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Preparative reversed-phase liquid chromatography of peptides Isocratic two-step elution system for high loads on analytical columns

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Abstract

We have developed further our novel sample displacement chromatography (SDC) methodology to carry out preparative separations on analytical equipment and 15-cm analytical columns for sample loads ≤200 mg. Thus, a two-step isocratic SDC protocol was developed and applied to the purification of important biologically active peptides, i.e. bradykinin antagonists of 10 and 11 residues. Following sample loading in 100% aqueous solvent at a concentration of ~7–10 mg/ml (with sample loads varying from 67 to 200 mg) onto a small C_{18} column (150×4.6 mm I.D., made up of three 50-mm columns attached in series), we applied isocratic elution with aqueous acetonitrile at two concentrations, the first (lower concentration) to displace hydrophilic impurities off the column and the second (higher concentration) to displace pure product from the column; hydrophobic impurities remain trapped on the column. This modified SDC approach promises to allow great flexibility in purifying peptides, at high yield of pure product (>99% purity), and encompassing a range of sample hydrophobicities as well as sample loads (≤200 mg) varying by as much as a factor of three.

Keywords: Preparative chromatography; Sample displacement chromatography; Peptides

1. Introduction

The growing therapeutic importance of synthetic peptides in recent years has led to a concomitant increase in the need for rapid and efficient peptide purification procedures. Considering that the impurities encountered during peptide synthesis (deletion, terminated or chemically modified peptides) are usually closely related structurally to the peptide of interest and, hence, often pose difficult purification problems, most preparative separations of synthetic peptides take advantage of the excellent resolving power and volatile mobile phase of reversed-phase chromatography (RP-HPLC) in gradient elution mode [1–4]. However, the elution mode of RP-HPLC is handicapped by relatively poor utilization of the stationary and mobile phases [5]. Hence, large-scale gradient elution separations of closely-related peptides necessitate the use of increasing larger columns in order to maintain satisfactory levels of product purity and yield leading, in turn, to higher operating costs in terms of packings, equipment and solvents. Most researchers would likely desire to carry out laboratory scale preparative separations on existing analytical equipment. This, in turn, tends to limit the research to the use of columns of analytical (e.g. 150×4.6 mm I.D.) or semipreparative (e.g. 250×10 mm I.D.) dimensions. How-

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ever, under conditions of conventional linear gradient-elution (0.5% to 2.0% organic modifier/min), a considerable number of reversed-phase runs with relatively low sample loads may have to be performed before the desired yield of pure product is obtained, due to the aforementioned inefficient utilization of column capacity.

Significantly more efficient use of hydrophobic stationary phases was achieved by applying the displacement mode of chromatography to reversed-phase preparative-scale separations of several classes of compounds, including peptides and proteins [5–14]. Thus, traditional displacement chromatography, designed to separate relatively large amounts of material on columns and instrumentation designed primarily for analytical work, involves sorption of a sample mixture near the inlet of the column, following its application in a carrier solvent that has low affinity for the stationary phase. A solution of a displacer, which has greater affinity for the stationary phase than any of the sample components, is then pumped slowly into the column. The sample components are thereby displaced from the surface of the stationary phase and move down the column preceding the displacer front, forming adjacent zones of purified solutes traveling at the same velocity (displacement train). Despite the advantages of displacement over elution chromatography in terms of sample load, the method is difficult to optimize in terms of choice of displacer, displacer concentration, and flow-rate. In fact, the displacer molecules, themselves tend to be somewhat toxic, an undesirable quality if a preparative technique is to be applied to, for example, pharmaceutical products. A requirement for easier and more reliable approaches to preparative reversed-phase separations of peptides, as well as expanding the researcher's options for solving a particular preparative separation problem, prompted the development in the authors' laboratory of RP-HPLC in sample displacement mode (or sample displacement chromatography; SDC) [15–20]. This novel approach to preparative-scale purification on analytical columns and instrumentation maximizes both the separation and loading potential of a reversed-phase packing, enabling rapid separation of a single peptide component, in high yield, from a complex multi-component mixture. In addition, the novel aspect of this approach is highlighted by the main separation process taking place in the absence of organic modifier. Several successful SDC separations have been reported [15–20], including the purification of a therapeutically important synthetic peptide representing part of the sequence of luteinizing hormone-releasing hormone (LHRH) [19]. Recently, SDC has also been adapted to modular solid-phase extraction (SPE) technology for development of a rapid, simple and cost-effective procedure for the efficient and parallel purification of multiple peptide mixtures [20]. An interesting application of anion-exchange chromatography in sample displacement mode for purification of proteins has also been reported [21].

The object of the present study was to develop reversed-phase SDC methodology further to carry out laboratory-scale preparative separations on existing analytical columns and equipment where the researcher has $<$200 mg of crude peptide to purify, such loads being perhaps too small to utilize traditional SDC to its fullest potential. In addition, in developing the SDC approach still further, it was also deemed important to highlight again the utility and suitability of SDC for purification of peptide therapeutics. Thus, the present study demonstrates the advantages of SDC when applied as a two-step isocratic protocol to the purification of important biologically active peptides, i.e. 10- and 11-residue bradykinin antagonists. Bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg) is involved in blood pressure regulation, pain sensation, inflammation and stimulation of growth [22,23]. Bradykinin antagonists are important tools for investigation of normal and pathophysiological functions of bradykinin, and are under development as potential drugs for inflammation and cancer [22,23].

2. Experimental

2.1. Materials

HPLC-grade water was prepared by an E-pure water purification system from Barnstead. Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (River Edge, NJ, USA). Acetonitrile was obtained from EM Science (Gibbstown, NJ, USA).

