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OPTIMIZED PHASE SYSTEM RESULTING FROM A COMPARISON OF ENDCAPPED AND NON-ENDCAPPED CHEMICALLY BONDED SILICA FOR THE HYDROPHOBIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES

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SUMMARY

An optimized, generally applicable mobile and stationary phase system is identified for the hydrophobic high-performance liquid chromatography (HPLC) of a wide variety of peptides. This reversed-phase system is arrived at as a result of an investigation in which endcapped and non-endcapped, chemically bonded, silica-based supports are compared in terms of their effect on the elution properties of the peptide solutes. Among the peptide solutes included in the study are enkephalins, neurotensins, angiotensins, oxytocin, phenylalanine homopolymers, and products of enzymatic digestion. Some of the features of the optimized phase system are reduced band broadening, improved peak symmetry, excellent resolution, low UV absorbance and volatility of the mobile phase. The retention data that are obtained aid in defining the mechanistic influence that residual silanols have on the elution behavior of peptides chromatographed under hydrophobic HPLC conditions.

INTRODUCTION

The applicability of reversed-phase high-performance liquid chromatography (HPLC) is undergoing a tremendous expansion into the analytical domains of biochemistry and biotechnology. The gain in popularity of reversed-phase HPLC (or hydrophobic HPLC) among life scientists is evidenced by not only the proliferation of literature on the topic[1–3] but also by the emergence of highly acclaimed and successful symposia devoted largely to this scientific discipline[4].

The increasing utility of reversed-phase HPLC to biochemical analysis, particularly as it pertains to peptide research, is due to several factors. First, there is a large array of commercially available HPLC instrumentation[5]. This wide selection of equipment permits users to choose HPLC systems that meet both specific technical as well as singular monetary requirements. The performance characteristics of these systems are, generally, quite good. For example, one study reports the attainment of less than 1% relative standard deviation in reproducibility of retention times of biochemical solutes under reversed-phase HPLC conditions[6].

A second reason why hydrophobic HPLC is being utilized more and more by

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biochemists is the large variety of available column materials[4]. Most of the currently used packing materials are silica-based, porous micro particles that have been chemically modified with alkyl silanes of various chemical properties. Many of these columns are presently offered on the market[4,7,8], while others are experimental[7,9]. The bonded residues on the columns include octadecyl, hexyl, cyano, phenyl and/or diol moieties. Equally diverse are the mobile phases being employed, which include perchlorate, phosphate, formate, acetate or trifluoroacetate anions with corresponding hydrogen, alkylammonium, sodium or pyridinium cations, among others[2,3].

The aim of this work was to develop a simple, optimized, generally applicable mobile and stationary phase system for the hydrophobic HPLC of numerous peptides. Such a phase system should provide reduced band broadening and enhanced peak symmetry, two attributes that lead to greater chromatographic resolution. In addition, the mobile phase should have a low UV absorbance and high volatility, the former asset allowing greater detectability and the latter easier post-HPLC operations. High volatility facilitates, *e.g.*, evaporation and lyophilization, which are post-HPLC operations that simplify micro-sequencing procedures. Also, mobile phases that are readily volatilized expedite purification and recovery of synthetic or natural peptide products. The following is a report on how such an optimized hydrophobic HPLC phase system was developed for peptides, through the investigation of endcapped and non-endcapped stationary phases and through a judicious combination of mobile phase constituents.

EXPERIMENTAL

Apparatus and operation

The liquid chromatography system consisted of an IBM Instruments LC/9533 ternary gradient liquid chromatograph (IBM Instruments, Danbury, CT, U.S.A.), an IBM Instruments LC/9523 variable-wavelength detector and an IBM Instruments LC/9540 chromatography data integrator. The IBM LC/9533 is equipped with a Rheodyne (Cotati, CA, U.S.A.) 7125 sample injector. A 9550 heating–cooling fluid circulator (IBM Instruments) maintained the temperature of the column and injector compartment at 23°C. The chromatograph was operated at a flow-rate of 1.0 ml/min. The integrator was operated at a chart speed of 1.0 cm/min in all experiments, except some of those in which band width and asymmetry were measured. For these experiments, a chart speed of 3.0 cm/min was utilized. The detector was set to 200 nm and either 0.2 or 0.5 a.u.f.s.

