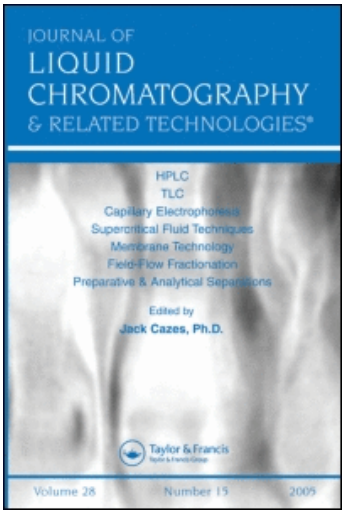

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A Novel Approach to Reversed-Phase Preparative High-Performance Liquid Chromatography of Peptides

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A NOVEL APPROACH TO REVERSED-PHASE PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES

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ABSTRACT

In this study, we describe a novel approach to preparative liquid chromatography which takes advantage of the different relative hydrophobicities of components of a sample mixture, so that when a column is optimally loaded with an aqueous solution of the sample mixture, there is competition among the sample components for the adsorption sites on the hydrophobic stationary phase. The more hydrophobic components compete more successfully for these sites than more hydrophilic components, which are displaced and immediately eluted from the column. Thus, the major separation takes place in water. Subsequent treatment with an aqueous organic modifier is only required to wash retained components off the column and takes no part in the major separation process. This approach was applied to the preparative purification of mixtures of closely-related peptides, representing the crude peptide mixtures typically obtained from solid-phase peptide synthesis. The excellent separation profiles and high yields of pure peptide products on analytical columns reported in this study demonstrate that this methodology has great potential for preparative separation of a major component from hydrophilic and/or hydrophobic impurities.

INTRODUCTION

The excellent resolving power and separation time of reversed-phase chromatography has led to its becoming the dominant mode of high-performance liquid chromatography for preparative separations in recent years. Preparative liquid chromatography is carried out primarily for the purification of a mixture for further characterization or study and may be performed on standard analytical (50-250 mm x 4 mm I.D.) or semipreparative (250 mm x 10 mm I.D.) columns, as well as preparative columns with internal diameters of 2.2 cm and higher. Maximum resolution is obtained on small particle size packings, the most common analytical packings ranging from 5-10 μm. The cost of preparative columns containing these packings can be prohibitively expensive and a compromise may have to be reached between performing several sample injections on smaller diameter columns or one injection on a larger column. It is essential to improve resolution around the component(s) of interest in order to maximize the amount of sample per injection. The maximum quantity of sample that can be separated is dependent upon the criteria established for the fraction of a component that is recovered as homogeneous product (1). For example, a desired recovery of greater than 0.9 means that more than 90% of the component must be obtained in homogeneous form after removal of fractions cross-contaminated with components on either side of the peak of interest[1]. When this criteria is not met (i.e. <90% recovery of homogeneous product), a condition of sample-related overload has occurred. Thus, the objective of preparative chromatography is to obtain maximum resolution and sample load for any given column under the constraints of the investigator's established yield of homogeneous product in the desired time frame.

Synthetic peptides are a class of compounds with increasing therapeutic importance and, thus far, reversed-phase gradient elution HPLC has been the chosen method of purification (2). Although an efficient peptide synthesis should result in only a small number of synthetic impurities, these impurities are usually closely related to the peptide of interest (deletion, terminated or chemically modified peptides). The most common analytical method used in reversed-phase chromatography of peptides involves a linear AB gradient (1% B/min at a flow-rate of 1 ml/min), where solvent A is 0.1% aqueous trifluoroacetic acid and solvent B is 0.1% trifluoroacetic acid in acetonitrile (3). The organic modifier is essential for removing peptides from the reversed-phase support during linear gradient elution and the components are resolved due to differences in hydrophobicity. An increase in peptide hydrophobicity results in a decreased

partitioning rate and an increase in retention time. Thus, the components of a mixture travel through the column at different velocities depending upon their hydrophobicities. Sample load may be increased tremendously in preparative scale HPLC compared to analytical separations and, in elution mode, both resolution and retention time are related to sample load and the concentration of organic modifier used for elution. Hence, large scale, gradient elution separations of closely related peptides necessitate the use of increasingly larger column volumes in order to maintain satisfactory levels of product purity and yield. This, in turn, leads to much higher operating costs in terms of packings, equipment and solvents.

Recently, Horváth and others (1, 4-8) have explored the potential of reversed-phase chromatography in the displacement mode for preparative-scale separations of several classes of compounds including peptides and proteins (6, 8). In contrast to the elution mode of chromatography, which is handicapped by relatively poor utilization of the stationary and mobile phases (1), displacement chromatography makes significantly more efficient use of the resolving capabilities of column packings. This enables the separation of relatively large amounts of material on columns and instrumentation designed primarily for analytical work. The displacement technique involves applying the sample in a carrier solvent that has low affinity for the stationary phase. Following adsorption of the sample mixture on the inlet section of the column, a solution of a displacer compound, which has greater affinity for the stationary phase than any of the sample components, is pumped into the column. The sample components are thereafter displaced from the stationary phase surface and move down the column preceding the displacer front, forming adjacent zones of purified solutes travelling at the same velocity (displacement train). Though displacement chromatography has advantages of sample load over elution chromatography, the method is extremely difficult to optimize (1,6) in terms of choice of displacer, displacer concentration and flow-rate. In addition, the need for column regeneration, an operational step not contributing directly to separation, is an undesirable feature of the technique (1).

Obviously there is a need for easier and more reliable methods of preparative reversed-phase chromatography. Our objective in the present study was to develop a method for the rapid and facile preparative separation of a single peptide component from a complex multi-component mixture in high yield. In this paper, we describe a novel approach to preparative chromatography applicable for general use on analytical columns and instrumentation. Preliminary results suggest that the speed, simplicity and loading capacity of reversed-phase

analytical columns operated in sample displacement mode (SDM) will be immensely advantageous in the research field.