2.2. Columns

Preparative separations were carried out on three Discovery C_{18} reversed-phase columns (50×4.6 mm I.D.) connected in series (5 μm particle size, 180 Å pore size, 12.3% carbon loading, 3.0 $\mu mol/m^2$ ligand density; Supelco, Bellefonte, PA, USA). Analytical runs were carried out on a Zorbax SB300-C_8 reversed-phase column (150×4.6 mm I.D., 5 μm, 300 Å) from Agilent Technologies (Little Falls, DE, USA), using a linear A–B gradient (2% B/min), at a flow-rate of 1 ml/min, where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile.

2.3. Instrumentation

Analytical and preparative RP-HPLC runs were carried out, respectively, on an Agilent 1100 Series liquid chromatograph and a Beckman Coulter System Gold HPLC system.

2.4. Peptide synthesis

Peptides B-9430 (D-Arg–Arg–Pro–Hyp–Gly–Igl–Ser–D-Igl–Oic–Arg) and B-10238 (pentafluorocinnamoyl-B-9430), both potent bradykinin antagonists with drug potential, were synthesized as described in Ref. [22]; Hyp, *trans*-4-hydroxyproline; Igl, α-(2-indanyl) glycine; Oic, octahydroindole-2-carboxylic acid. The structures of these two bradykinin antagonists are shown in Fig. 1.

3. Results and discussion

The column size selected was 150×4.6 mm I.D., made up of three individual columns of dimensions 50×4.6 mm I.D. attached in series (Fig. 2). Apart from the flexibility of such a multi-column arrangement [17,19], an analytical stationary phase volume of this size is suitable for peptide sample loads of <200 mg which, although small in industrial terms, is of considerable size for most laboratory-based researchers.

3.1. Conventional SDC of a crude peptide mixture

3.1.1. Principles of SDC

Conventional reversed-phase HPLC was conceived as a novel method for highly efficient, preparative-scale purification of peptides on high-performance analytical columns and instrumentation, where the only variable was sample load, i.e. without the addition of organic modifier or displacer to the mobile phase [15–17]. Since peptides favour an adsorption–desorption mechanism of interaction with a hydrophobic stationary phase, under normal analytical load conditions an organic modifier is typically required for their elution from a reversed-phase column. However, when such a column is subjected to high loading of a peptide mixture dissolved in a 100% aqueous mobile phase, there is competition by the sample components for the adsorption sites on the reversed-phase sorbent, resulting in solute–solute displacement during washing with 100% aqueous mobile phase. A more hydrophobic peptide com-

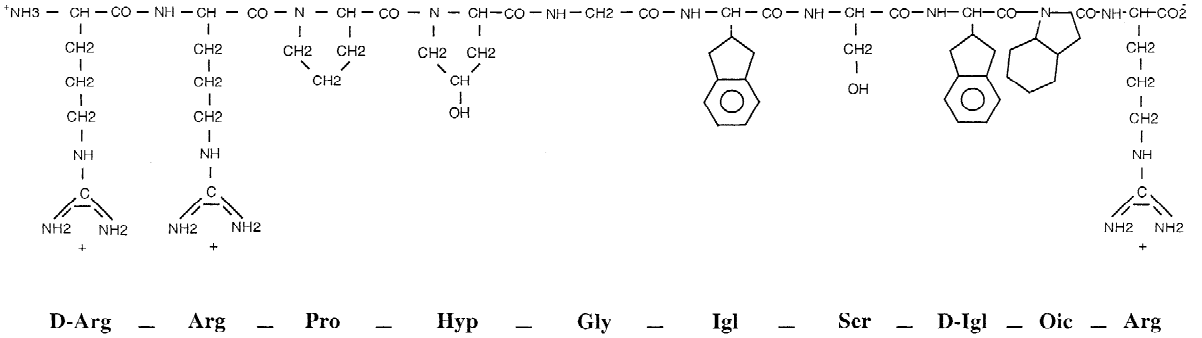

Fig. 1. Structure of bradykinin antagonist B-9430. The structure of B-10238 is the same as B-9430, save for the addition of 2,3,4,5,6-pentafluorocinnamic acid at its N-terminus.

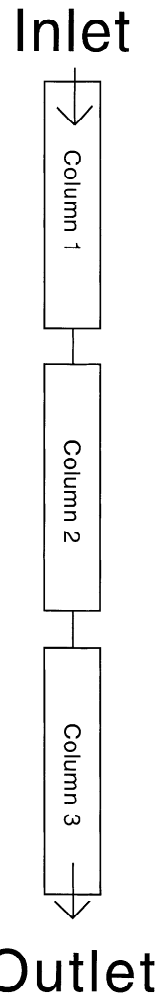

Fig. 2. Schematic representation of the three-column set-up for preparative purification of peptides. The analytical C_{18} columns used are described in Section 2. Arrows denote direction of eluent flow.

ponent competes more successfully for these sites than a less hydrophobic component, which is thus displaced ahead of the more hydrophobic solute, i.e. the sample components act as their own displacers. The SDC approach is thus simply application of the well-established general principles of displacement chromatography without the need for a separate displacer. This mode of operation, a hybrid scheme of frontal chromatography followed by elution, is characterized by a marked reduction in solvent consumption, minimal elution volumes, and the collection of fewer fractions for product isolation than in conventional RP-HPLC, with consequent reductions in time and handling.

3.1.2. Conventional reversed-phase SDC of peptide B-9430

Fig. 3 (top) shows the analytical elution profile of crude peptide B-9430. The large peak, denoted P, is the desired peptide component, representing ~58% product yield. Both hydrophilic and hydrophobic impurities are present in the crude peptide mixture, a number of which are eluted close to the peptide of interest, thus offering a good test for conventional SDC purification.