Columns

Four IBM Instruments columns were used: octyl endcapped (8635272), octyl (8635309), methyl (8635310) and silica (8635307). Each column, 250 × 4.5 mm, was prepared from the same production batch of silica. The 8635272 column is derived from a batch of 8635309 material by endcapping, *i.e.*, by a secondary silanization reaction with monochlorotrimethylsilane. The packing material is 5 μm in diameter and spherical.

Mobile phases

Acetonitrile (ACN) was HPLC grade (Burdick & Jackson Labs., Muskegon,

MI. U.S.A.). Trifluoroacetic acid (TFA) was 99+% pure (No. 67362, Pierce, Rockford, IL, U.S.A.). Triethylamine (TEA) was T0886 (Sigma, St. Louis, MO, U.S.A.). Water was purified with an Organicpure™ system (Sybron/Barnstead, Boston, MA, U.S.A.).

Solutes: preparation and injection

All proteins and peptides were purchased from Sigma. The following abbreviations are employed: TUFTSIN = tuftsin diacetate, T-6135; LYS-PHE = lysylphenylalanine, L-5877; $(PHE)_2$ = diphenylalanine; $(PHE)_3$ = triphenylalanine, T-8004; $(PHE)_4$ = tetraphenylalanine, T-6129; METENK = met-enkephalin, M-6638; OXYT = oxytocin, O-3251; GLUTH = glutathione, G-4626; ELEDRPEP = eledoison-related peptide, E-3253; NEURFRG = neurotensin fragment 1–8, N-0509; NEUR = neurotensin; BSA = bovine serum albumin, A-7638; LEUENK = leu-enkephalin, L-9133. Typically, a solution of 1–5 mg of each solute in 0.2–1.0 ml of solvent A of the gradient was freshly prepared on the day of injection. Samples were refrigerated when not in use. One to fifteen μl of each solution were injected, *i.e.* generally 1–10 μg. Exceptions were secretin and the enzymatic digest of BSA, of which 50–100 μg were injected. All experiments were performed at least in duplicate, the averages being reported.

RESULTS AND DISCUSSION

Octyl phases

Separations of a variety of peptides ranging in molecular weight from 500 to 1100 daltons are shown in Fig. 1. The octyl-endcapped and octyl-non-endcapped column materials are compared. Overall, there is little difference between these supports in terms of their influence on the elution of the selected peptides under the conditions described. Retention times (volumes) are essentially equivalent for both stationary phases for those peptides that exhibit reasonable symmetry, *e.g.*, the phenylalanine homopolymers, the enkephalins, OXYT and GLUTH. The equivalence in retention between the endcapped and non-endcapped octyl supports is further illustrated in Table I, in which the retention volumes measured from the chromatograms in Fig. 1 are listed.

The largest difference in retention volumes that does occur characterizes those peptides that show the largest band broadening and asymmetry, *e.g.*, TUFTSIN, LYS-PHEN and ELEDRPEP. Their peaks are broad and grossly tailed, as is evident in Fig. 1, on the non-endcapped as well as the endcapped columns. The non-endcapped packing causes a small but, nonetheless, measurable increase in the retention for these peptides. The void volumes of the two columns were determined by the use of 0.01 *M* potassium nitrate and the results are cited in Table I. The difference in the void volumes of the two columns is too small, *i.e.*, 0.02 ml, to account for the disparity in retention volumes, which range from 0.5 to 0.8 ml for these three very asymmetric bands.