EXPERIMENTAL

Materials. HPLC-grade water and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC-grade trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.).

Peptides. Peptides 1-5 were synthesized on a Beckman Model 990 peptide synthesizer (Beckman Instruments, Berkeley, CA, U.S.A.) using the general procedure for solid-phase synthesis described by Parker and Hodges (9). Crude peptides were purified on an Aquapore RP 300 (C_8) analytical column (220 x 4.6 mm I.D., 7 μm particle size, 300 A pore size) (Pierce, Rockford, IL, U.S.A.), using linear AB gradients (0.1% B/min) at a flow-rate of 1 ml/min, where solvent A was 0.1% aqueous TFA and solvent B was 0.05% TFA in acetonitrile.

Apparatus. The HPLC instrument consisted of a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) coupled to a Hewlett-Packard (Avondale, PA, U.S.A.) HP1040 A detection system, HP85B computer, HP9121 disc drive, HP2225 A Thinkjet printer and HP7470 plotter. Samples were injected with a 2.0 ml injection loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Column. Preparative and analytical separations were performed on an Aquapore RP 300 (C_8) analytical column (30 x 4.6 mm I.D., 7 μm particle size, 300 A pore size) (Pierce, Rockford, IL, U.S.A.).

Gradient delay time (t_g). Gradient delay time is defined in this work as the time for the gradient to reach the detector from the proportioning value via pump, injection loop, and column.

SAMPLE DISPLACEMENT MODE IN PREPARATIVE CHROMATOGRAPHY

Preparative chromatography, whether it is carried out in elution or displacement mode, requires the optimization of several parameters in order to maximize separation and yield. Gradient steepness, flow-rate, choice of organic modifier and, particularly, sample load are all critical factors in the efficient application of gradient elution to preparative separations. In displacement chromatography, column length, mobile phase flow-rate, sample concentration, the choice of displacer and displacer concentration are all important factors in producing the correct (isotachic) conditions required to form the solute displacement train (1, 4-8). Mobile phase flow-rates are typically, and necessarily (1,

4-8), low in displacement chromatography (0.1-0.2 ml/min), often leading to excessively long run times. The optimization procedure for reversed-phase preparative chromatography would be greatly simplified if the only variable was sample load, i.e. could the major separation process of removing impurities from the component of interest be performed in water without the use of organic modifier and added displacer? This major separation process would involve the components of a mixture to be separated displacing each other from a reversed-phase column. When a reversed-phase column is optimally loaded with a sample mixture dissolved in a 100% aqueous mobile phase, there is competition by the sample components for the adsorption sites on the hydrophobic stationary phase. The more hydrophobic components would compete more successfully for these sites than less hydrophobic components, which should be displaced and immediately eluted from the column. Subsequent treatment with an aqueous organic modifier would only be required to wash retained components off the column and would take no part in the major separation process. Column regeneration to a 100% aqueous mobile phase would be very rapid. Thus, under conditions of optimal sample load, either the component of interest would be used to displace the impurities from the column or the impurities would be used to displace the component of interest. In the latter case, the component of interest is isolated in water and the organic modifier is used only to wash the column free of impurities. In the former case, the organic modifier is used to elute the component of interest after the separation in water is complete. Flow-rates of 1 ml/min on analytical columns (4 mm I.D.) should be possible in SDM, thereby allowing rapid separations, since the time required for the initial major separation in water should be considerably less than that to produce ideal (isotachic) conditions in traditional displacement chromatography. Operation in SDM is simply using the well-established general principles of displacement chromatography without using the displacer.

RESULTS AND DISCUSSION

Separation of a major peptide component from various peptide impurities was an ideal model system for testing the viability of SDM as a preparative tool, since it reflected separation difficulties frequently encountered by researchers involved in peptide synthesis and purification. Thus, SDM was applied to various mixtures of closely-related synthetic peptides (Fig. 1), designed to simulate the crude peptide mixtures typically produced by solid-phase peptide synthesis. The five decapeptides shown in Fig. 1 are closely related in hydrophobicity: between peptides 2 and 3 there is an increase of only one carbon atom, between peptides

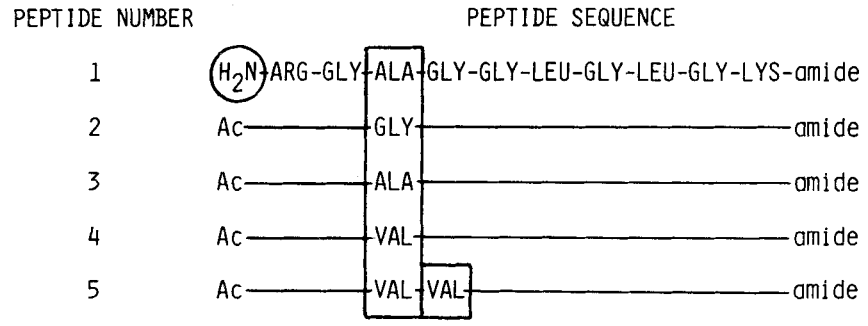

Figure 1. Sequences of peptides used in this study. Ac = N^{α}-acetyl; amide = c^{α}-amide.

3 and 4 there is an increase of two carbon atoms, and between peptides 4 and 5 there is an increase of three carbon atoms. When referring to peptide product (P) or peptide impurity (I) in the sample mixtures (see below), the subscripts 1-5 denote peptides 1-5 (Fig. 1), respectively.

A short (30 mm) analytical reversed-phase column was used to test the potential value of SDM in order to limit the amount of material required to saturate the hydrophobic stationary phase, and also to demonstrate the impressive yields of pure peptide products obtainable even on these small columns.