Fig. 4 profiles the results of preparative SDC of 200 mg of crude peptide B-9430 on the three-column system shown schematically in Fig. 2. Panel A, an

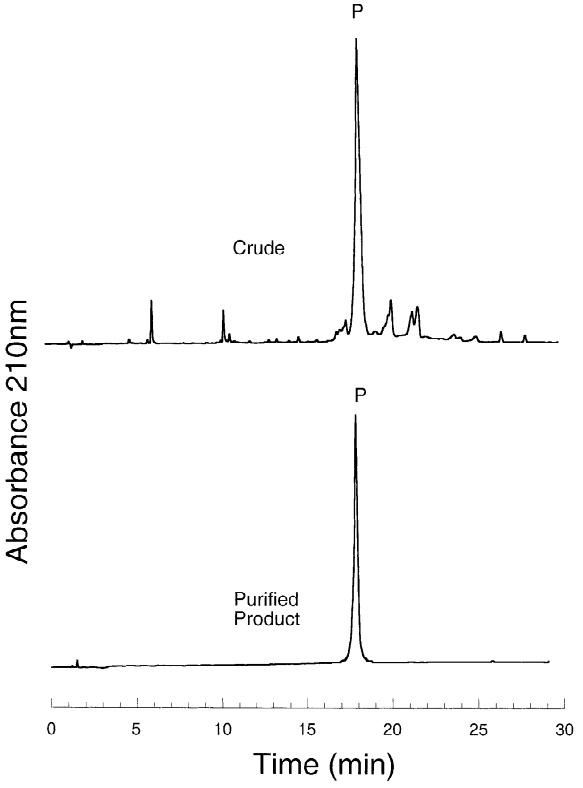

Fig. 3. Analytical RP-HPLC of (top) crude synthetic peptide B-9430 and (bottom) purified B-9430 following reversed-phase SDC of 200 mg of crude peptide (see Fig. 4 for details). Analytical RP-HPLC column and conditions shown in Section 2; injection volume, 10 μl. P denotes desired product.

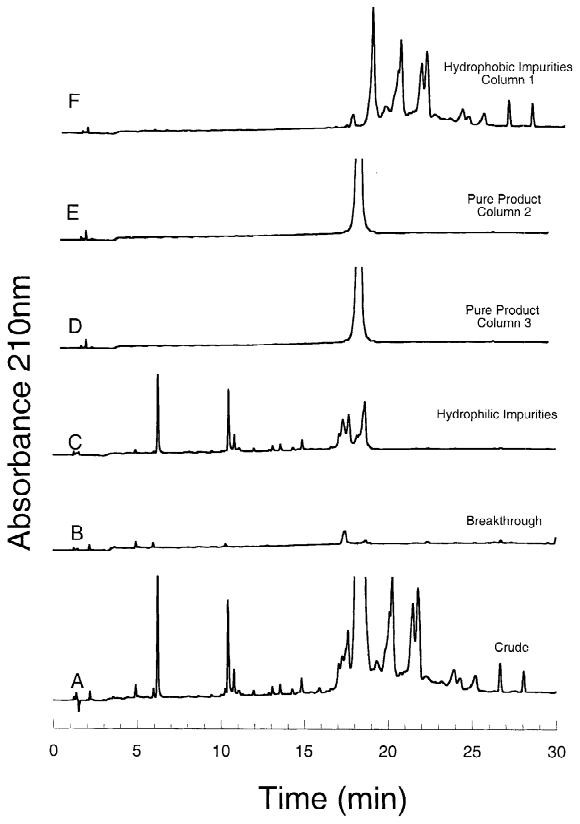

Fig. 4. Reversed-phase SDC of crude synthetic peptide B-9430. Sample (200 mg in 20 ml 0.05% aqueous TFA, i.e. 10 mg/ml) was loaded onto the three-column system (Fig. 2) at 0.5 ml/min in 4×5 ml sample loads. The columns were then washed with 15 ml of 0.05% aqueous TFA at 1 ml/min (1-ml fractions collected), separated, and individually eluted with 5 ml of 60% aqueous acetonitrile containing 0.05% TFA. Panel A, analytical RP-HPLC of crude peptide; panel B, analytical RP-HPLC of breakthrough fraction (from sample loading); panel C, pooled hydrophilic impurities displaced from columns during 15-ml wash with 0.05% aqueous TFA; panels D, E and F, components retained by columns 3 (purified product), 2 (purified product) and 1 (product contaminated with hydrophobic impurities), respectively. Analytical RP-HPLC column and conditions shown in Section 2; injection volume, 10 μl.

expanded version of the analytical elution profile shown in Fig. 3 (top), shows more clearly the extent of the hydrophilic and hydrophobic impurities contaminating the desired peptide product. Under optimal sample load and elution volume of 0.05% aqueous TFA, all hydrophilic impurities should be displaced from the columns and all hydrophobic impurities should be retained by a single column (column 1, Fig. 2), ideally with purified product only retained on the remaining columns (columns 2 and 3) (a discussion of the effect of run parameters on multicolumn SDC may be found in Ref. [17]). The SDC separation illustrated in Fig. 4 represents an optimized purification of peptide B-9430. Thus, the ''breakthrough'' fraction (panel B) represents components of the crude peptide mixture displaced from and/or unretained by the columns during sample loading. Clearly, relatively negligible material is displaced from the columns despite loading fully 200 mg of crude peptide onto three analytical columns with combined dimensions of only 150×4.6 mm, a testament to the efficient use of the stationary phase capacity. Indeed, previous SDC runs (data not shown) had determined the full sample load capacity of this three-column system as 200 mg of the 10-residue B-9430 peptide. Panel C now represents the analytical elution profile of the pooled fractions of all components displaced from the hydrophobic stationary phase during the 15-ml wash with 0.05% aqueous TFA, i.e. during the conventional SDC purification in the absence of organic modifier. Only a small amount of product (6.5% of total product yield) was found in this displaced fraction, the remainder of the components in this fraction representing hydrophilic impurities displaced by the more hydrophobic product. Indeed, the presence of even this small amount of product in this fraction implies that all of the hydrophilic impurities have been effectively displaced from the columns. As shown in panels D and E, this is indeed the case with only pure product found on columns 2 (panel E; 40.0% of total product yield) and 3 (panel D; 37.4% of total product yield). All of the hydrophobic impurities have been concentrated on column 1 (also containing the remaining 16.1% of product), these impurities being responsible for the successful displacement of the bulk of the desired product onto columns 2 and 3.