The compositional sequences of all the peptides in Fig. 1 are presented in Table II[10,11]. Insight into the chromatographic mechanisms influencing the elution of the peptides can be gained by inspection of their sequences. The three peptides that exhibit excessive peak broadening and tailing are the only peptides in Table II that

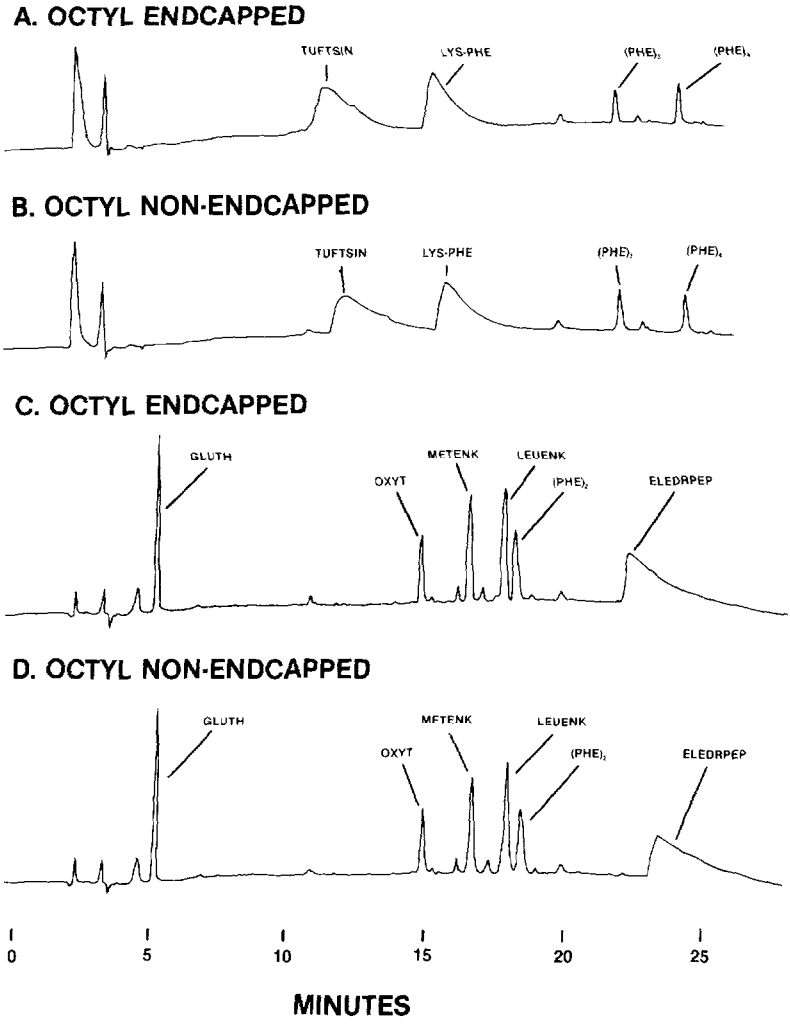

Fig. 1. Reversed-phase HPLC of peptides on endcapped and non-endcapped octyl supports. Mobile phase conditions: linear gradient, from 5 to 80% B in 24 min, A = 0.05% (v/v) TFA in water, B = 0.05% (v/v) TFA in ACN.

contain an amino acid with a strongly basic side chain. Specifically, TUFTSIN contains a terminal arginine, ELEDRPEP a terminal lysine and, of course, LYS–PHE a terminal lysine.

It is hypothesized that the protonated amino function of basic solutes can undergo strong interaction with residual silanols on chemically modified silica supports, resulting in broadening and distortion of bands[12–15]. Even with exhaustive silanization and secondary silanization (endcapping) of the silica matrix, a considerable fraction of the support surface remains unreacted[16]. Therefore, it is not surprizing that tailing occurs on both the endcapped and non-endcapped octyl phases when solutes containing highly accessible amino functions are chromatographed. It

TABLE I

PEPTIDE ELUTION VOLUMES (ml) ON ENDCAPPED AND NON-ENDCAPPED STATIONARY PHASES

Mobile phase conditions: same as Fig. 1.

