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Short Communication

Application of an enzyme-based stationary phase to the determination of enzyme kinetic constants and types of inhibition

New high-performance liquid chromatographic approach utilizing an immobilized artificial membrane chromatographic support☆

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ABSTRACT

The application of an immobilized enzyme HPLC column to the qualitative and quantitative determination of enzyme kinetics has been investigated. The enzyme used in this study was α-chymotrypsin (ACHT) which was immobilized by absorption into a commercially available immobilized artificial membrane (IAM) interphase. The resulting IAM-ACHT phases were enzymatically active and catalyzed the hydrolysis of L-tryptophan methyl ester to L-tryptophan. The interaction between the IAM-ACHT phase and known reversible inhibitors of ACHT has been studied with hydrocinnamic acid (HCA) and β-phenylethylamine (BPEA), and the results demonstrate that displacement chromatography can determine the type and degree of enzyme/inhibitor interactions. In addition, an inhibition constant (K_I) of 1.8 m*M* for the competitive inhibition by HCA was calculated which is consistent with the previously reported value of 4.5 m*M* determined using non-immobilized ACHT. For BPEA the calculated K_I was 8.5 m*M* and the inhibition was mostly noncompetitive. This indicates that the IAM-ACHT can be used to quantitatively determine the enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions. The same immobilized enzyme was repeatedly used over a 10-day period to study enzyme kinetics.

INTRODUCTION

In the past few years there has been a rapid growth of high-performance liquid chromatography (HPLC) chiral stationary phases (CSPs) based upon immobilized proteins [1–4]. The success of immobilized protein supports as

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HPLC phases has prompted the investigation of the chromatographic utility of another class of biopolymers, enzymes. For example, Wainer and co-workers have covalently immobilized α-chymotrypsin (ACHT-CSP) [5–7] and trypsin (TRYP-CSP) [8] on a silica-based HPLC support. The resulting phases were used in the stereochemical resolution of amino acids, amino acid derivatives and dipeptides. The observed chiral resolutions were based upon two mechanisms: (1) stereoselective enzymatic hydrolysis of only one of the isomers of an enantiomeric substrate, *e.g.* hydrolysis of L-tryptophanamide but not D-tryptophanamide; (2) relative stabilities of the diastereomeric enzyme/pseudo-substrate complexes formed during the chromatographic process [6].

It was also reported [6] that the hydrolytic activity of the immobilized ACHT could be inhibited by injecting 4-nitrophenyl trimethylacetate (4-NTA) onto the ACHT-CSP. 4-NTA is a reversible inhibitor of ACHT and the enzymatic activity of the ACHT-CSP was regenerated by washing the column with the appropriate phosphate buffer. These results indicate that the covalently immobilized ACHT-CSP retained its sensitivity to enzyme inhibitors.

One of the problems associated with the covalent immobilization of enzymes on silica-based HPLC supports is the possible restriction of the conformational mobility of the enzyme by the immobilization process. In order to avoid this potential problem, Chui and Wainer [9] immobilized ACHT and TRYP on an immobilized artificial membrane (IAM) HPLC support to create the IAM-ACHT and IAM-TRYP phases.

The IAM support was developed by Pidgeon *et al.* [10], and produced through the covalent immobilization of a diacylphosphatidylcholine on an aminopropyl silica. The synthetic lipid was 1-myristoyl-2-[(13-carboxyl)tridecanoyl]-*sn*-3-glycerophosphocholine which was covalently attached to the silica support through the ω-carboxyl group on the C-2 fatty acid chain. In the resulting support (IAM·PC), the phosphatidylcholine headgroups form the surface of the support and the hydrocarbon side chains produce a hydrophobic interphase which extends from the charged headgroup to the surface of the aminopropyl silica. The residual amino groups in the IAM stationary phase may be free (IAM·PC), or endcapped with methylglycolate (IAM·PC·MG).