Fig. 5 shows analytical elution profiles of three of the 15 1-min fractions collected during elution of the columns with 0.05% aqueous TFA following sample loading (Fig. 4, panel C represents the analytical elution profile of the combined 15 fractions). The components in these three fractions represent hydrophilic impurities (relative to product) displaced from the columns by more hydrophobic components of the

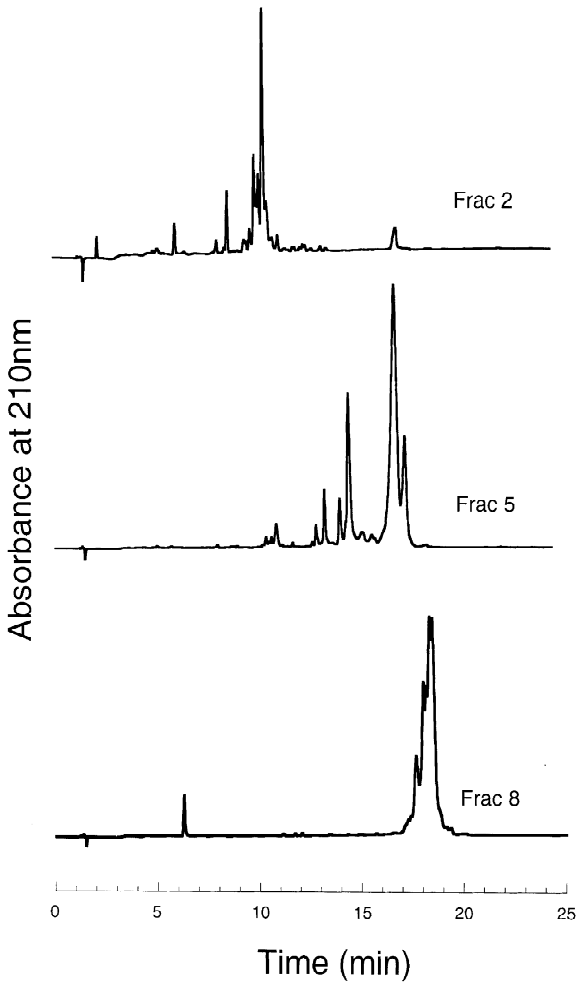

Fig. 5. Analytical RP-HPLC of fractions (1 ml) collected during 15-min column wash with 0.05% aqueous TFA following sample loading (see Fig. 4). Analytical RP-HPLC column and conditions shown in Section 2; injection volume, 10 μl.

crude peptide mixture during SDC, illustrating well the principles of displacement effects within the mixture at high sample load (or ''overload''). Thus, during loading of 200 mg of peptide B-9430, more hydrophobic components displace more hydrophilic components from adsorption sites on the hydrophobic stationary phase and effectively ''push'' more hydrophilic components ahead of them. Concomitantly, components are concentrated on the hydrophobic stationary phase resulting in adjacent zones of concentrated sample components throughout the stationary phase, approximating the ''isotachic train'' of concentrated sample zones characteristic of traditional displacement chromatography [5–14] but requiring neither the displacer nor the generally long run times of displacement chromatography. From Fig. 5, fractions 2, 5 and 8 all contain hydrophilic impurities from the crude peptide mixture, but clearly the displacement effect has fractionated even these components into groups of impurities of varying hydrophobicities. Thus, fraction 2, the earliest fraction shown, contains components of the peptide mixture displaced very early from the column during elution with 0.05% aqueous TFA, i.e. the most hydrophilic of the impurities; fraction 5 contains later displaced impurities less hydrophilic than those of fraction 2; finally, fraction 8 contains the least hydrophilic (most hydrophobic) of the impurities in the fractions shown in Fig. 5.

From the distribution of purified product shown in Fig. 4 and the excellent purity (>99%) of the pooled product shown in Fig. 3 (bottom), the success of this conventional SDC approach to purification is quite clear. Thus, from Fig. 4, columns 2 and 3 contained purified peptide B-9430 representing 40.0% and 37.4%, respectively, of total product yield, i.e. 77.4% of product yield was >99% purity. As noted above, the separation shown in Fig. 4 represents results obtained under optimized sample load and run conditions, i.e. loads <200 mg were not optimum for taking full advantage of the SDC effect and purified product yields were less than the 77.4% obtained here (data not shown). This decrease in purified peptide yields at lower sample loads is due to a lessening of the sample displacement effect, i.e. the concentrations of various sample components during peptide loading and subsequent washing with 0.05% aqueous TFA are not enough to (1) displace product maximally from column 1 to column 2 (i.e. more product is left on column 1 contaminated with hydrophobic impurities) or (2) to displace hydrophilic impurities from column 3 (i.e. product on column 3 remains contaminated with hydrophilic impurities). Thus, the best result that can be achieved is a much lower yield of purified product, obtained only on column 2. Despite the success of the separation shown in Fig. 4, we wished to develop the SDC approach still further to improve even this high yield of purified product. Specifically, we wished to increase the flexibility of SDC to allow purification

of peptides encompassing both a range of sample hydrophobicities and sample loads ≤200 mg. Simply removing a single column from the three-column set-up (Fig. 2) for lower sample loads is not a practical option since this would increase considerably the difficulty of run and sample load optimization to ensure that purified sample only was present on the second column. Thus, the overall flexibility of the system would be seriously compromised.

