations will be achieved when the substrate concentration is many times higher than the Michaelis-Menten constant (K_m) and the enzyme concentration. Consider an enzyme reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Since the initial rate of an enzyme reaction is proportional to the concentration of ES complex, we can write:

$$v = k_2[ES]$$

When the substrate concentration is so high that essentially all the enzyme in the system is present as the [ES] complex, we reach the maximum velocity $V_{max.}$.

$$v = k_2[E]$$

At saturating conditions, the reaction velocity is proportional to the enzyme concentration and is zero-order with respect to substrate concentration.

Similarly, if [S] is much greater than K_m, the reaction will reach its maximum velocity and be zero-order with respect to substrate concentration. It can be shown in the Michaelis-Menten equation that when $[S] \gg K_m$ then $v = V_{max.}$. As substrate S is depleted, the reaction becomes first order with respect to substrate concentration, and under these conditions, the assay is no longer solely dependent on enzyme concentration[8].

Sensitivity of the detector at a given enzyme concentration is obviously related to the amount of product formed. Product formation in an enzyme assay with zero-order reaction kinetics is proportional to the turn-over rate of the enzyme, the temperature and the reaction time. Since the turn-over number of an enzyme is a constant, enzyme detector sensitivity may be varied with residence time and temperature in the flow-through detector.

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out on either a Micromeritics Model 7000 liquid chromatograph (Micromeritics Instrument Company, Norcross, Ga., U.S.A.)

or an ISCO Model 384 gradient system (Instrument Specialties, Lincoln, Nebr., U.S.A.). The substrate pump was an ISCO Model 314. Injections were made with a Disc Model 706 sample injection valve with a 0.069-ml loop (Disc Instruments, Costa Mesa, Calif., U.S.A.). The two detectors used were a Perkin-Elmer LC-55 variable-wavelength detector (Perkin-Elmer, Norwalk, Conn., U.S.A.) and an Aminco Fluoro-Monitor (American Instrument Company, Silver Springs, Md., U.S.A.).

Reagents

5,5′-Dithio-(2-nitrobenzoic acid) (DTNB), nicotine adenine dinucleotide (NAD, grade AA), *p*-nitrophenol standard solution, L-lactate solution, alcohol dehydrogenase from yeast, lactate dehydrogenase (LDH; Type III from bovine heart and Type IX from bovine muscle), calf intestine alkaline phosphatase (AP; Type I) and trypsin Type IX were from Sigma (St. Louis, Mo., U.S.A.). *p*-Nitrophenyl phosphate (NPP) and DL-lactic acid were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). *p*-Nitrophenyl-*p*′-guanidinobenzoate·HCl (NPGB) was obtained from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.). Creatine phosphokinase (CPK) reagents were obtained in tablet form from Smith, Cline Instruments (Palo Alto, Calif., U.S.A.). To prepare the creatine phosphokinase reagents in bulk solution, the following were obtained from Sigma: phosphocreatine, adenosine diphosphate, glucose, adenosine monophosphate, nicotine adenine dinucleotide phosphate, glutathione, albumin, hexokinase and glucose-6-phosphate dehydrogenase.

Preparation of anion-exchange support

DEAE-Glycophase/CPG was synthesized as described previously[2,3].

Supports for post-column reaction vessel

Non-porous glass beads with a mean diameter of 40 μm were a generous gift from Dr. F. Rabel of Whatman (Clifton, N.J., U.S.A.). Non-porous glass beads in four ranges of particle sizes were also donated by the Cataphote Company (Jackson, Miss., U.S.A.). Columns were dry packed by the tap-fill procedure[9].

Protein assays

The Lowry and biuret assays were used for protein determination[10].

Enzyme assays

Activity of AP preparations was determined at four different substrate concentrations with a 0.05 *M* Tris–HCl buffer at pH 8.5 containing 0.005 *M* $MgCl_2$. The substrate was NPP in the above buffer. After pre-incubation of an aliquot of enzyme solution containing 58 μg of biuret protein in 2 ml of buffer, an equal volume of substrate solution was added to the cuvette with subsequent mixing by inversion. After a 30-sec interval, the change in transmittance with time was monitored with a Bausch & Lomb 70 spectrophotometer.

Following pre-dialysis against phosphate buffer an aliquot of beef muscle LDH containing 87 μg of Lowry protein per ml was diluted 100-fold with a solution of 0.025 *M* Tris–HCl (pH 8.3) containing 1 m*M* ethylene diamine tetracetic acid, 2 m*M* dithiothreitol and 1% bovine serum albumin. A substrate–buffer solution containing 2.5 m*M* NAD, 50 m*M* L-lactate in 0.025 *M* Tris–HCl at pH 8.3 was added to

the diluted enzyme solution and the mixture pre-incubated for 3 min. The assay was performed at ambient temperature (24°–26°) by measuring the 340-nm absorbance initially and after 10 min. The LDH enzyme from beef heart was assayed in the same manner with an undiluted enzyme solution containing 180 μg of biuret protein per ml.