In addition to ACHT and TRYP, the IAM·PC chromatographic support has been used to immobilize lipase [11] and hepatic microsomes [12]. Both phases were enzymatically active. For example, the microsome-based immobilized enzyme reactor was active in catalysing on-line production of phase I and phase II biotransformation metabolites including 7-hydroxycoumarin formed by O-deethylation of 7-ethoxycoumarin in the presence of NADPH (P-450 catalyzed monooxygenation) [12] and glucuronides produced by O-glucuronidation of 7-hydroxy-4-methylcoumarin and 4-nitrophenol in the presence of uridine 5′-diphosphoglucuronic acid (UDPGA) [13].

The IAM-ACHT and IAM-TRYP supports also retained the hydrolytic activity of the native enzymes —IAM-TRYP catalyzed the hydrolysis of Nα-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and IAM-ACHT catalyzed the hydrolysis of a number of substrates including L-tryptophan methyl ester [9]. The hydrolytic activity of the immobilized enzyme could be determined from the resulting substrate/product ratios obtained either directly from IAM-ACHT chromatograms or from chromatograms produced by a coupled column system. In addition, the activities of both supports were decreased by known enzyme inhibitors and the activity of the IAM-ACHT was affected by changes in pH and temperature [9].

The results of the initial study suggested that IAM-ACHT and IAM-TRYP could be used as chromatographic probes of enzyme/substrate and enzyme/inhibitor interactions. The present study continues this investigation and examines the utility of the IAM-enzyme columns in the determination of the qualitative and quantitative aspects of enzyme kinetics. In particular, the hydrolysis of L-tryptophan methyl ester on the IAM-ACHT was examined alone and in the presence of eight known reversible ACHT inhibitors.

The results of this study indicate that the

IAM-ACHT can be used to qualitatively determine the type and degree of enzyme/inhibitor interactions and quantitatively determine the enzyme kinetic constants associated with enzyme/substrate and enzyme/inhibitor interactions.

EXPERIMENTAL

Chemicals

α-Chymotrypsin (ACHT, 57 U/mg protein, 84% protein) and the ACHT inhibitor indole were purchased from ICN Biochemicals (Cleveland, OH, USA). L-Tryptophan methyl ester·HCl (L-Trp-OMe) was purchased from American Chemicals (Montreal, Canada). D-Tryptophan methyl ester·HCl (D-Trp-OMe), L-tryptophan (L-Trp), ACHT inactivated with DFP (diisopropylphosphofluoridate), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and the remaining seven ACHT inhibitors tested: hydrocinnamic acid (HCA), β-phenylethylamine·HCl (BPEA), indole-3-propionic acid, α-acetamidocinnamic acid, hippuric acid, N-benzoyl-DL-methionine and N-benzoyl-DL-phenylalanine were all obtained through Sigma (St. Louis, MO, USA).

Apparatus

The chromatographic experiments were performed with a modular HPLC system which consisted of a Beckman 110B solvent module pump (Beckman Instruments, Houston, TX, USA), a Rheodyne 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA), a 783 programable UV absorbance detector (ABI Analytical, Ramsey, NJ, USA) and a DataJet integrator (Spectra-Physics, San Jose, CA, USA).

Chromatographic columns

The frontal elution experiments were performed with 15 cm × 4.6 mm I.D. IAM·PC (12 μm, 300 Å) chromatographic columns. For the displacement chromatography experiments, chromatographic cartridges (1 cm × 3.0 mm I.D.) and columns (3 cm × 4.6 mm I.D.) packed with the same IAM·PC support were used. The samples from the experiments with non-immobilized ACHT were analyzed on a 15 cm × 4.6 mm I.D. Rexchrom Octyl (5 μm, 100 Å) column. All the columns and cartridges were obtained from Regis (Morton Grove, IL, USA).