3.2. Isocratic two-step preparative purification of peptides of high sample load

In our effort to extend the flexibility of the SDC approach to crude peptides of varying load and hydrophobicity, we wished to maintain and/or improve the quality of purified product as well as product yield. Thus, our criteria included maintaining at least 99% of product purity at overall pure product yields of ≥85%.

3.2.1. Principles of isocratic two-step purification

Our favoured approach to modifying the conventional multi-column SDC approach demonstrated above (Fig. 4) was to change the role of the three-column system from being both a trap for hydrophobic impurities (column 1) and retention of purified product (columns 2 and 3) to that of solely a trap for hydrophobic impurities during the displacement effect. Thus, hydrophilic impurities and purified product would be displaced, in a two-step operation, from the columns, thereby laying greater emphasis on efficient fraction collection. Such an approach would require a means of complementing the displacement effect with a second sample desorption effector, i.e. isocratic elution with an organic modifier. The effect of low levels of organic modifier on SDC purification of large-scale peptide separations has previously shown potential [19] for specific separations, and we now wished to develop this approach further to maximize separation efficiency for much lower sample loads of a wide range of crude peptide mixtures.

An outline of the two-step isocratic elution protocol, termed TS-SDC for two-step SDC, subsequently developed is as follows.

1. Load sample in 0.05% aqueous TFA.
2. Apply, as a rule of thumb, isocratic elution with an acetonitrile concentration 20% less than that required to elute the product during linear gradient elution; hydrophilic impurities are displaced from the column(s) and the sample displacement effect is also allowed to continue on the column(s).
3. Now apply isocratic elution with a 5% higher concentration of acetonitrile (i.e. 15% less than that required to elute the product during linear gradient elution) to displace purified product from the column(s); hydrophobic impurities remain trapped on the column(s).
4. Regenerate columns with a wash of 60% acetonitrile containing 0.05% aqueous TFA.

Accurate calculation of the % acetonitrile required to elute the desired product from the column(s) requires knowledge of the gradient elapse time (t_g) of the chromatographic system [1]. However, even an approximation of this value will generally suffice given the rule-of-thumb nature of the isocratic acetonitrile concentrations required. It should be noted that, since the acetonitrile concentration used for isocratic elution of a peptide mixture depends solely on the hydrophobicity of the peptide of interest, this two-step isocratic approach may be used for crude peptides of widely varying hydrophobic characteristics; only a single analytical gradient scouting run is required prior to preparative purification. Theoretically, this two-step approach to sample displacement purification could be carried out on a single reversed-phase column (e.g. a single 150 mm×4.6 mm I.D. column) instead of the three-column system illustrated in Fig. 2. However, if a three-column system is retained, the researcher may tailor the separation approach (i.e. conventional multi-column SDC or a two-step isocratic elution protocol) to the purification requirements of the specific peptide mixture under consideration. Thus, the purification options of the researcher are more flexible.

3.2.2. Two-step SDC of peptide B-9430: 67-mg sample load

Fig. 6 illustrates the results of preparative two-step SDC of 67 mg of crude peptide B-9430 on the three-column system (Fig. 2). As noted previously, the analytical elution profile of the crude peptide mixture is shown in Fig. 3 (top) with the desired