Selectivity of stationary phase. Before carrying out preparative chromatography, it is important to verify that the stationary phase of choice has sufficient selectivity towards the components of the mixture so that there is promise of developing a preparative separation with adequate resolution. This is generally carried out by gradient elution of an analytical injection of the sample to be resolved. Table I demonstrates the resolution obtained on an Aquapore RP 300 C_8 column (30 x 4.6 mm I.D.) when eluting an analytical sample mixture of the five synthetic peptides (Fig. 1) with a linear AB gradient (1% B/min) at 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile. The resolution (r) between two peaks is defined by: $r = 2\Delta t/W_1 + W_2$, where Δt is the difference in retention times of two components, and W_1 and W_2 are the widths of the two component peaks at 10% above baseline. The resolution values of the five peptides (Table I) range from 1.2 to 3.2 and are representative of the type of values obtained between

TABLE I

Resolution of Peptide Mixture by Analytical Gradient Elution

Column: Aquapore RP 300 C_1 (30 x 4.6 mm I.D.) Conditions: linear AB gradient (1% B/min), where A is 0.05% aqueous TFA and B is 0.05% TFA in acetonitrile; flow-rate, 1 ml/min. Sequences of peptides 1-5 are shown in Fig. 1.

Peptides	Resolution[a]
1 - 2	1.23
2 - 3	1.32
3 - 4	3.54
4 - 5	3.16

[a] The resolution (r) between two peaks is defined by the expression: $2\Delta t/W_1 + W_2$, where Δ is the difference in retention times of two components, and W_1 and W_2 are the widths of the two component peaks at 10% above baseline.

the main component of a peptide synthesis and more hydrophobic and/or hydrophilic impurities. To achieve a preparative separation with little overlap between peaks, a minimum resolution of 1.0 is desirable. Scaling up a gradient elution separation of a mixture of the five peptides (Fig. 1) to preparative loadings rapidly leads to a degeneration of the resolution values demonstrated in Table I, and this degeneration is representative of the kinds of difficulties encountered in the purification of synthetic peptides.

Optimization of sample load. At optimal loading, SDM should maximize resolution between the component of interest and any impurities. Fig. 2 demonstrates the relationship between the relative hydrophobicities of peptides 1-5 (Fig. 1) and the maximum loads of these peptides that can be applied to an analytical column (Aquapore RP 300, 30 x 4.6 mm I.D.) and not be eluted with 0.05% aqueous TFA in 10 or 40 min. Relative hydrophobicity values were calculated from analytical linear AB gradient elution runs (see Table I for conditions) of the five peptides on the column. Peptide 4 was assigned a hydrophobicity value of 1.0. The relative hydrophobicities of the other four peptides were then

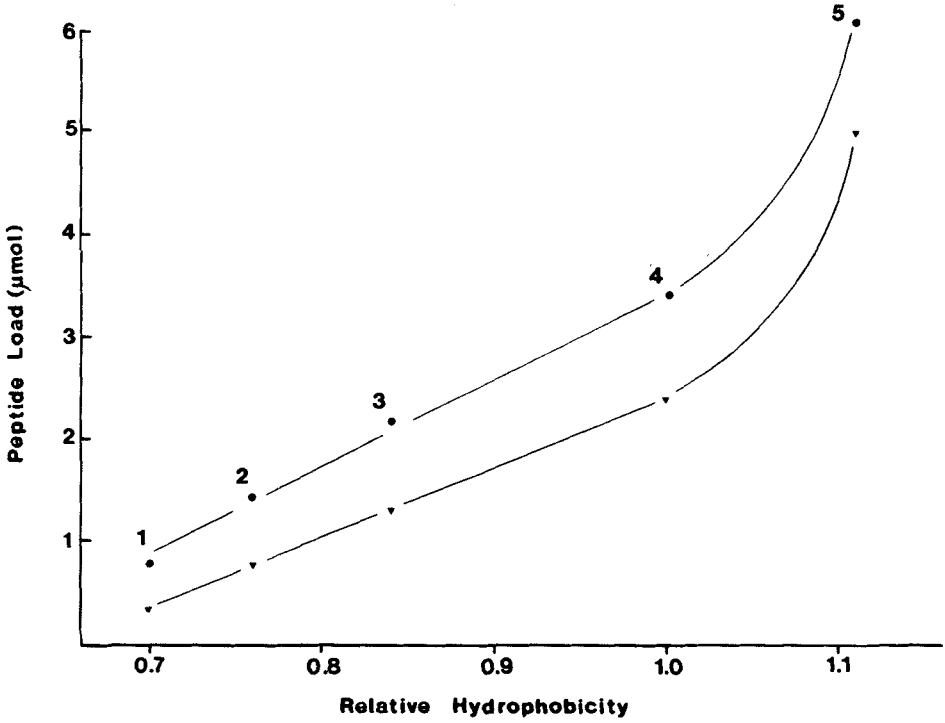

Figure 2. A plot of maximum peptide load versus relative hydrophobicity of peptides. Column: Aquapore RP 300 C_1 (30 x 4.6 mm I.D.). The symbols (• and ▲) denote the maximum load of a peptide that can be applied to the column without elution of the peptide by, respectively, 10 or 40 min isocratic elution with 0.05% aqueous TFA at a flow-rate of 1 ml/min. Relative hydrophobicity of a peptide (x) was determined by the expression $(R_t^{(x)} - t_g) / (R_t^{(4)} - t_g)$, where $R_t^{(x)}$ and $R_t^{(4)}$ denote retention times of peptide x and peptide 4, respectively, on the above C_1 column (linear AB gradient [1% B/min] at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile), and t_g denotes gradient delay time (see Experimental).

expressed as the percentage of acetonitrile in the mobile phase required to elute these peptides compared to the percentage required to elute peptide 4: $(R_t^{(x)} - t_g)/(R_t^{(4)} - t_g)$, where $R_t^{(x)}$ and $R_t^{(4)}$ denote retention times of peptide x and peptide 4, respectively, and t_g denotes gradient delay time (see Experimental). The purpose of this plot was twofold: firstly, to establish load capacities for each of the five peptides used to test SDM; secondly, to establish a standard curve for the column, so that load capacity for other peptides could be determined from their retention times. The appropriate sample loads of mixtures of the five peptides (Fig. 1) were estimated from the 40 min curve of Fig. 2. This enabled maximum, i.e. baseline, resolution between the displacer peptide(s), which should not begin to significantly elute before 40 min isocratic elution in water, and displaced peptide(s) which elute immediately in water. The load of the component(s) that was to play the role of displacer was the critical factor. In addition to the investigator's established criteria of overload as described in the introduction, a column in SDM mode is definitely overloaded when the displacer starts to elute in the column dead time.