Chromatographic conditions

On the IAM·PC columns the mobile phase consisted of a sodium phosphate buffer (0.1 *M*, pH 6.8), and the flow-rate was 0.5 ml/min. L-Tryptophan and L-tryptophan methyl ester were quantitated by UV absorption at 280 nm. On the octyl column (used for the determination of substrate conversion with the non-immobilized enzyme), the mobile phase consisted of 0.1 *M* sodium dihydrogenorthophosphate adjusted to pH 3.0 with orthophosphoric acid–acetonitrile (9:1, v/v). The flow-rate was 0.5 ml/min. The experiments were performed at ambient temperature.

Experiments with the immobilized ACHT

Frontal affinity chromatographic approach. The IAM·PC-ACHT (15 cm × 4.6 mm I.D.) column was prepared as described earlier [9]. For each inhibitor, a range of concentrations in a sodium phosphate buffer (0.1 *M*, pH 6.8) were perfused through the column and the elution volume (V) determined for each concentration from the breakthrough elution profile [14]. After each experiment, the inhibitor was washed out from the column using the sodium phosphate buffer (0.1 *M*, pH 6.8).

Identical sets of experiments were performed on an IAM·PC column without the enzyme and on an IAM·PC-ACHT·DFP column. The latter column was prepared in a same way as IAM·PC-ACHT, except that the ACHT used was irreversibly inactivated with diisopropylphosphofluoridate (DFP). An IAM stationary phase containing ACHT irreversibly inactivated with TPCK, was prepared to conduct a single frontal elution experiment with N-benzoyl-DL-phenylalanine.

The enzymatic activity in all the tested columns was checked by injecting 20 μl of 1 m*M* L-tryptophan methyl ester.

Displacement chromatography approach. A range of L-tryptophan methyl ester concentrations (0.5, 1, 2, 3 and 4 m*M*) were injected onto

an IAM · PC-ACHT 1-cm cartridge connected in series to an IAM · PC 3-cm column. The experiment was repeated with several fixed inhibitor concentrations (1, 2, 5 and 10 m*M* HCA; 5 and 10 m*M* BPEA) in the mobile phase. The product formed (L-tryptophan) and the unhydrolyzed substrate were monitored at 280 nm. The injected sample volume was 20 μl, the mobile phase flow-rate was 0.5 ml/min, and all the experiments were performed at room temperature (25°C).

Calculations

The experimentally obtained data (average values from 2–5 HPLC determinations) were processed in terms of Michaelis–Menten kinetics. Kinetic constants were calculated from double reciprocal Lineweaver–Burk plots $1/s$ *versus* $1/v$ (where s is the substrate concentration and v the rate of enzymatic reaction). The initial L-tryptophan methyl ester concentrations were plotted against the specific activity of ACHT. These parameters were determined by the rate of the enzymatic conversion of L-tryptophan methyl ester to L-tryptophan. The rate of enzymatic reaction is presented by the amount of L-tryptophan formed (or the decrease in L-tryptophan methyl ester) during the reaction time. The reaction time is defined by the time the substrate resides in the IAM-ACHT chromatographic column, which is dependent upon the flow-rate in the chromatographic system. The flow-rate was held constant at 0.5 ml/min throughout the study.

RESULTS AND DISCUSSION

The experimental approaches used in this work included both frontal elution and displacement chromatography techniques. The results of the frontal affinity chromatographic experiments with the eight inhibitors indicated that the calculated affinities (K_M) of the inhibitors for the IAM · PC are as great or greater than the K_M calculated for the supports containing active or inactivated enzymes.

Pidgeon *et al.* [10] have demonstrated that retention on the IAM support involves both hydrophobic and electrostatic interactions and both moieties are present in the inhibitors used in this study. From the experimental results with the enzymatically active and inactivated form of ACHT, it appears that the interactions between the IAM support and these compounds are so great that they mask the interactions between the immobilized enzyme and the solutes. In the ideal situation the chromatographic support for frontal affinity chromatography should be negligibly retentive or not retentive at all for the compounds tested. Thus, the frontal elution approach could not be used with the IAM · PC-ACHT to determining the K_M of the inhibitors for ACHT. However, new supports under development by Pidgeon [15] should address this problem and will be tested when they are available.