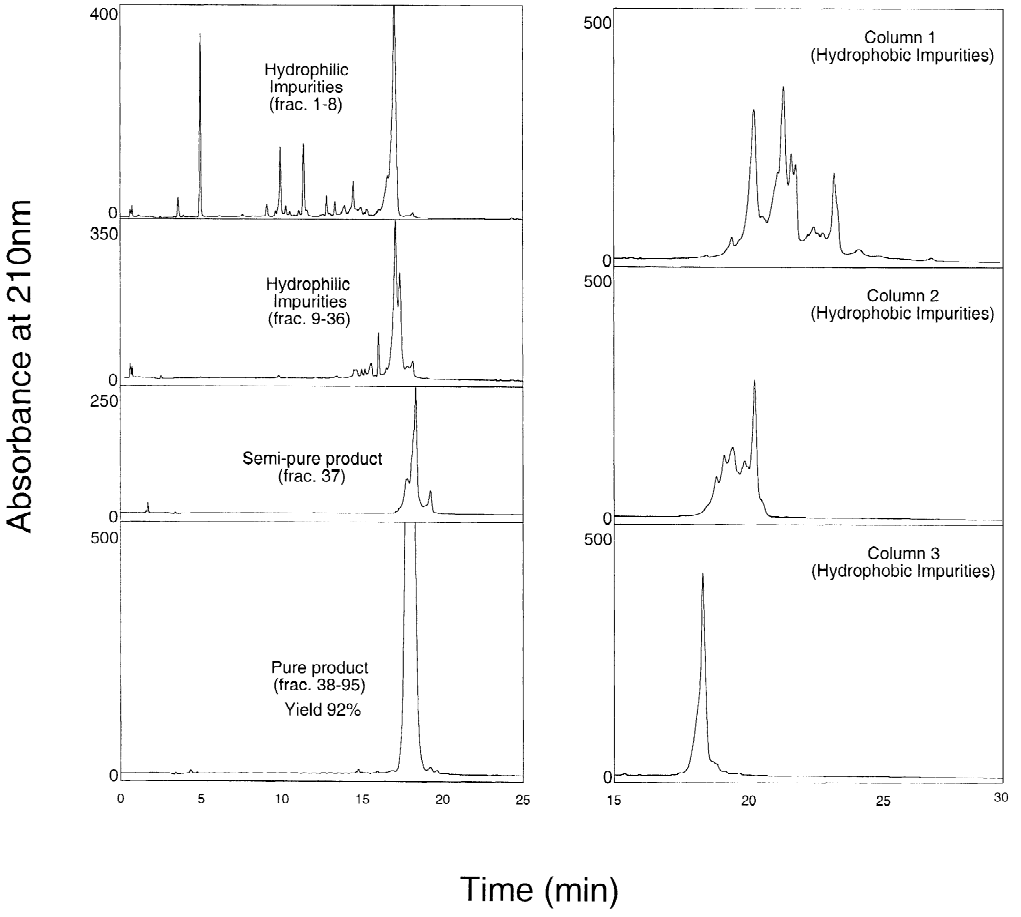

Fig. 6. Two-step isocratic RP-HPLC purification of peptide B-9430: 67 mg sample load. Sample (10 mg/ml in 0.05% aqueous TFA) was loaded onto the three-column system (Fig. 2) at 0.5 ml/min. The columns were then eluted at 1 ml/min with two isocratic washes: (1) 10% aqueous acetonitrile containing 0.05% TFA for 30 min, followed by (2) 15% aqueous acetonitrile containing 0.05% TFA for 70 min. Fractions were collected every minute during isocratic elution with aqueous acetonitrile. The columns were then separated and individually eluted with 5 ml of 60% aqueous acetonitrile containing 0.05% TFA. Analytical RP-HPLC column and conditions for individual fraction analysis and analysis of pooled fractions shown in Section 2; injection volume, 25 μl.

product representing ~58% crude peptide yield. Following sample loading (no sample components detected in breakthrough eluent) in 0.05% aqueous TFA, the columns were subjected to an isocratic wash with 10% aqueous acetonitrile containing 0.05% TFA (this acetonitrile concentration being calculated as 20% less than that required to elute the peptide during linear gradient elution), with concomitant start of fraction collection (100 1-min fractions). After 30 min, an isocratic wash with 15% aqueous acetonitrile containing 0.05% TFA was applied for a further 70 min. The columns were then separated and individually eluted with 60% aqueous acetonitrile containing 0.05% TFA. Note that identical conditions of sample loading, isocratic washes and fraction collection were applied to all three sample loads of this peptide.

From Fig. 6, the initial 30-min isocratic wash (10% acetonitrile) displaced most of the hydrophilic impurities off the columns, with the second wash (15% acetonitrile) displacing the remainder of these impurities as well as the product. Purified product (representing an excellent 92% of total product yield; Table 1) was found in fractions 38–95, with only a

Table 1
Comparison of SDC and TS-SDC of crude peptide mixtures

Preparative run	Yield of[a] purified product (%)	Figure(s)
SDC of B-9430 (200 mg)[b]	77.4	3–5
TS-SDC of B-9430 (67 mg)[c]	92.0	6
TS-SDC of B-9430 (133 mg)[c]	96.0	7
TS-SDC of B-9430 (200 mg)[c]	100.0	8
TS-SDC of B-10238 (157 mg)[c]	85.0	9 and 10

[a] Denotes product obtained at ≥99% purity.
[b] Denotes conventional multi-column reversed-phase sample displacement chromatography.
[c] Denotes two-step isocratic elution SDC.

single fraction (fraction 37) representing a crossover fraction with product (just 8% of total product yield) significantly contaminated with impurities. In a similar manner to the fractions collected during conventional SDC (Fig. 5), the earliest fractions collected contain the most hydrophilic components of the crude peptide mixture which are thus concentrated and displaced first from the columns (fractions 1–8). Less hydrophilic (i.e. more hydrophobic) components are displaced in order of decreasing hydrophilicity, with purified product being eluted last. As was desired, all hydrophobic impurities were retained by the three columns. Note that, in a similar manner to the other peptide components (hydrophilic impurities and product), the concentration and displacement effects of the hydrophobic impurities are dictated by their relative hydrophobicities. Thus, the most hydrophobic components are displaced the least and remain on the column (column 1) closest to the