Calculations

The number of moles of product that were present in a peak were determined from graphical integration of the peak using the half-width times peak height formula. By converting this number into absorbance units in the calculated peak volume, it was possible to find the moles of product when the extinction coefficient of the product was known.

The slopes and correlation coefficients for many graphs were simultaneously determined using the equations and computer programs of Bevington[11].

RESULTS AND DISCUSSION

Packing selection

It was noted in the Theoretical section of this paper that reactor packing materials should have a marked effect on band spreading. Additionally, it was pointed out that any association of enzyme, substrate, or product molecules with the post-column reactor material would result in both demixing and band spreading.

The suitability of a series of both porous and non-porous inorganic supports as reactor packing materials was examined and the results presented in Table I. Compounds with a relative retention time of 1 were totally non-retained by the support.

TABLE I

THE RELATIVE RETENTION TIMES OF COMPOUNDS ON VARIOUS SUPPORT MATERIALS IN POST-COLUMN REACTORS

A series of compounds were pumped through 0.48 × 25 cm stainless-steel columns packed with various support materials at a flow-rate of 1.25 ml/min. TAME = tosylarginine methyl ester, PDA = *p*-phenylenediamine, NP = *p*-nitrophenol, TS = *p*-toluenesulfonic acid, NPP = nitrophenylphosphate, Myo = myoglobin, Try = trypsin, Ab = albumin, AP = alkaline phosphatase. 40-Å Pore CPG and borosilicate glass beads were obtained from Corning Glass Works. 100-Å Pore Lichrosorb was obtained from EM Labs. (Elmsford, N.Y., U.S.A.).

Compound tested	*Porous material*		*Non-porous material*			
	40-Å Pore CPG	*100-Å Pore Lichrosorb*	*Borosilicate*		*Whatman glass beads*	
			Native	*GPS coated*	*Native*	*GPS coated*
TAME	2.38	3.61	>5.00	—	1.04	1.03
PDA	2.29	2.96	>5.00	2.94	1.01	1.01
NP	1.20	1.60	1.28	1.24	1.01	1.01
TS	1.06	1.51	1.13	1.07	1.02	1.02
NPP	1.02	1.34	1.03	1.02	1.01	1.02
Myo	1.05	>5.00	1.06	1.01	1.04	1.01
Try	1.02	1.25	1.02	1.02	—	—
Ab	1.00	1.00	1.00	1.00	1.00	1.00
AP	1.00	1.00	1.01	1.01	1.00	1.00

An ideal post-column reactor support would have relative retention times of 1 for all compounds. It will be seen in Table I that the retention times of small molecules were larger than those of proteins on the 40-Å, and 100-Å pore diameter supports. This is due to differential penetration of solutes into porous materials on the basis of solute size. It is interesting to note that 40-Å CPG shows less size discrimination than 100-Å Lichrosorb. This is due to the very small pore volume of the 40-Å glass (<0.1 ml/g).

The abnormally high retention times of tosylarginine methyl ester (TAME) and *p*-phenylenediamine (PDA) shown in Table I are probably the result of adsorption of these positively charged solutes to the surface of the supports. This effect was even more pronounced with borosilicate glass. Although solute adsorption was partially controlled by a glycerylpropylsilyl (GPS) coating[1] on borosilicate glass, it was still not reduced to a tolerable level in the case of PDA. Whatman non-porous support, on the other hand, showed little discrimination between any of the solutes. On the basis of these findings, the uncoated Whatman support was chosen for most studies. Some solutes, such as trypsin, adsorbed on virtually all surfaces, so solutions with albumin or high salt concentrations were pumped concurrently to control adsorption.

Reactor design

In order to gain the benefits of sensitivity and selectivity afforded by a chemically selective detector, the resolution achieved in the chromatographic system must be maintained in the post-column reactor. This implies that band spreading must be kept at a minimum. The theoretical analysis of band spreading in both capillary tubing and columns packed with non-porous spheres suggested that the packed columns would be superior as a post-column reactor and that the particle size in the packed column would be relatively unimportant. Fig. 2 confirms that band spreading in a 50 ft. × 0.02 in. I.D. capillary is indeed greater than in a 600 × 4.1-mm column packed with non-porous spheres of 40-μm mean diameter. The capillary column provides less than 1/3 of the number of plates obtained with a packed column.

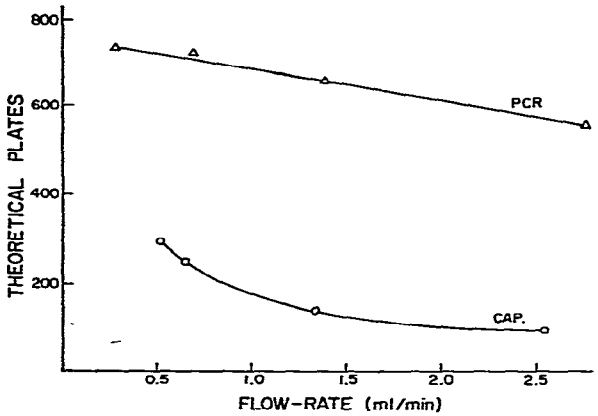


Fig. 2. The comparison of band spreading between a capillary tubing (CAP) and a packed column reaction (PCR) vessel. The capillary tubing was a 50-ft. coil of 0.02-in. I.D. while the packed column was a 600 × 4.1-mm precision bore tube. The column was packed with Whatman glass spheres. The solute was 0.5 m*M* NP 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.