Bearing in mind that chromatographic supports are always more or less retentive under applied conditions, it should also be mentioned that injecting a mixture of a substrate and an (reversible) inhibitor onto a column with immobilized enzyme will give us a real picture of their interaction only if the contact between all the "reactants" is maintained through the whole length of the chromatographic column. The enzyme being uniformly present in the chromatographic support, this means that both the substrate and the inhibitor should have the same retention time.

These problems were circumvented by the use of displacement chromatography. In this approach, the inhibitor is added to the mobile phase and equilibrated with the chromatographic support, *i.e.* the enzyme. In this technique, the substrate displaces the inhibitor from the enzyme and the extent of the observed enzymatic reaction is a function of both the K_I of the inhibitor and the K_M of the substrate. Different affinities of the substrate and the inhibitor for the chromatographic support itself will not affect the rate of reaction, because the inhibitor is supplied in constant concentration in the mobile phase. The reaction time is equal to the retention time of the substrate in the column with the immobilized enzyme, and is generally not affected by the presence of inhibitors. Thus, the product formed is the real picture of the enzyme/inhibitor relationship in the chromatographic column.

The necessity of placing the inhibitors in the mobile phase eliminated from further study all but two of the inhibitors, HCA and BPEA. Unlike the other inhibitors, these compounds do not display a significant UV absorption at 280 nm, the wavelength used to monitor the substrate and product. Although not an impossible task, the background absorption of the high inhibitor concentration in the mobile phase could make difficult to monitor the subtle increase in the UV absorbance generated by the enzymatically produced product peak in the initial stage of the enzymatic reaction. The substrate chosen for this study was L-Trp-OMe which is converted by ACHT to L-Trp. While L-Trp-OMe is a substrate for ACHT, D-Trp-OMe is not enzymatically cleaved. However, both enantiomers hydrolyze spontaneously in 0.1 *M* sodium phosphate buffer pH 6.8 which can be a source of error in these studies. To overcome this problem, the extent of hydrolysis of D-Trp-OMe was used as a blank for the spontaneous hydrolysis of the L-enantiomer and storing the L- and D-Trp-OMe standard solutions at −20°C reduced the spontaneous hydrolysis to less than 1% in 10 days.

In order to follow the Michaelis–Menten kinetics of the ACHT hydrolysis of L-Trp-OMe, not more than 30% of the initial substrate should be hydrolyzed during each experimental observation; this keeps the experimental conditions within the linear range of the enzyme kinetics [16]. The amount of ACHT immobilized on the 15 cm × 4.6 mm I.D. column was too large for the substrate and inhibitor concentrations used in these studies and a column with a less ACHT was necessary. The required immobilized enzyme concentration was achieved using a 1-cm IAM · PC cartridge. The amount of ACHT on the IAM cartridge was adjusted using a 1 mg/ml solution of ACHT which was either perfused through the cartridge or introduced by repeated bolus injections. After each loading, the enzymatic activity was checked using L-Trp-OMe and the amount of ACHT was adjusted to keep the maximum hydrolysis of the substrate less than 30%.

A baseline separation of L-Trp and L-Trp-OMe could not be achieved on the 1-cm IAM · PC-ACHT which acts as an enzymatic reactor rather than a chromatographic column. In order to obtain a total chromatographic resolution of the substrate and product, a 3-cm IAM · PC column was attached in series. Fig. 1 presents some typical chromatograms from this coupled column system.

The results of the displacement chromatographic studies were analyzed using Lineweaver–Burk plots which are presented in Fig. 2. The K_I values for HCA and BPEA and the K_M values for L-Trp-OMe determined from the Lineweaver–Burk plots are presented in Table I.