For experimental purposes, sample loadings are generally expressed by weight instead of molarity. Thus, the peptide loadings in the SDM separations described below are expressed in mg (1 μmol of a decapeptide is equivalent to approximately 1 mg).

Separation of desired component from hydrophobic impurities. Fig. 3 demonstrates SDM of a peptide mixture, where peptide 2 is the desired "product" (P_2) and peptides 3-5 (I_3, I_4, I_5) represent hydrophobic impurities. An analytical separation profile of the four peptides is shown in Panel A. The peptides were eluted by a linear AB gradient (1% B/min) at 1 ml/min, where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile. The ratio (w/w) of "product" to "impurities" was 3:1:1:1 (P_2:I_3:I_4:I_5). This mixture, representing a somewhat poor synthesis yield of P_2 (50%), would be difficult to resolve by preparative gradient elution. The protocol for preparative SDM of the peptide mixture involved displacement in water (0.05% aqueous TFA) of P_2 by the more hydrophobic "impurities" (I_3,I_4,I_5), which should remain bound to the column for at least 40 min of isocratic elution in water. Thus, the loads of the displacer peptides (I_3,I_4 and I_5) had to be at a high enough level so that they were sufficient to displace P_2, but not so high that they were eluted within 40 min of isocratic elution in water. From Fig. 2, the 40 min elution maximum loads of peptides 3, 4 and 5 were ~ 1.8 mg, 3.5 mg and 7.0 mg, respectively. These are the maximum loads of each peptide when applied alone. Hence, to

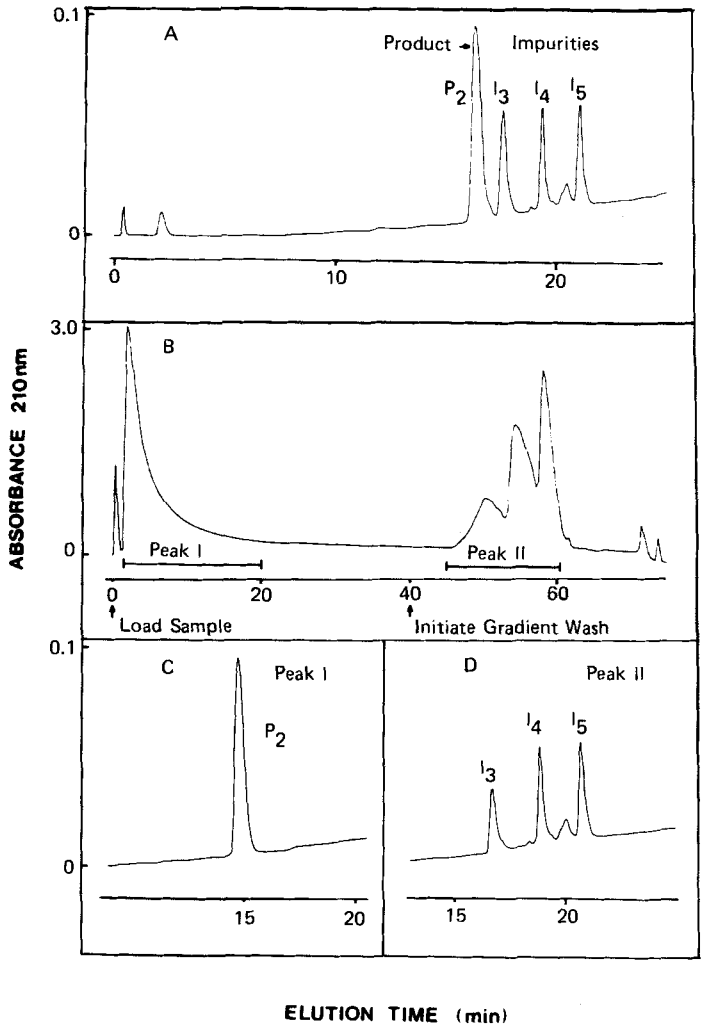

Figure 3. Separation by reversed-phase chromatography operated in sample displacement mode of peptide "product" (P_2) from hydrophobic peptide "impurities" (I_3,I_4,I_5). Column: Aquapore RP 300 C_8 (30 x 4.6 mm I.D.). Panel A: analytical separation profile of peptide mixture; conditions, linear AB gradient (1% B/min) at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile. Panel B: preparative separation profile of peptide mixture; conditions, isocratic elution with 100% solvent A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min; sample load, 6.0 mg consisting of 3.0 mg of P_2 and 1.0 mg of each of I_3, I_4 and I_5 dissolved in 100 μl of solvent A. Panels C and D demonstrate analytical elution profiles (see Panel A for conditions) of Peaks I and II (Panel B), respectively. The subscripts of P_2,I_3,I_4 and I_5 denote peptides 2-5, respectively.