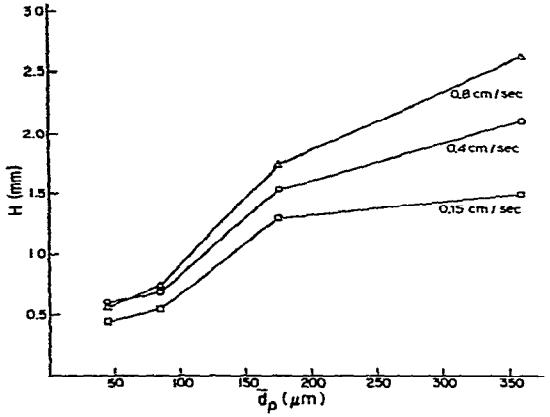


Fig. 3. Effect of mean particle diameter on plate height for 3 linear velocities. Solute, benzene; mobile phase, isopropanol; detector, ultraviolet (UV), 254 nm; temperature, room temperature; column, 4.1 × 300 mm stainless steel; pressure, 100–200 p.s.i.

The packed columns also permitted longer reaction times with less band spreading.

The study of the effect of particle size on efficiency for non-porous spheres is shown in Fig. 3. Reduction in plate height with decreasing particle size diminishes below 100 μm mean particle diameter. This suggests that the increased efficiency to be gained by using microparticulate non-porous supports would be minimal. Supports in the 40–100-μm range have the relative advantages over 5–10-μm supports of (a) negligible pressure drop (several hundred p.s.i.) even at very high linear velocities, (b) lower cost, and (c) ease of packing. The 5–10-μm non-porous spheres are particularly difficult to slurry pack because they are 5–10 times more dense than porous supports.

The effect of column diameter on band spreading is also important. As shown in Fig. 4, columns with 4.1-mm I.D. had much greater efficiencies than those of 3.2

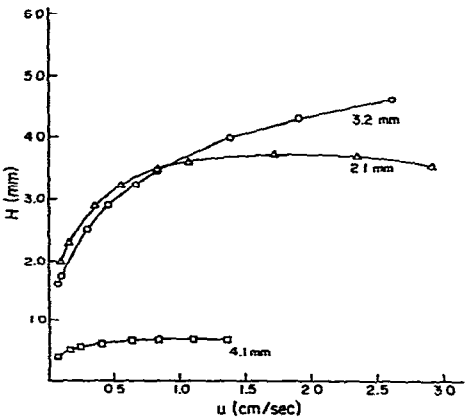


Fig. 4. Plate height curve for non-porous spheres with benzene as the solute for 3 column diameters. Mobile phase, isopropanol; detector, UV, 254 nm; temperature, room temperature; particle diameter, 28–53 μm; pressure, 200 p.s.i.; columns, 4.1 × 300 mm, 2.1 × 300 mm, 3.2 × 250 mm stainless steel.

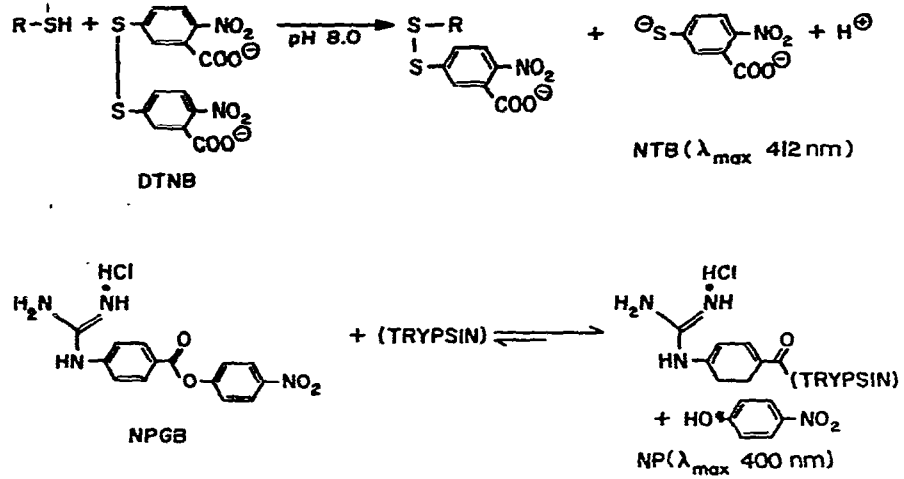


Fig. 5. Colorimetric reactions of DTNB with sulfhydryl groups and NPGB with trypsin.