The initial qualitative examination of the data revealed a competitive type of ACHT inhibition for HCA which is consistent with the previously reported mode of HCA inhibition [17,18]. The K_I values determined in this study, 1.8 and 6.1 m*M* (Table I), are also consistent with the previously reported value of 4.5 m*M* [17,18]. The literature value was calculated using a single concentration of the substrate, acetyl-L-tyrosinamide, a pH of 7.8, 25°C and the assumption of competitive inhibition. The kinetic measurements reported in this paper were performed using a range of L-Trp-OME concentrations (0.5 to 4.0 m*M*), a pH of 6.8 (the previously determined optimal value for the IAM-ACHT column) and 25°C.

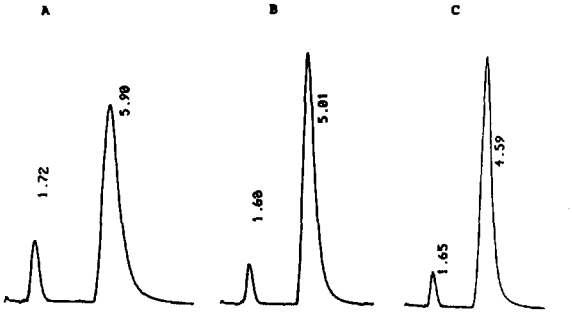

Fig. 1. Chromatograms following injection of L-tryptophan methyl ester 3 m*M* onto an IAM · PC-ACHT (1 cm × 3.0 mm I.D.) cartridge connected in series to an IAM · PC (3 cm × 4.6 mm I.D.) column. The first eluting peak corresponds to the product, L-tryptophan, the second eluting peak corresponds to L-tryptophan methyl ester. (A) No inhibitor in the mobile phase; (B) mobile phase containing 5 m*M* β-phenylethylamine; (C) mobile phase containing 10 m*M* β-phenylethylamine. Values at peaks are retention times in min. See text for chromatographic conditions.