	Octyl endcapped	*Octyl non-endcapped*	*Methyl*	*Silica*	*Octyl* endcapped*
GLUTH	5.56	5.42	4.67	4.22	4.10
TUFTSIN	11.52	12.27	11.48	--	6.17
OXYT	15.28	15.32	15.44	10.53	15.00
LYS-PHE	15.75	16.23	13.75	8.01	13.28
METENK	17.11	17.18	15.03	8.65	16.27
LEUENK	18.42	18.50	16.41	10.00	17.52
$(PHE)_2$	18.68	18.90	16.44	7.40	17.89
$(PHE)_3$	22.43	22.64	20.24	–	21.39
ELEDRPEP	23.26	23.94	21.42	15.75	19.54
$(PHE)_4$	24.82	25.05	21.62	14.70	23.78
0.01 *M* KNO_3**	1.81	1.79	1.81	1.72	–

* Mobile phase contains 0.05% triethylamine.
** Mobile phase equals 70:30 (v/v) methanol–water.

would be interesting to compare the elution properties of peptide solutes containing amino acid side chains of various degrees of accessibility (*e.g.*, terminal *versus* internal). Work in this direction is in progress.

The remaining peptides in Table II do not contain amino acids with basic side chains. Consequently, silanoliphilic interactions involving these peptides would not be expected to be significant. Indeed, this is experimentally supported by the fact that the retention times are virtually identical and band shapes are symmetrical for the remaining peptides when the endcapped and non-endcapped octyl columns are compared (Fig. 1 and Table I).

Methyl and silica phases

Table I also lists the elution volumes of the peptides when they are chromatographed on a methyl-bonded packing. In every case, the retention on the methyl column is less than that on the endcapped and non-endcapped octyl columns. An

TABLE II

SEQUENCE AND MOLECULAR WEIGHT OF PEPTIDE SOLUTES

GLUTH	Glu-Cys-Gly	656
TUFTSIN	Thr-Lys-Pro-Arg	621
OXYT	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly	1010
LYS-PHE	Lys-Phe	455
METENK	Tyr-Gly-Gly-Phe-Met	634
LEUENK	Tyr-Gly-Gly-Phe-Leu	616
$(PHE)_2$	Phe-Phe	312
$(PHE)_3$	Phe-Phe-Phe	460
ELEDRPEP	Lys-Phe-Ile-Gly-Leu	780
$(PHE)_4$	Phe-Phe-Phe-Phe	607

interpretation of this result is that hydrophobic forces play a predominant role in determining retention of the peptides, as already indicated by others[7,17,18]. However, the silanoliphilic interaction is still influential, since the peptides that contain the terminal basic side chain exhibit marked band broadening and asymmetry on the methyl phase, as was the case on the endcapped and non-endcapped octyl phase. An example of the band broadening and tailing on the methyl column is given in Fig. 2 for LYS-PHE. TUFTSIN and ELEDRPEP yield similar peak shapes (not shown). The remaining peptides show bands that are reasonably symmetrical, as exemplified in Fig. 2 by the enkephalins and tetraphenylalanine.

The bottom chromatogram in Fig. 2 is an illustration of hydrophobic HPLC performed on silica. It is clear that METENK and $(PHE)_4$ are more broadened on the silica phase than on the methyl phase. These findings will be discussed later in greater detail.

The retention times on the methyl and silica phases are compared in Table I. Every peptide exhibits its smallest retention on the silica phase. Furthermore, the elution order for all four phases (octyl endcapped, octyl non-endcapped, methyl and silica) remains invariant. This is more evidence that the predominant mechanistic force affecting retention is hydrophobic in nature, even with silica as the stationary phase. Indeed, with no bonded alkyl ligand, silica could be anticipated to generate the least retention[19].

Peak shape and interpretation of mechanistic interactions

Table III is a listing of the bandwidths and asymmetries of the peptides that were chromatographed on the columns discussed thus far. The data in Table III are extracted, in part, from the chromatograms shown in Figs. 1 and 2. The measurement of band width is by the tangent method, which is inherently characterized by some