and 2.1 mm. This is probably due to wall effects in the narrow bore columns and the ease of packing wider columns. The data in this study were fitted with a least squares fit to a semi-empirical equation used by Kennedy and Knox[12]:

$$h = \frac{2\gamma}{v} + Av^{0.33} + Cv$$

where h is the reduced plate height (H/d_p), H is the plate height, d_p is the particle diameter, v is the reduced velocity (ud_p/D_m), u is the linear velocity, D_m is the diffusion constant of the solute in the mobile phase, γ is an obstruction factor equal to 0.6 for non-porous materials and A and C are constants. The correlation coefficients (R) obtained by linear regression for these data ranged from 0.9 to 0.97 which was regarded as good confidence in the fit.

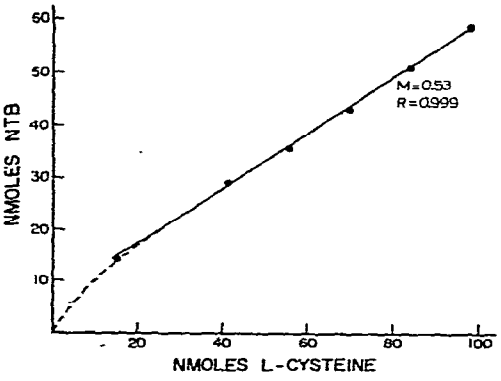


Fig. 6. The reaction of DTNB with cysteine in a packed column reactor. The column was a 600 × 4.1-mm precision bore tube packed with Whatman glass spheres. The main pump was filled with 0.1 M NaH_2PO_4 buffer (pH 8.0), and the reagent pump with 10 mM DTNB in the same buffer. Both pumps were driven at equal rates generating a total flow-rate of 1.41 ml/min. The reaction product, NTB, was detected at 412 nm. Each point was determined from the mean of duplicate injections. M = slope.

Colorimetric reactions

Before post-column reactors were studied in the detection of enzymes, their use for simple colorimetric reactions was investigated. The reaction of DTNB with sulfhydryl groups is fairly rapid and can be used to make a sulfhydryl-selective detection system. Fig. 5 shows the reaction of DTNB with a sulfhydryl-containing amino acid or protein. Both molecules are detected linearly in the analyzer. The reaction of cysteine with DTNB, as seen in Fig. 6, did not reach completion since the amount of nitrothiobenzoate (NTB) detected was less than the amount of cysteine applied. The linearity of response was nonetheless above the 95% confidence limit. Fig. 7 shows that the sulfhydryl-containing protein gave a linear response and closely reflected the known stoichiometry of the reaction as evidence by a slope of almost unity.

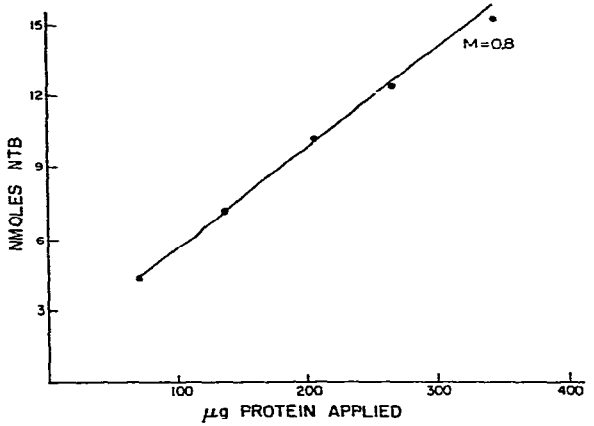


Fig. 7. The reaction of DTNB with yeast alcohol dehydrogenase in a packed column reactor. The column was a 600 × 4.1-mm precision bore tube packed with Whatman glass spheres. The main pump was filled with 0.1 *M* NaH_2PO_4 buffer (pH 8.0), and the reagent pump with 10 m*M* DTNB in the same buffer. Both pumps were driven at the same rate, generating a total flow-rate of 1.41 ml/min. Each point was the mean of duplicate injections.

Concentrations of some enzymes may also be determined colorimetrically. Recently a number of stoichiometry reagents have been developed that titrate the active site of an enzyme[13]. These reagents make it possible to determine the concentration of an enzyme solution with an endpoint reaction. A very effective active site titrant for trypsin is NPGB[14]. NPGB reacts only with active trypsin (Fig. 5) and is insensitive to other enzymes and even to the inactive forms of trypsin. Using this reagent, it is possible to quantitate trypsin in a flow-through reactor as shown in Fig. 8. Since trypsin adsorbs strongly to many surfaces, it was necessary to include 0.5 *M* NaCl in the buffer to prevent adsorption. The linearity is quite remarkable in view of the difficulty of mixing an organic solvent containing NPGB and high ionic strength buffers containing the enzyme.