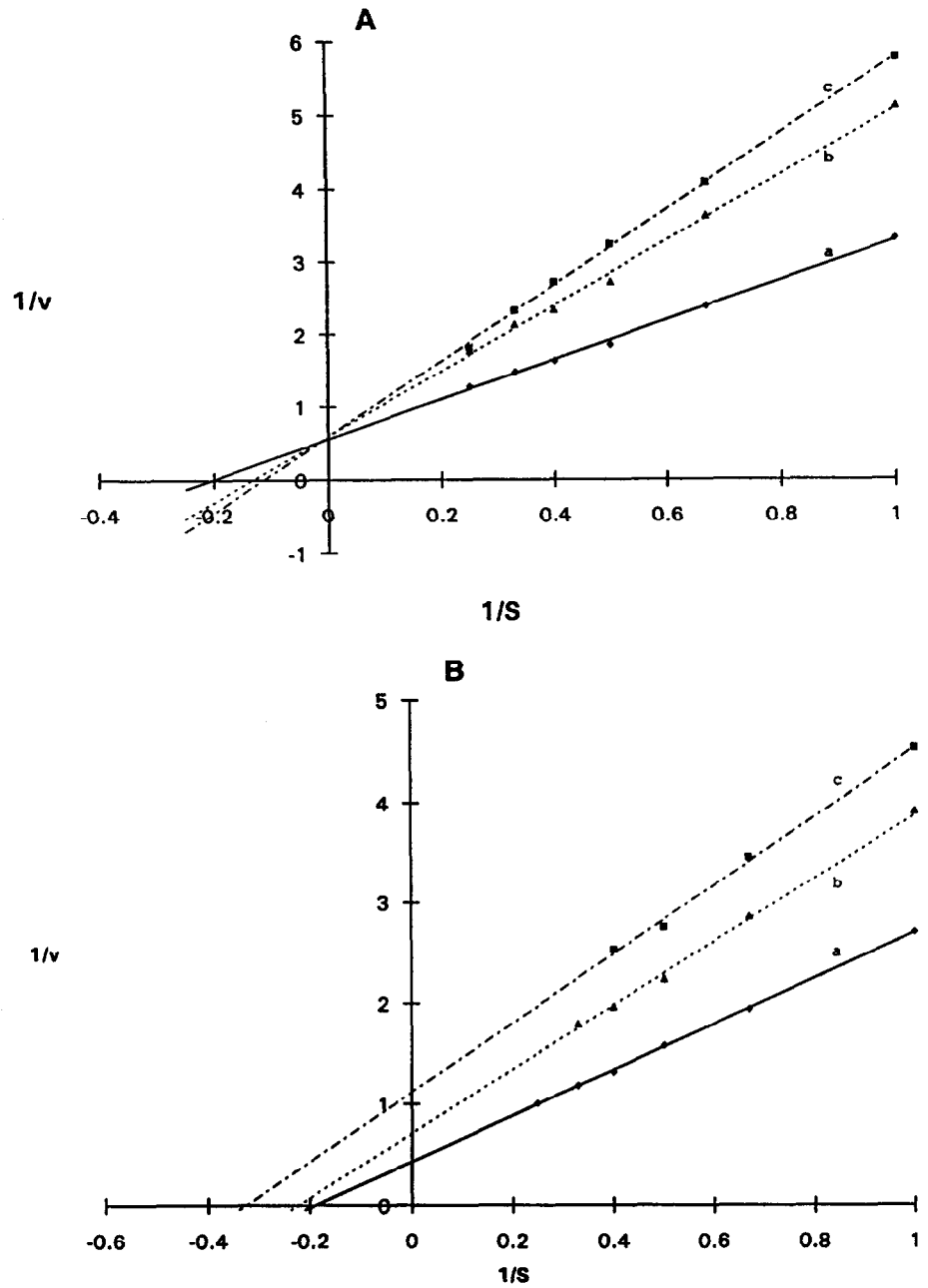

Fig. 2. (A) Lineweaver–Burk plots of the inhibition of enzymatic hydrolysis of L-tryptophan methyl ester by HCA on an IAM · PC-ACHT column. a = Control without addition of inhibitor to the mobile phase; b = mobile phase containing 1 m*M* HCA; c = mobile phase containing 5 m*M* HCA. Abscissa in 1/m*M*, ordinate in min/m*M*. (B) Lineweaver–Burk plots of the inhibition of enzymatic hydrolysis of L-tryptophan methyl ester by BPEA on an IAM · PC-ACHT column. a = Control without addition of inhibitor; b = mobile phase containing 5 m*M* BPEA; c = mobile phase containing 10 m*M* BPEA. Abscissa in 1/m*M*, ordinate in min/m*M*.

TABLE I

CALCULATED AFFINITY CONSTANTS FROM STUDIES WITH THE IAM-ACHT STATIONARY PHASE

Inhibitor and concentration (m*M*)	L-Trp-OMe K_M [a] (m*M*)	V_{max} (mmol/min)	K_I (calc.) (m*M*)	K_I (lit.) (m*M*)	Type of inhibition
HCA 0	4.9	1.8			
1	(7.6)[b]	(1.7)[b]	1.8	4.5 [17,18]	Competitive
5	(8.8)[b]	(1.7)[b]	6.1		
Day 1 (24%)[c]					
BPEA 0	5.3	2.3			
5	(4.7)[b]	(1.5)[b]	8.5	Not	Mixed
10	(3.1)[b]	(0.9)[b]	6.3	available	
Day 11 (11%)[c]					
BPEA 0	19.4	2.7			
5	(22.0)[b]	(1.8)[b]	10.2		Non-competitive
10	(23.0)[b]	(1.6)[b]	15.0		

[a] K_M for L-Trp-OMe calculated using non-immobilized ACHT = 1.5 m*M*.
[b] Apparent values of K_M and V_{max} in the presence of inhibitor.
[c] Activity of immobilized ACHT reported by percent of substrate (L-Trp-OMe, 4 m*M*) converted to L-Trp.