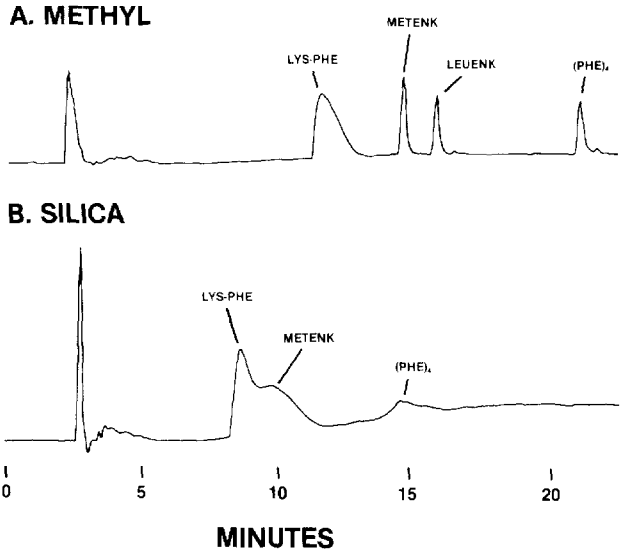

Fig. 2. Reversed-phase HPLC of peptides on methyl and silica supports. Mobile phase conditions as in Fig. 1.

TABLE III

BAND WIDTH AND ASYMMETRY ON ENDCAPPED AND NON-ENDCAPPED STATIONARY PHASES

Mobile phase conditions as in Fig. 1. Format of data: band width (ml)/asymmetry factor.

	Octyl endcapped	*Octyl non-endcapped*	*Methyl*	*Silica*	*Octyl* endcapped*
GLUTH	0.3/1.1	0.3/1.1	0.3/1.5	0.3/1.4	0.2/1.0
TUFTSIN	1.5/13.5	1.4/10.8	1.7/11.3	–	0.4/2.7
OXYT	0.2/1.3	0.2/0.9	0.3/1.4	–	0.2/1.3
LYS-PHEN	1.3/7.8	1.4/12.4	1.3/4.6	1.2/3.3	0.4/2.5
METENK	0.2/1.2	0.2/0.8	0.3/1.2	3.0/1.9	0.2/1.1
LEUENK	0.2/1.2	0.2/1.1	0.3/1.2	2.2/1.9	0.2/1.2
$(PHE)_2$	0.3/1.4	0.3/1.3	0.3/1.4	2.9/3.7	0.2/1.2
$(PHE)_3$	0.2/1.4	0.3/1.5	0.3/1.6	–	0.2/1.3
ELEDRPEP	2.2/18.0	2.1/15.6	1.1/8.3	2.1/3.5	0.4/1.9
$(PHE)_4$	0.2/1.6	0.3/1.4	0.3/1.5	2.5/3.2	0.2/1.3

* Mobile phase contains 0.05% (v/v) triethylamine.

error when the band profiles are not Gaussian[20]. For the purpose of this work, however, only gross differences in bandwidths are considered. The measurement of asymmetry is performed according to conventional methodology at 10 per cent of the peak height[20].

The data in the first two columns of Table III reinforce the observations made earlier regarding the endcapped and non-endcapped octyl phases. When the peptide contains a terminal amino acid with a strongly basic side chain, large peak widths (*i.e.*, 1.3–2.2 ml) and asymmetry factors (*i.e.*, 7.8–18.0) result. In contrast, when there is no such basic side chain on any of the amino acids in the peptide sequence, the bandwidths and asymmetry factors are greatly reduced (*i.e.*, 0.2–0.3 ml and 0.8–1.6, respectively). Similar observations concerning peptides with *versus* without basic side functions are made for the methyl column, too.

The data for the silica phase are especially noteworthy for their impact on mechanistic interpretation. The peaks of the peptides that contain a basic side chain are measurably more symmetrical on the silica phase than on either of the octyl columns (see LYS-PHE and ELEDRPEP, Table III). This is interpreted to mean that the predominant factor causing tailing of these peptides is not the interaction *per se* of the side chain amino moiety with the silica, but rather the heterogeneity of the stationary phase[21]. In the case of the octyl phases, the non-polar alkyl ligands and the polar residual silanols render the stationary phase surface quite heterogeneous, resulting in mixed retention mechanisms and, therefore, tailing[22].

On the other hand, since it contains no bonded alkyl ligands, the silica phase is expected to be a more homogeneous surface. Thus it permits a more uniform retention mechanism for these two solutes which are so sensitive (by virtue of their basic side group) to residual silanols on the surface of the stationary phase. As a result, less tailing is observed on the silica phase.