Enzyme reactions

These studies focused primarily on three clinically important enzymes found in human serum: AP which is a hydrolase, LDH which is a reductase and CPK which

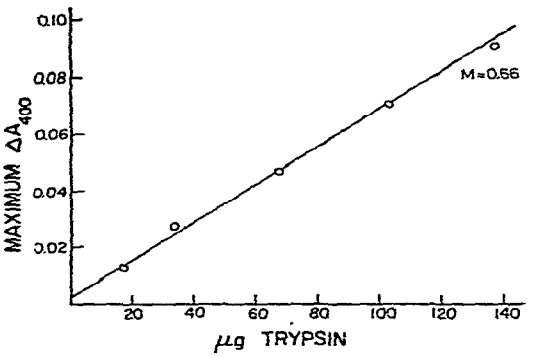
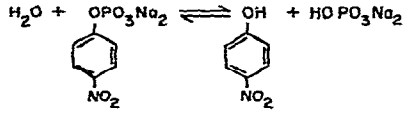


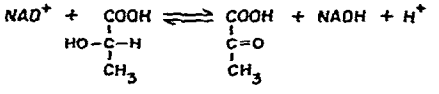
Fig. 8. The reaction of trypsin with the trypsin active site titrant, NPGB, in a packed column reactor. The column was a 300 × 4.1-mm precision bore tube packed with Whatman glass spheres. The main pump was filled with 0.05 *M* Tris–HCl buffer (pH 8.2) containing 0.5 *M* NaCl and 0.005 *M* $CaCl_2$, and the reagent pump was filled with 1 m*M* NPGB in abs. methanol. The reagent pump was driven at 0.82 ml/min and the main pump at 1.90 ml/min. The reaction product, NP, was detected at 400 nm. Each point was the mean of duplicate injections.

ENZYME REACTIONS

1. ALKALINE PHOSPHATASE



2. LACTATE DEHYDROGENASE



3. CREATINE PHOSPHOKINASE

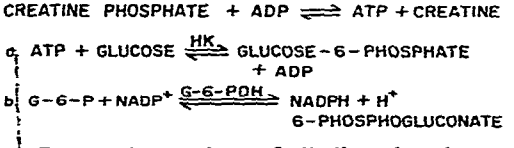


Fig. 9. Enzymatic reactions of alkaline phosphatase, lactate dehydrogenase, and creatine phosphokinase.

is a kinase. The reactions employed in the kinetic assay of these enzymes are diagrammed in Fig. 9.

Factors that affect the response of the continuous-flow analyzer were investigated in the absence of an analytical column. From the integrated Michaelis-Menten equation in Fig. 10 it is apparent that the amount of product formed in an enzymatic reaction depends on the reaction time. AP, an enzyme with fairly simple kinetics, was used to examine linearity of the kinetics in the flow-through system. Fig. 11 shows that product formation as a function of time is linear, having a correlation coefficient of 0.99. The minimum reaction time in a continuous-flow enzyme detector

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

MICHAELIS-MENTEN EQUATION

$$-\frac{d(S)}{dt} = \frac{k_2 (E)(S)}{K_m + (S)}$$

INTEGRATED MICHAELIS-MENTEN EQUATION

$$(S_0) - (S_t) + K_m \ln ((S_0)/(S_t)) = k_2 (E)t$$

LET:

$t = R_t$

$(S_0) - (S_t) = (P_t)$

ASSUME:

$(S_0)/(S_t) \cong 1$

THEN:

$(P_t) = k_2 (E)R_t$

WHERE PRODUCTS AND ENZYMES OCCUPY SAME VOLUME

$P_t = k_2 E_0 R_t$

Fig. 10. Use of the integrated Michaelis-Menten equation to establish the kinetic basis for the proportionality between the amount of applied enzyme and the amount of detected products. (S_0) is the original substrate concentration, (S_t) is the substrate concentration at time t, (P_t) is the product concentration at time t, R_t is the reaction time or residence time in the reaction vessel, and E_0 is the original amount of enzyme.

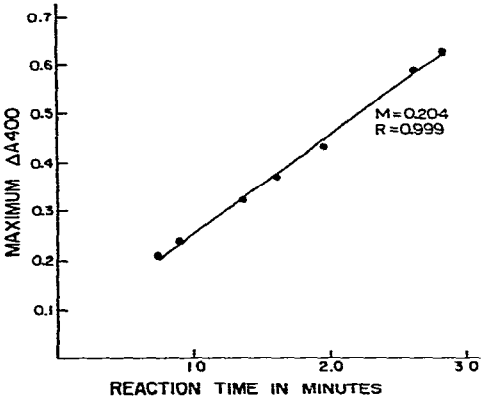


Fig. 11. The effect of reaction time on the alkaline phosphatase catalyzed formation of NP in the packed column reaction vessel. The column was a 300 × 4.1-mm precision bore tube filled with Whatman glass spheres. The main pump was filled with 0.05 *M* sodium borate buffer (pH 8.5) containing 0.005 *M* $MgCl_2$, and the reagent pump was filled with 4 m*M* PNP in the same buffer. Both pumps were driven at equal rates, and the overall flow-rate varied from 0.678 to 2.65 ml/min. The enzymatic product, NP, was detected at 400 nm. Each point was determined from duplicate injections of 33 μg of an alkaline phosphatase preparation as determined by Biuret analysis.