ensure that all three "impurities" remain bound to the column for at least 40 min, these levels must be lowered. Although peptides I_3,I_4 and I_5 are all present in the same proportion in the sample mixture, I_3 has the lowest allowable maximum load and so will determine the sample loads of all three "impurities". It was estimated that a load of 1 mg of I_3, with the presence of I_4 and I_5, would be sufficient to rapidly displace the bulk of P_2 in water, while the "impurities" themselves remained on the column. The total sample load was calculated to be 6 mg. If the percentage of P_2 in the mixture was higher, the total sample load would increase, i.e. the total sample load is controlled only by the percentage of displacing "impurities". For example, if the level of P_2 was 90%, the total sample load would be 30 mg (10% = 3.0 mg of "impurities" [I_3,I_4,I_5]; total load = $\frac{100}{10} \times 3.0 = 30$ mg, i.e. 27 mg of P_2). Panel B of Fig. 3 demonstrates the SDM elution profile of the sample mixture. Following isocratic elution with 0.05% aqueous TFA at 1 ml/min, a gradient wash was initiated after 40 min (1% acetonitrile/min) to remove remaining sample impurities from the column. The fractions pooled as Peaks I and II (Panel B) were subjected to gradient elution analysis and the results are shown in Panels C and D, respectively. Peak I (Panel C) was pure P_2 and contained 99% of recovered P_2 (Table II), demonstrating excellent SDM separation. The remaining 1% was found in the 20-40 min region of the preparative run profile (Panel B), where it was contaminated with I_3. Panel D demonstrates that the bulk of I_3 and all of I_4 and I_5 had, as expected, remained bound to the column during elution with 0.05% aqueous TFA, and were only removed by the addition of acetonitrile to the mobile phase.

TABLE II

Yields of Purified Peptides

Peptide "Product"	Recovery (%)[a]
P_2 (Fig. 3)	99
P_3 (Fig. 4)	93
P_2 (Fig. 5)	76
P_2 (Fig. 6)	96
P_4 (Fig. 7)	86

[a] Recovery is reported as the amount of pure "product" recovered as a percentage of the total "product" recovered

Separation of desired component from hydrophilic impurities. Fig. 4 demonstrates SDM of a peptide mixture, where peptide 5 is the desired "product" (P_5) and peptides 1-4 (I_1,I_2,I_3,I_4) represent hydrophilic impurities. An analytical separation profile of the five peptides is shown in Panel A (run conditions identical to those used for Fig. 3, Panel A). The ratio (w/w) of "product" to "impurities" was 1:1:1:1:2 (I_1:I_2:I_3:I_4:P_5), i.e. P_5 represented only 33.3% of the sample mixture. Sufficient P_5 was required to displace all four hydrophilic "impurities" during elution with water, while itself remaining bound to the column. From Fig. 2, the 40 min elution maximum load for peptide 5 was ~ 7.0 mg. Thus, the peptide mixture loaded onto the column contained 21 mg which included 7.0 mg P_5 and 3.5 mg of each of I_1,I_2,I_3 and I_4. Since the displacer is now the "product", the sample load is controlled by the percentage of P_5, i.e. if the percentage of P_5 was less, the total sample load applied would be increased. For example, if the level of P_5 was only 5%, the total sample load would be 140 mg ($7.0 \text{ mg} \times \frac{100}{5} = 140 \text{ mg}$). Panel B of Fig. 4 demonstrates the SDM elution profile of the sample mixture. Subsequent analysis of the fractions denoted Peaks I and II (Panels C and D, respectively) demonstrated excellent separation of P_5 in high yield from the four hydrophilic "impurities". Peak II (Panel B), removed by gradient elution with acetonitrile after 40 min isocratic elution with water, was pure P_5 and accounted for 93% of total P_5 recovered (Table II). The remaining 7% eluted prior to 47 min of the preparative run (Panel B) and was contaminated with I_4. The bulk of I_1-I_4 was, as expected, eluted as an unretained fraction (Peak I) during isocratic elution with 0.05% aqueous TFA.

Separation of desired component from hydrophilic and hydrophobic impurities. The separations demonstrated in Figs. 3 and 4, where the desired peptide product is the most hydrophilic or hydrophobic component, respectively, represent situations where SDM is most easily optimized to produce maximum product yield. Fig. 5 demonstrates a more difficult separation by SDM of a peptide mixture, where the desired "product" (P_2) is contaminated by both hydrophilic (I_1) and hydrophobic (I_3,I_4,I_5) "impurities". An analytical separation profile of the five peptides is shown in Panel A (run conditions identical to those used for Fig. 3, Panel A, with the exception of a faster gradient [2% B/min]). The ratio (w/w) of "product" to "impurities" was 1:3:1:1:1 (I_1:P_2:I_3:I_4:I_5), i.e. P_2 represented approximately 43.0% of the sample mixture. The protocol for initial preparative separation of P_2 from most of the contaminating peptides involved displacement of I_1 and P_2 by the more hydrophobic I_3,I_4 and I_5. Except for the addition of I_1, the sample mixture was identical to that of Fig. 3. Thus, the level of peptide 3 (I_3) is again the determining factor for sample loading of the