However, it should be mentioned that the consecutive increases in HCA concentration in the mobile phase from 2 to 5 to 10 m*M* did not result in a corresponding increase of ACHT inhibition, as monitored by the product formation in the HPLC column. This effect could be ascribed to either a mass-transfer limitation which has been observed in other immobilized enzyme systems [19], or, less probably, to a mixed type of inhibition.

The inhibition of ACHT by BPEA is depicted in Fig. 2B and the Lineweaver–Burk plots indicated a mixed type of inhibition. With 5 m*M* BPEA in the mobile phase, the inhibition appeared to be non-competitive; when 10 m*M* BPEA was present in the mobile phase, a straight line almost parallel to the one obtained for 5 m*M* BPEA was obtained which is characteristic of uncompetitive inhibition. The K_I calculated from these experiments were 8.5 and 6.3 m*M* (Table I). Unfortunately, there was no previously reported K_I value for BPEA inhibition of ACHT. BPEA has been studied, but the K_I was not determined due to background interference [17,18].

When the BPEA experiment was repeated 10 days later in the same column, the different results were obtained. In the interim, the activity of the IAM · PC-ACHT support had decreased from a 24% conversion of substrate to 11% (Table I). This resulted in changed Lineweaver–Burk plots and consequently different K_I values of 10.2 and 15.0 m*M*. The K_I values were not the only parameters which had changed, the initial K_M values calculated for L-Trp-OMe from the experiments with HCA and BPEA were 4.9 and 5.3 m*M*, respectively, relative to a K_M of 1.5 m*M* calculated using the non-immobilized ACHT. At day 11, this value had risen to 19.4 m*M*.

The addition of inhibitors to the mobile phase affected k' as well as enzymatic activity; the addition of 10 m*M* HCA to the mobile phase increased the k' of L-Trp-OMe by 5% while the presence of 10 m*M* BPEA in the mobile phase decreased the k' of L-Trp-OMe by 30%. These results are consistent with two observations by Pidgeon and co-workers [10,20] regarding chromatographic retention on the IAM · PC phase: (1) It was observed that an important aspect of the retention mechanism on the IAM · PC phase is due to ionic interactions with uncapped primary amines on the aminopropyl silica and that

capping these sites increases the retention; possibly by allowing the solute to penetrate deeper into the hydrophobic cavities. Thus addition of the anionic HCA to the mobile phase should increase k' by blocking some or all of the uncapped primary amines through ionic bonding. (2) It was also observed than an important aspect of the retention mechanism on the IAM·PC was solute adsorption to the polar head groups which contain both anion- and cation-exchange sites. The addition of a cationic modifier, *i.e.* BPEA, to the mobile phase should reduce the interactions between the positively charged solute and negatively charged phosphonate moiety on the stationary phase.

Based upon the results of this study, it is clear that the properties of IAM·PC-ACHT phase will change over time. However, the observed changes between days 1 and 11 in enzymatic activity and the resulting changes in the calculated kinetic constants (Table I) are not that dramatic when one considers the fact that the column was in constant use over the 10-day period. Thus, the IAM·PC-ACHT appears to be relatively stable.

It has been reported that the IAM·PC phase can be regenerated by washing the soluble proteins off the support with detergents [10]. Our experience confirmed this and we have reloaded regenerated IAM·PC supports with ACHT and other enzymes producing enzymatically active phases. In addition, we have found the immobilized enzyme supports to be relatively stable and several 15 cm × 4.6 mm I.D. IAM·PC columns containing ACHT and one column containing lipase have been in constant use in our laboratory as immobilized-enzyme reactors for more than four months.

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