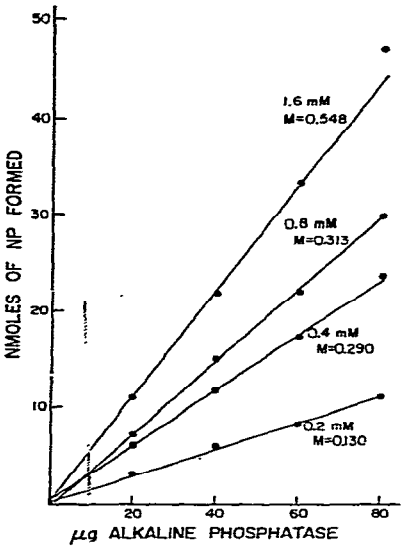


Fig. 12. The effect of substrate concentration (m*M*) on the alkaline phosphatase catalyzed formation of NP in the packed column reaction vessel. The column was a 300 × 4.1 mm-precision bore tube filled with Whatman glass spheres. The main pump was filled with 0.05 *M* borate buffer (pH 8.5) containing 0.005 *M* $MgCl_2$, and the reagent pump was filled with 4 m*M* PNP in the same buffer. Both pumps were driven at the same rate and generated an overall flow-rate of 1.16 ml/min. The enzymatic product, NP, was detected at 400 nm. The amount of applied alkaline phosphatase reflected the total amount of Biuret protein in the injected sample, and each point was the mean of duplicate injections.

is that time required to generate a signal that is about one order of magnitude above the background noise of the detector. Actually, reaction times greater than 5–10 min are not practical in a post-column reactor. Long reaction times require lengthy reactor columns and band spreading increases in proportion to column length.

As noted in the Theoretical section, enzyme reaction rate is dependent on the substrate concentration until saturating conditions are reached. Fig. 12 shows that the amount of product generated by AP in a fixed time can be increased 4-fold by increasing the substrate concentration in the reactor 8-fold. In the case of AP, it is seen that the reaction is linear even below saturating substrate concentrations. However, maximum sensitivity in a continuous-flow enzyme analyzer is obtained only when the system is operated at saturating substrate concentration.

LDH is a tetrameric enzyme composed of two different types of subunits. LDH 5, the isoenzyme that is predominant in skeletal muscle, is composed of four equivalent subunits of type M. LDH 1, the isoenzyme found mainly in heart tissue, is composed only of type H (ref. 15). The three remaining isoenzymes are the possible tetrameric hybrids of the H and M type of subunit. As seen in Fig. 13, the detected response to increasing concentrations of both M_4 and H_4 LDH isoenzyme is linear. The reaction of LDH with lactic acid is diagrammed in Fig. 9.

Applications

Chromatographic resolution of LDH isoenzymes using continuous-flow enzyme detection is seen in Fig. 14. The identities of LDH 1–5 eluting from these DEAE

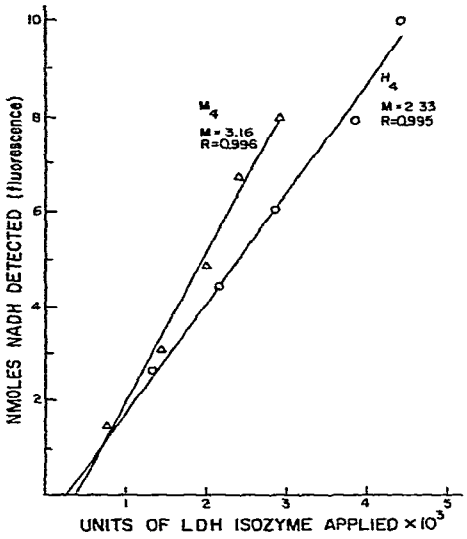


Fig. 13. Response of the continuous-flow analyzer to varying concentrations of the LDH 1 (H_4) and the LDH 5 (M_4) isoenzymes. The post-column reaction vessel was a 600 × 4.1-mm precision bore tube packed with Whatman glass spheres. The main pump was filled with 0.025 *M* Tris–HCl (pH 8.3) containing 0.2 *M* NaCl, and the reagent pump with 100 m*M* L-lactate and 5 m*M* NAD in the same buffer. Both pumps were driven at the same flow-rate for each determination, but the overall flow-rate for the M_4 determination was 1.32 ml/min and for the H_4 determination it was 1.44 ml/min. The resulting NADH was detected with the Fluoro-Monitor. Each point represents the mean of duplicate injections.