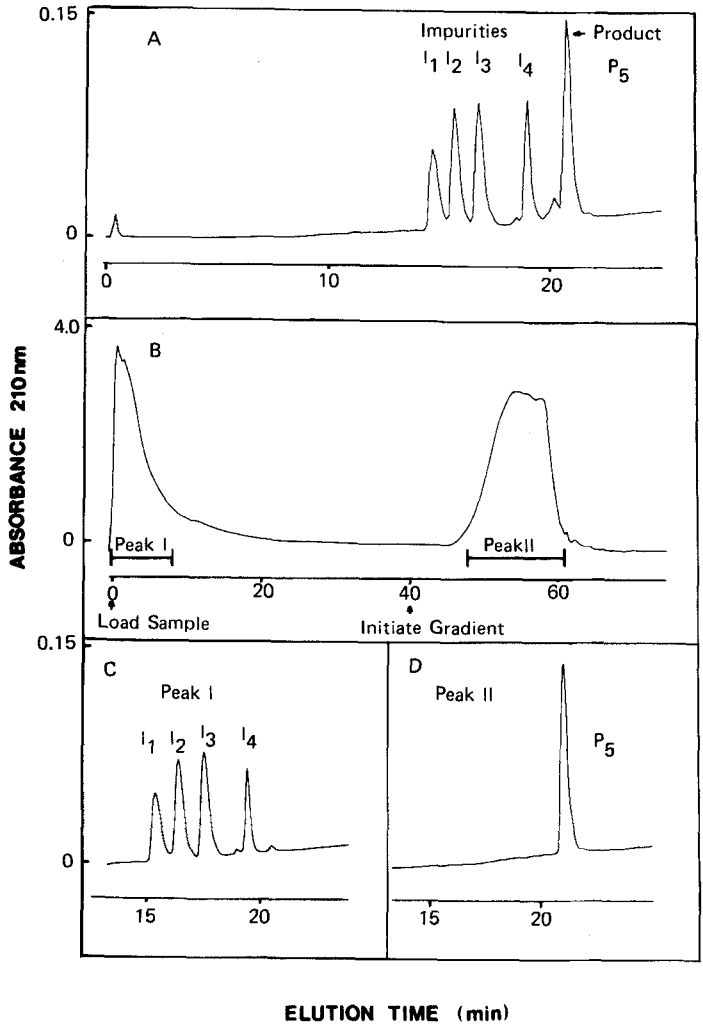

Figure 4. Separation by reversed-phase chromatography of peptide "product" (P_5) from hydrophilic peptide impurities (I_1,I_2,I_3,I_4) operated in sample displacement mode. Column: Aquapore RP 300 C_8 (30 x 4.6 mm I.D.). Panel A: analytical separation profile of peptide mixture; conditions, linear AB gradient (1% B/min) at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile. Panel B: preparative separation profile of peptide mixture; conditions, isocratic elution with 100% solvent A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min; sample load, 21 mg consisting of 7.0 mg of P_5 and 3.5 mg of each of I_1,I_2,I_3 and I_4 dissolved in 500 μl of solvent A. Panels C and D demonstrate analytical elution profiles (see Panel A for conditions) of Peaks I and II (Panel B), respectively. The subscripts of I_1,I_2,I_3,I_4 and P_5 denote peptides 1-5, respectively (see Fig. 1).

three hydrophobic "impurities" and, hence, displacement of I_1 and P_2. The peptide sample contained 3.0 mg of P_2 and 1.0 mg of each of I_1,I_3,I_4 and I_5 for a total sample load of 7.0 mg. The preparative run was carried out exactly as described above (Fig. 3 and 4) and the elution profile is demonstrated in Fig. 5, Panel B. Fraction analysis of the displaced components (fractions a,b,c) and the components removed from the column by the gradient wash (fraction d) are presented in Panel C. Fraction "c" was pure P_2 and contained 76% of recovered P_2 (Table II). Fraction "b" contained the remaining 24% of P_2, contaminated with I_1. Fraction "a" contained pure I_1, while fraction "d" contained the bound, displacer components (I_3,I_4 and I_5) uneluted by water from the column. It is interesting to note that the displaced components, I_1 and P_2, eluted in order of their relative hydrophobicities, i.e. P_2 eluting just behind I_1. In fact, there was only a small overlap between the I_1 and P_2 elution zones, producing only one small fraction "b" which contained both I_1 and P_2.

Although respectable, a yield of 76% of pure product may not be sufficient. Assuming that a higher yield is desired, fractions a, b and c (Fig. 5, Panel B) were pooled, lyophilized and submitted to a further round of SDM (Fig. 6). From Fig. 2, it can be seen that the maximum loading of peptide 2 on the column without elution in water prior to 10 min was ~ 2 mg. The level of P_2 in fractions a-c of Fig. 5 was approximately 3 mg. Thus, it may be expected that application of the total pooled fraction would produce overloading of the column with P_2, thereby not only displacing I_1, but also causing some P_2 to elute with water. The results presented in Fig. 6 confirmed that this was, indeed, the case. Panel A shows the analytical profile of the pooled, displaced components a-c from Fig. 5, Panel B (run conditions identical to those used for Fig. 5, Panel A). Panel B shows the preparative separation of this entire pooled fraction. The gradient was initiated at 20 min instead of 40 min in order to hasten the elution of P_2 from the column. Fraction analysis of Pools I and II (Panel B) demonstrated that only I_1 was present in Pool I (Panel C), while Pool II was pure P_2 (Panel D) and contained 99% of recovered P_2. The remaining 1% of recovered P_2, contaminated with I_1, was detected in the 6-8 min region and comprised the only overlap between the I_1 and P_2 elution zones. These results demonstrate that, in a simple two-component system, excellent separation by SDM may still be achieved even when the more hydrophobic component is overloaded by our criteria as defined previously (Optimization of sample load).

Fig. 7, Panel A, demonstrates the analytical elution profile (run conditions identical to those used for Fig. 5, Panel A) of a peptide sample