It should be pointed out that on the methyl column LYS-PHE and ELEDRPEP exhibit tailing that is intermediate between that on the silica and octyl col-

umns. The shortness of the methyl chain makes the methyl phase less non-polar (or more polar) than the octyl phase, with its longer octyl ligand. This diminished non-polarity of the shorter methyl chain prevents the creation of a surface as heterogeneous as that created when the longer, more non-polar octyl chain is covalently attached to the polar silica matrix.

These basic peptides, as well as the other peptides, nonetheless exhibit finite tailing on the silica phase, presumably due to some inherent heterogeneity characterizing the distribution of adsorption sites on the silica surface[22]. Consistent with this hypothesis is the fact that the "non-basic" peptides, *e.g.*, the enkephalins and phenylalanine homopolymers, show very little tailing on the methyl phase. The methyl ligand, in spite of its shortness, seems capable of masking the native heterogeneity of the silica surface, as long as the peptides cannot undergo strong interaction with residual silanols.

Finally, the silica phase is marked by the greatest band broadening, as compared to the other phases. This is generally true of all the peptides tested, and, hence is interpreted to mean that the chemical and physical nature of the silica causes a decreased rate of adsorption and desorption under these conditions.

Optimized phase system

The rational design of an optimized phase system intended for the hydrophobic HPLC of peptides should account for the molecular interaction of the silica surface with these peptides. One approach to taking such effects into account is through the judicious selection of mobile phase constituents. It is commonly known that the addition of an alkylammonium cation to the mobile phase can reduce significantly both band broadening and asymmetry of peaks corresponding to solutes —not unlike some peptides— containing ionizable amine functions[23]. It is suggested that the alkylammonium cation, by its distribution to the stationary phase, masks the residual silanols and suppresses silanoliphilic interactions[23].

A volatile mobile phase maintains the ease and readiness of post-HPLC operations frequently desired and/or required in many biochemical applications. More-

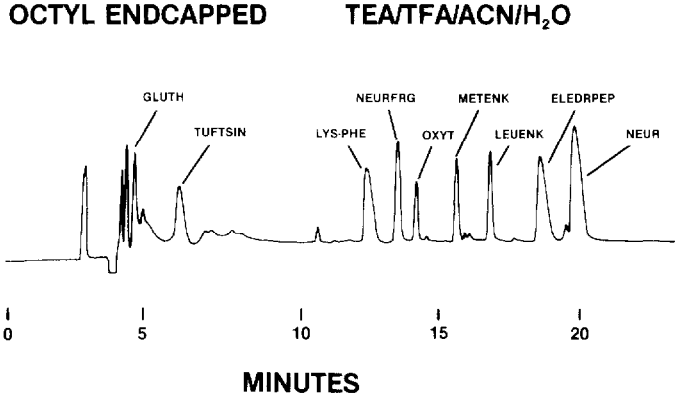

Fig. 3. Reversed-phase HPLC of peptides on an endcapped octyl support. Mobile phase conditions: linear gradient, from 5 to 80% B in 24 min, A = 0.05% (v/v) TFA and 0.05% (v/v) TEA in water, B = 0.05% (v/v) TFA and 0.05% (v/v) TEA in ACN.

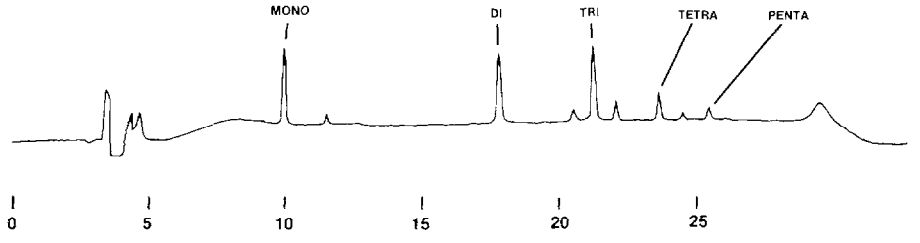

Fig. 4. Reversed-phase HPLC of phenylalanine homopolymers on an endcapped octyl support. Mobile phase conditions as in Fig. 3.

over, the preferred alkylammonium cation is essentially transparent in the UV region. This allows high detectability without the necessity of utilizing fluorescent detection, which typically mandates additional procedures and instrumentation. Meeting these requirements, TEA was selected to be included with TFA in the mobile phase. Fig. 3 shows a separation of peptides chromatographed with this mobile phase on the octyl-endcapped column, which was selected as the stationary phase on the basis of its extremely high stability and reproducibility[24]. The effects of the TEA are dramatic, as explained below.