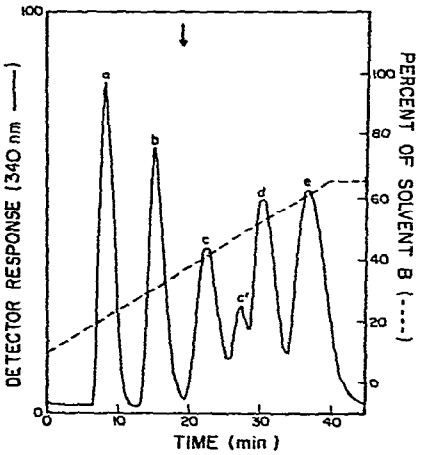


Fig. 14. The separation of 5 LDH isoenzymes on 37–74-μm 250-Å pore diameter DEAE-Glycophase/CPG. The main pumping system was a gradient system operated as specified. The reagent pump was filled with a solution of 0.05 *M* Tris–HCl (pH 8.8) containing 0.279 *M* D,L-lactate, 1 m*M* NAD, and 1% bovine serum albumin. The post-column reaction vessel was a 600 × 4.1-mm precision bore tube packed with 37–74-μm non-porous glass particles that were coated with a glycerol polymer. The reagent pump was driven at half the overall flow-rate for the main pumping system. The resulting NADH was detected at 340 nm. Column, 600 × 4.8 mm stainless steel. Solvents: A, 0.02 *M* Tris (pH 8.0); B, 0.025 *M* Tris (pH 8.0)–0.2 *M* NaCl. Flow-rate, 2.25 mm/sec; pressure, 100 p.s.i. Peaks: a, LDH 5; b, LDH 4; c, LDH 3; d, LDH 2; e, LDH 1.

columns have been confirmed electrophoretically. The anomalous peak c′ sometimes results from the freeze-thaw hybridization of beef heart LDH 1 and rabbit muscle LDH 5 and is considered to be an artifact of the method of preparation[16].

In an effort to increase the sensitivity of enzyme detection, the LDH reaction

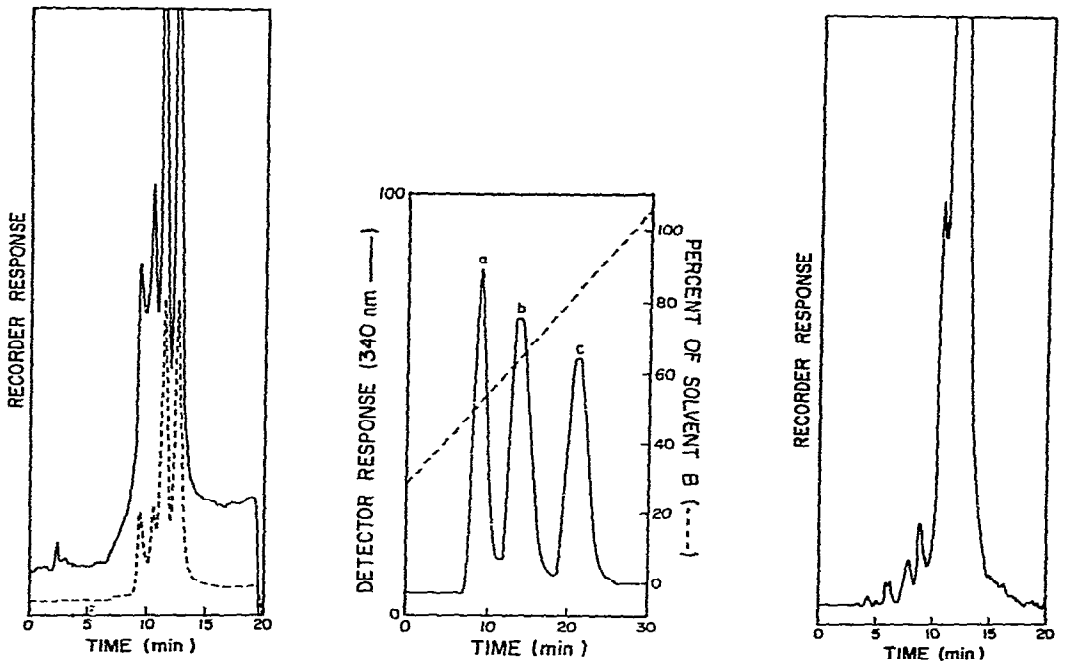


Fig. 15. The comparison of fluorescence with absorption detection in the the high-speed separation of 4 LDH isoenzymes. The analytical column was packed with 5–10-μm 250-Å pore diameter DEAE-Glycophase/CPG, the post-column reaction vessel was a 600 × 4.1-mm precision bore tube and was packed with Whatman glass spheres. The total pressure at the head of the system varied between 1500 and 2000 p.s.i. Buffer pumps: A, 0.025 *M* Tris (pH 8.3); B, 0.025 *M* Tris (pH 8.3)–0.2 *M* NaCl, 10 min linear gradient, flow-rate 1.5 ml/min. Substrate pump: 10 mm NAD, 100 mm lactate solution (pH 8.3) at a flow-rate of 50 ml/h. Detection: ———, fluorescence; – – –, absorbance at 340 nm, 0.05 V f.s.