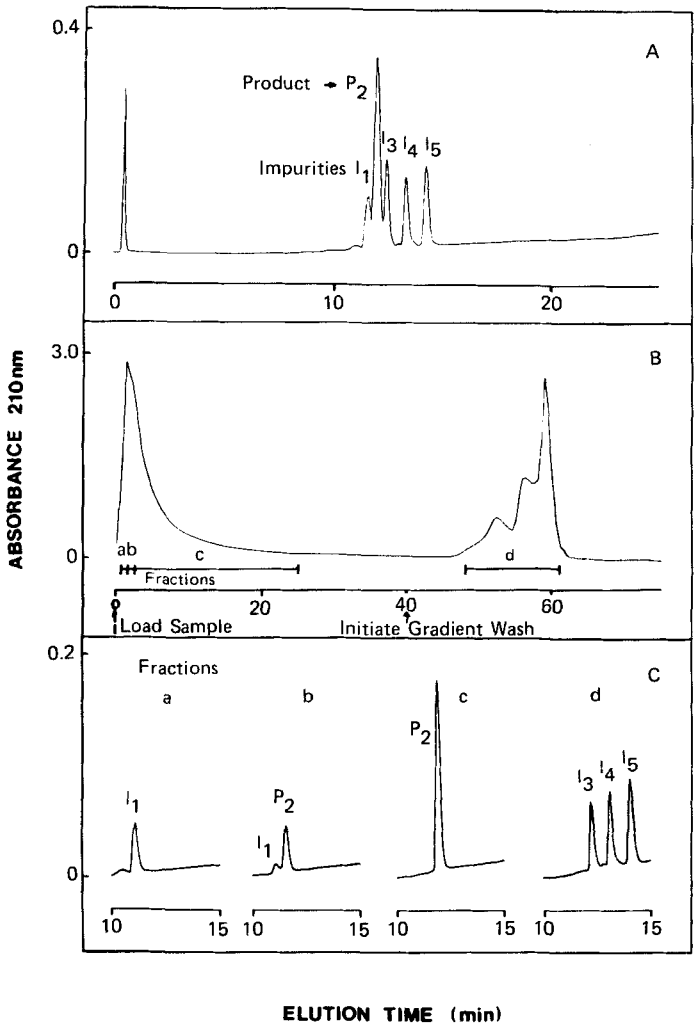

Figure 5. Separation by reversed-phase chromatography of peptide "product" (P_2) from hydrophobic (I_3,I_4,I_5) and hydrophilic (I_1) peptide "impurities" operated in sample displacement mode. Column: Aquapore RP 300 C_4 (30 x 4.6 mm I.D.). Panel A: analytical separation profile of peptide mixture; conditions, linear AB gradient (2% B/min) at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile. Panel B: preparative separation profile of peptide mixture; conditions, isocratic elution with 100% solvent A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min; sample load, 7.0 mg consisting of 3.0 mg of P_2 and 1.0 mg of each of I_1,I_3,I_4 and I_5 dissolved in 150 μl solvent A. Panel C demonstrates analytical elution profiles (see Panel A for conditions) of fractions a-d (Panel B). The subscripts of I_1,P_2,I_3,I_4 and I_5 denote peptides 1-5, respectively (see Fig. 1).

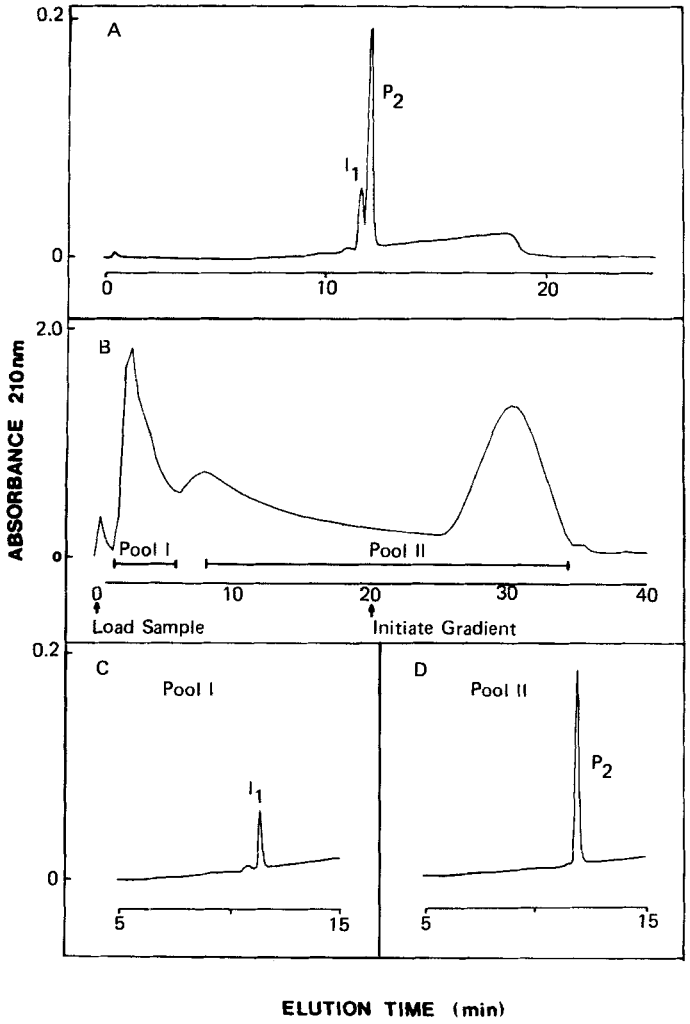

Figure 6. Separation by reversed-phase chromatography of peptide "product" (P_2) from a hydrophilic peptide "impurity" (I_1) operated in sample displacement mode. Column: Aquapore RP 300 C_8 (30 x 4.6 mm I.D.). Panel A: analytical separation profile of peptide mixture; conditions, linear AB gradient (2% B/min) at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile. Panel B: preparative separation profile mixture; conditions, isocratic elution with 100% solvent A for 20 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min. Panels C and D demonstrate analytical elution profiles (see Panel A for conditions) of Pools I and II, respectively (Panel B). The subscripts of I_1 and P_2 denote peptides 1 and 2, respectively (see Fig. 1). Sample load was approximately 3 mg of P_2 and 1 mg of I_1 dissolved in 150 μl of solvent A.