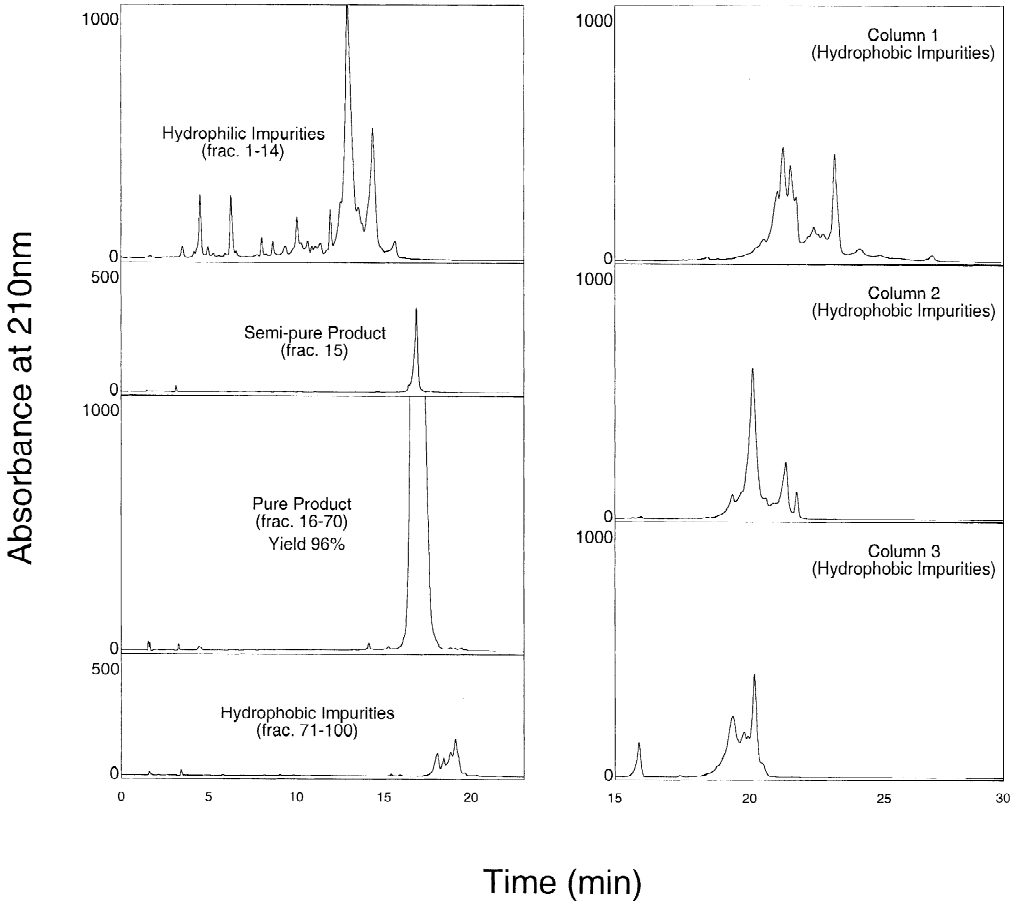

Fig. 7. Two-step isocratic RP-HPLC purification of peptide B-9430: 133 mg sample load. Experimental details described in Fig. 6.

inlet; in contrast, the least hydrophobic components of these impurities are displaced to, but remain on, the column (column 3) closest to the outlet.

3.2.3. Two-step SDC of peptide B-9430: 133-mg sample load

The two-step SDC approach was now applied to purification of 133 mg of peptide B-9430, i.e. double the 67-mg load shown in Fig. 6. From Fig. 7, the results at this higher sample load were similar to those obtained with 67 mg of sample (Fig. 6) with another excellent purified product yield of 96% (Table 1). In addition, a single fraction (fraction 15) contained product (4% of total product yield) contaminated with hydrophilic impurities. The same pattern of decreasing component hydrophilicity with increasing fraction number of displaced fractions can again be seen, with the additional appearance of hydrophobic impurities displaced from the columns late in the run (fractions 71–100). In addition, the retention pattern of the hydrophobic impurities on the three columns is also similar to that seen in Fig. 6. It is interesting to note that the first fraction containing product at this sample load was significantly earlier (fraction 15) compared to that of the 67-mg sample load (fraction 37), i.e. the product is displaced from the columns earlier as sample load is increased.

3.2.4. Two-step SDC of peptide B-9430: 200-mg sample load

Fig. 8 now shows the results of applying the two-step procedure to 200 mg of peptide B-9430. A successful separation at this sample load was signifi-

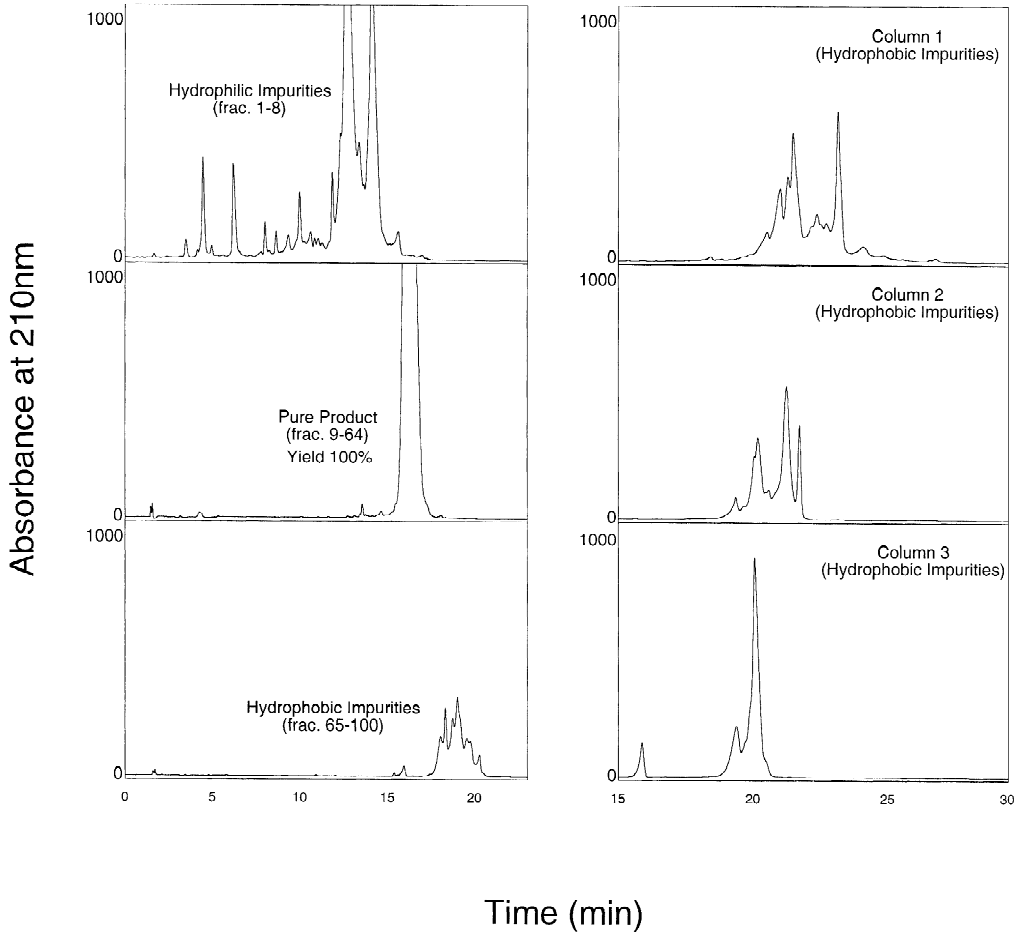

Fig. 8. Two-step isocratic RP-HPLC purification of peptide B-9430: 200 mg sample load. Experimental details described in Fig. 6.