Fig. 16 Separation of 3 CPK isoenzymes on a 37–74-μm 250-Å pore diameter DEAE/Glycophase columr. The main pumping system was a gradient system that was filled and operated as specified. The reagent pump was filled with the CPK reagent buffer described in the experimental section. The post-column reaction vessel was a 600 × 4.1-mm precision bore tube packed with 37–74-μm non-porous glass particles that were coated with a glycerol polymer. The flow-rate of the reagent pump was half that of the main pumping system. The product of the coupled enzymatic reaction, NADPH, was detected at 340 nm. Column, 600 × 4.8 mm stainless steel. Solvents: A, 0.05 *M* Tris–0.05 *M* NaCl–10^{-3} *M* mercaptoethanol, pH 7.5; B, 0.05 *M* Tris–0.3 *M* NaCl–10^{-3} *M* mercaptoethanol, pH 7.5. Flow-rate, 2.25 mm/sec; pressure, 100 p.s.i. Peaks: a, CPK_3; b, CPK_2; c, CPK_1.

Fig. 17. The high-speed separation of LDH isoenzymes from serum that was known to be elevated in LDH. The analytical column was packed with 5–10-μm 250-Å pore diameter DEAE/Glycophase CPG and the post-column reaction vessel was a 600 × 4.1 mm tube packed with Whatman glass spheres. Total pressure at the head of the system varied between 1500 and 2000 p.s.i. Buffer pumps: A, 0.025 *M* Tris (pH 8.3); B, 0.025 *M* Tris (pH 8.3)–0.2 *M* NaCl, 25 min linear gradient, flow-rate 1.5 ml/min. Substrate pump: 10 mm NAD, 100 mm lactate solution (pH 8.3) at a flow-rate of 50 ml/h. Fluorescence detection.

product, NADH, was monitored with a fluorescence detector. Fig. 15 shows both the absorbance and fluorescence response to the NADH produced by LDH isoenzymes in serum. Absence of LDH 5 in this sample was confirmed electrophoretically and was probably the result of LDH 5 instability with extended freezing[17]. The fluorescence detector was found to be 50-fold more sensitive for NADH than the absorbance detector. In the detection of M_4 isoenzyme, the working range was 6–60 ng of injected M_4 protein. Since each enzyme molecule generated multiple product molecules, there is a molar amplification of the signal generated. The chemical amplification occurring by the conversion of NAD to NADH averaged 1.8×10^4 at saturating substrate concentration. By increasing both temperature and reaction time this amplification could easily be increased to 10^5.

The assay of CPK is accomplished through a coupled enzyme assay as shown in Fig. 9. Through the use of hexokinase and glucose-6-phosphate dehydrogenase, CPK is coupled to produce NADPH. Fig. 16 shows that when the blend of substrates, enzymes, and cofactors necessary for the coupled assay of CPK are introduced through the substrate pumping system of the post-column reactor, CPK isoenzymes may be detected in a sample.

The high-speed analyses of partially purified preparations of known isoenzyme content indicate that the union of a high-pressure liquid chromatography column with a continuous-flow analyzer is feasible. However, a more difficult test of the total system is the analysis of a crude biological extract containing a multitude of components. Fig. 17 shows the separation of the five LDH isoenzymes in a human serum sample with an abnormally high total LDH level. Before injection, it was necessary to dialyze the sample against phosphate buffer to remove materials that strongly adhere to the analytical column and adversely affect resolution. The low levels of LDH 5 and LDH 4 observed in the chromatogram may be due to the greater sensitivity of these isoenzymes to freezing[17]. In normal serum the LDH 2 isoenzyme is present at higher levels than the LDH 1, but in cases of myocardial damage the reverse is usually true. The so-called LDH flip is very prominent in the above separation of serum LDH isoenzymes, and the elevated levels of both the LDH 1 and LDH 2 isoenzyme together indicate the strong possibility of myocardial damage.

CONCLUSIONS

It has been demonstrated that columns packed with non-porous spherical glass beads of less than 100 μm in mean particle diameter are suitable for use in countinuous-flow chemically selective detectors for liquid chromatography. These post-column reactors have three times the efficiency of 0.02-in.-I.D. open tubular capillaries and may be used to monitor colorimetric and enzymatic reactions. The detection system consists of a reagent or substrate pumping system, a packed post-column reactor, and a photometric detector. When used in enzyme-specific detection, the system will respond selectively and linearly to a single class of enzymes in the presence of a large number of other chemical species. Sensitivity of the enzyme detector is a function of the molar absorptivity of the product, turn-over number of the enzyme, reaction temperature, and reaction time. In the case of LDH, the lower limit of detection was 6 ng (4×10^{-14} moles). This sensitivity may be attributed to the fact that a catalyst is being detected by monitoring the reaction it catalyzes. Each molecule of LDH

generated 1.8×10^4 molecules of NADH during residence in the post-column reactor The detection system has ample sensitivity to monitor clinically significant enzymes found in serum.

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