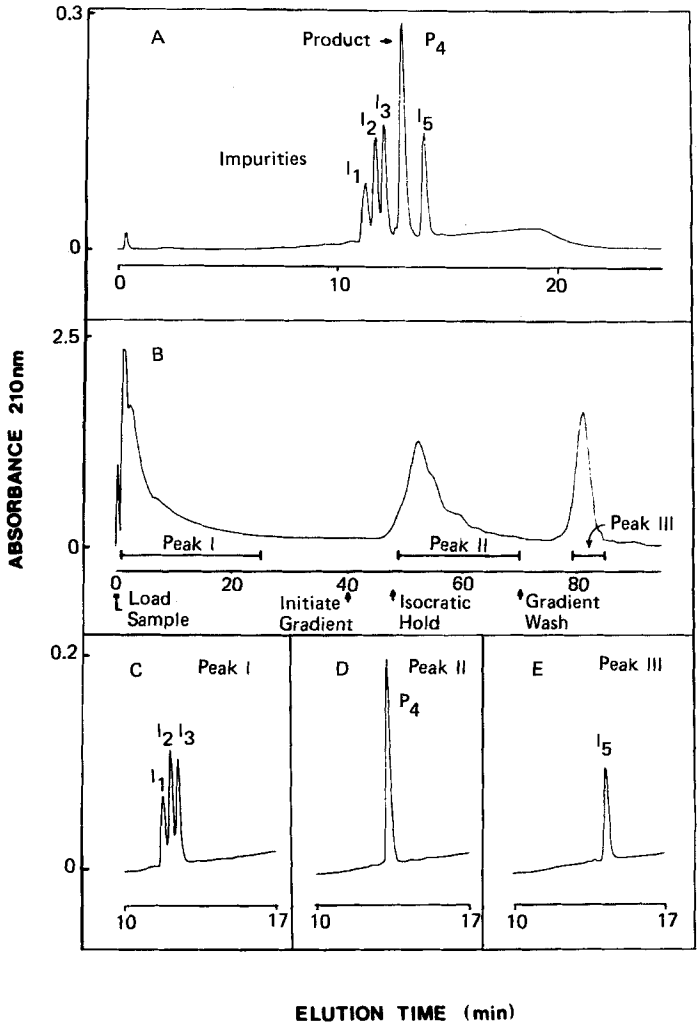

Figure 7. Separation by reversed-phase chromatography of peptide product (P_4) from hydrophobic (I_5) and hydrophilic (I_1,I_2,I_3) peptide impurities using multiple modes of chromatography. Column: Aquapore RP 300 C_8 (30 x 4.6 mm I.D.). Panel A: analytical separation profile of peptide mixture; conditions, linear AB gradient (2% B/min) at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile. Panel B: preparative separation profile of peptide mixture; conditions, elution with 100% A in sample displacement mode for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min, isocratic elution (7% B) from 47 min to 70 min, and, finally, continued linear gradient elution at 1% B/min; sample load, 7.8 mg consisting of 3.0 mg of P_4 and 1.2 mg of each of I_1, I_2, I_3 and I_4 dissolved in 150 μl of solvent A. Panels C, D and E demonstrate analytical elution profiles (see Panel A for conditions) of Peaks I, II and III (Panel B), respectively. The subscripts of I_1,I_2,I_3, P_4 and I_5 denote peptides 1-5, respectively (see Fig. 1).

mixture, where the desired "product" (P_4) is contaminated with three hydrophilic (I_1,I_2,I_3) and one hydrophobic (I_5) "impurities". The ratio of "product" to "impurities" was 1.2:1.2:1.2:3.0:1.2 (I_1:I_2:I_3:P_4:I_5), i.e. P_4 represented approximately 38% of the sample mixture. From Fig. 2, the 40 min elution loads for peptides 4 and 5 were 3.5 mg and 7.0 mg, respectively, with the level of peptide 4 being the determining factor for sample load. It was estimated that 3.0 mg of P_4 and 1.2 mg of I_5 would be sufficient to displace I_1,I_2 and I_3 (1.2 mg of each). Total sample load was 7.8 mg of peptide. The preparative separation of the peptide mixture is shown in Fig. 7, Panel B. Fraction analysis of Peak I (Panel C) confirmed that displacement of I_1,I_2 and I_3 in water had taken place. The removal and separation of P_4 and I_5, still bound to the column, could be approached in two ways. One approach would be to remove all of P_4 and I_5 with a gradient wash and, following lyophilization, reinject these components for a further round of separation in the sample displacement mode. More than one preliminary separation (Fig. 7, Panel B) would be required to obtain a sufficient amount of I_5 in the P_4/I_5 mixture for displacement of P_4 by I_5 to occur in this subsequent SDM step. An alternative approach, demonstrated in Fig. 7, Panel B, is to manipulate the gradient, following 40 min isocratic elution with 0.05% aqueous TFA, to separate P_4 from I_5. The conditions used in the present study (1% B/min gradient followed by isoratic elution at 7% B for 23 min and, finally, continued 1% B/min gradient elution) produced the excellent separation of Peak II (pure P_4; Panel D) and Peak III (pure I_5; Panel E) shown in Fig. 7, Panel B. Fraction analysis showed that 86% of recovered P_4 was found in Peak II. The remaining 14% of P_4 (contaminated with impurities) was found on either side of Peak II. These results clearly demonstrate the versatility of the SDM approach in that it can be combined with gradient elution and isocratic chromatography to achieve the separation shown in Fig. 7, Panel B in a single run. The alternative approach of two SDM runs may be easier and more convenient than optimizing a single run using combined modes of chromatography.

An important characteristic of the preparative separation profiles demonstrated in Fig. 7, as well as Figs. 3-6, is the simple visualization of the major solute zones following SDM and subsequent gradient elution. This is frequently not the case in traditional displacement chromatography, where the amount of material required for efficient development of the displacement train may overload the detector response, producing chromatographic profiles with no easily identifiable solute zones (6, 8). Extensive fraction analysis is then necessary, unlike the relatively few fraction analyses required for complete assignment of

components in the separation profiles presented in Figs. 3-7. To maintain the U.V. profile at very high loads, all that is required is to increase the wavelength for detecting peptide bonds from 210 to 220-230, or decrease the path length of the flow cell.

CONCLUSIONS

The experimental systems described in this study were designed to demonstrate the viability of sample displacement mode as a useful preparative tool. The results presented certainly indicate that SDM shows great promise for rapid separation of a single peptide component from closely-related impurities. The effects of various run parameters, such as flow-rate, column length and diameter, sample concentration and volume on peptide resolution and yield is currently under investigation.

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