For the peptides that contain the terminal amino acid with the basic side chain, there is a 3–9-fold improvement in symmetry, as compared to chromatography without TEA in the mobile phase. This observation is based on the data in Table III (see TUFTSIN, LYS-PHE and ELEDRPEP). Likewise, there is a 3- 5-fold reduction in band broadening for the same peptides. The remaining peptides cited in Table III maintain both their low band width and asymmetry factors when triethylamine is incorporated into the phase system. As indicated in Table I, all the peptides show a decrease in retention.

The wide applicability of the mobile and stationary phase system shown in Fig. 3 is underscored by the variety of separations illustrated in Figs. 4 7. Fig. 4 shows the separation of five phenylalanine homopolymers. Fig. 5 shows the separation of angiotensin-related peptides.

A commercially available sample of secretin, specified by the manufacturer not to be highly purified, was separated into its components, as illustrated in Fig. 6.

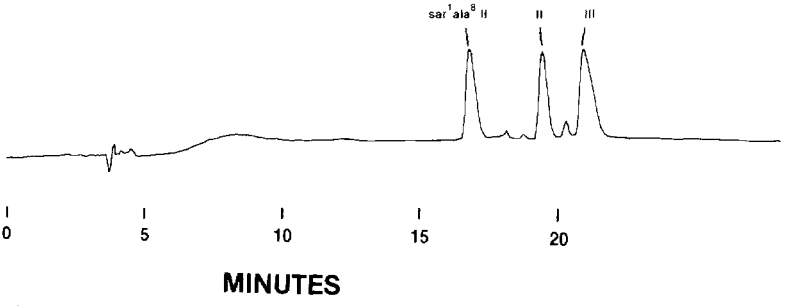

Fig. 5. Reversed-phase HPLC of angiotensins on an endcapped octyl support. Mobile phase conditions as in Fig. 3.

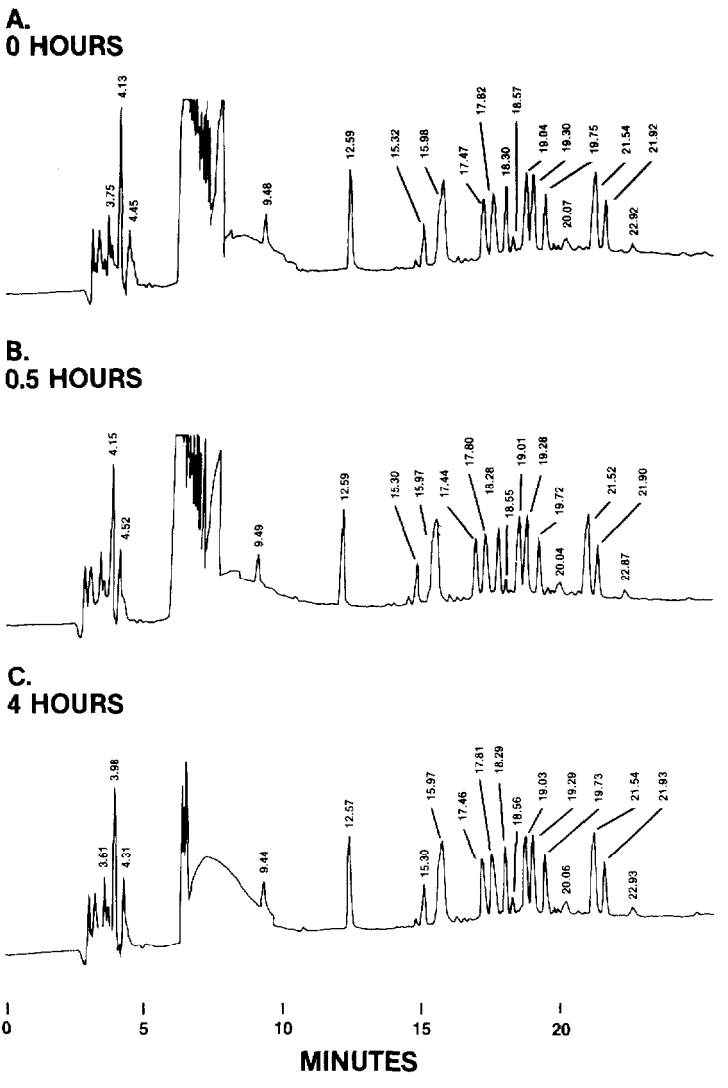

Fig. 6. Reversed-phase HPLC of crude porcine secretin on an endcapped octyl support. Mobile phase conditions as in Fig. 3.

There are more than ten peaks in these chromatograms, qualitatively indicating that the sample contains a significant number of impurities. Fig. 6A and B represent replicate injections, made as soon as the sample was prepared and 30 min later. The retention times of individual peaks were the same in both chromatograms as evidence for the high level of chromatographic reproducibility of the system. Fig. 6C is a chromatogram of the identical secretin sample injected 4 h after preparation. Between the 5–10-min elution period, the chromatographic profile has changed, implying ongoing modifications, *e.g.*, decompositional, conformational, etc., that the sample is experiencing.

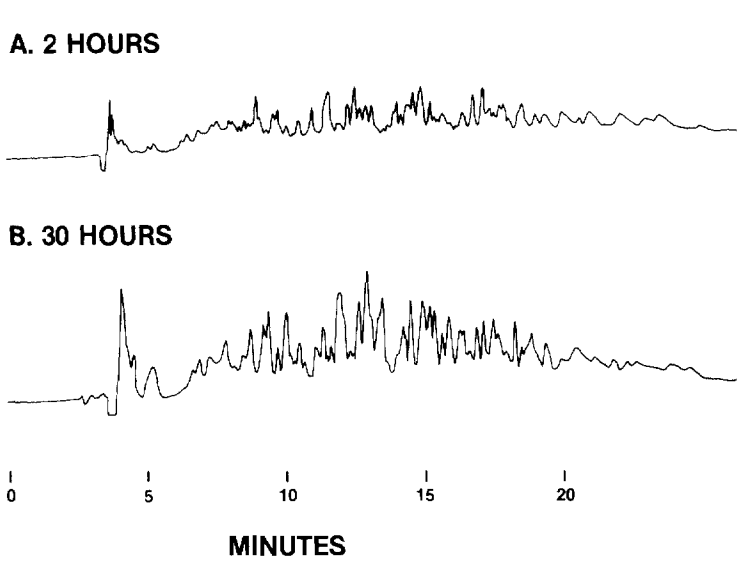

Fig. 7. Reversed-phase HPLC of a tryptic digest of BSA. Column: endcapped octyl. Mobile phase conditions as in Fig. 3.

Fig. 7 demonstrates the usefulness of this phase system for peptide mapping. A sample of BSA is subjected to enzymatic digestion with trypsin. Injection of the sample after 2 h of digestion is presented in Fig. 7A, while injection of the same sample after 30 h of digestion is presented in Fig. 7B. More than 20 peaks are readily identifiable.

CONCLUSIONS

A simple, optimized, generally applicable, hydrophobic HPLC system for the separation of a variety of peptides is obtained by using a mobile phase consisting of ACN, water, TFA and TEA. This system, when combined with an endcapped octyl column, features high resolution, reduced band widths, enhanced peak symmetries, volatility and high detectability. The system can be applied to the separation of enkephalins, neurotensins, amino acid homopolymers, angiotensins, oxytocin and other peptide compounds. In addition, it can be used to assess the purity of crude peptide mixtures and to perform peptide mapping of enzymatic digestions of proteins. The retention data generated in the study implicate both solvophobic and silanoliphilic interactions as being influential in the hydrophobic chromatography of peptides. The recognition of these interactions is used in part as a conceptual basis for the rational design of the optimized phase system.

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