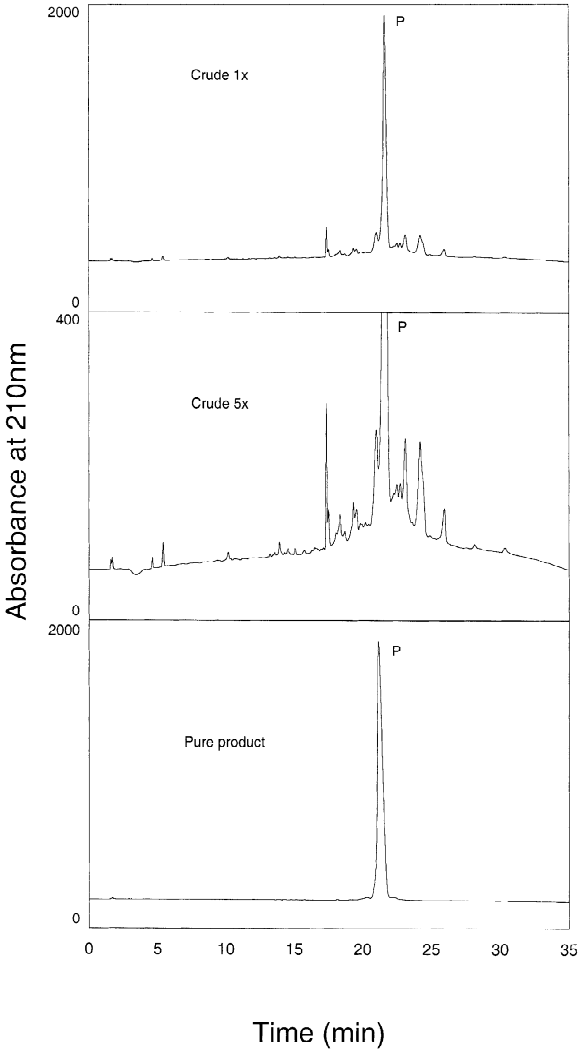

Fig. 9. Analytical RP-HPLC of (top and middle) crude synthetic peptide B-10238 and (bottom) purified B-10238 following two-step SDC of 157 mg of crude peptide (see Fig. 10 for details). Analytical RP-HPLC column and conditions shown in Section 2; injection volume, 10 μl. P denotes desired product.

cant both to demonstrate the value of this approach for a sample loading varying by as much as threefold (67 mg (Fig. 6)→200 mg (Fig. 8)) and to offer a direct comparison to conventional SDC at this sample load (Fig. 4).

From Fig. 8, the success of the two-step purification of 200 mg of the peptide is quite clear with fully 100% of total product yield purified to greater than 99% purity, compared to 77.4% purified product obtained by the conventional SDC approach (Fig. 4), (i.e. no significant cross-contamination in any collected fractions). Again, product is being displaced earlier from the column at this highest sample load, appearing first in fraction 9, compared to fraction 15 (133-mg load; Fig. 7) and fraction 37 (67-mg load; Fig. 6). In addition, hydrophobic impurities are also again being displaced from the columns, albeit first appearing earlier during fraction collection (fraction 65 for a 200-mg sample load; Fig. 8) compared to the 133-mg sample load (fraction 71; Fig. 7).

3.2.5. Two-step SDC of peptide B-10238

Having demonstrated the efficacy of this two-step isocratic displacement approach to purifying, with high yields of purified product, a crude peptide mixture varying by as much as threefold in sample amounts, it was now important to demonstrate its flexibility in terms of purification of peptides of varying hydrophobicity. Thus, this approach was now applied to the preparative purification of peptide B-10238 (Fig. 1), a more hydrophobic peptide than B-9430 (compare analytical retention times of 17.7 min for B-9430 (Fig. 3) and 22.0 min for B-10238 (Fig. 9)).

Fig. 9 (top panels) shows the analytical elution profile of the crude B-10238 peptide mixture, with the product, denoted P, representing ~58% of crude peptide synthesis yield. The results shown in Fig. 10, illustrating a successful preparative purification of B-10238, were obtained under identical conditions of sample loading, run conditions and fraction collection as described above (Figs. 6–8), except for the isocratic concentrations of acetonitrile. Thus, based on the rule-of-thumb of isocratic elution with an acetonitrile concentration 20% less than that required to elute B-10238 during linear gradient elution (Fig. 9), the initial 30-min column wash was carried out with 16% aqueous acetonitrile containing 0.05% TFA, which was then raised to 21% acetonitrile for the second, 70-min, isocratic elution. From Fig. 10, purified product, representing 85% of total product yield (Table 1), was successfully obtained in fractions 30–100. Interestingly, the greater majority of the hydrophobic impurities were concentrated on just two columns (columns 1 and 2), with some product (9.9% of total yield) still retained on column 3. Indeed, it is likely that the yield of purified product

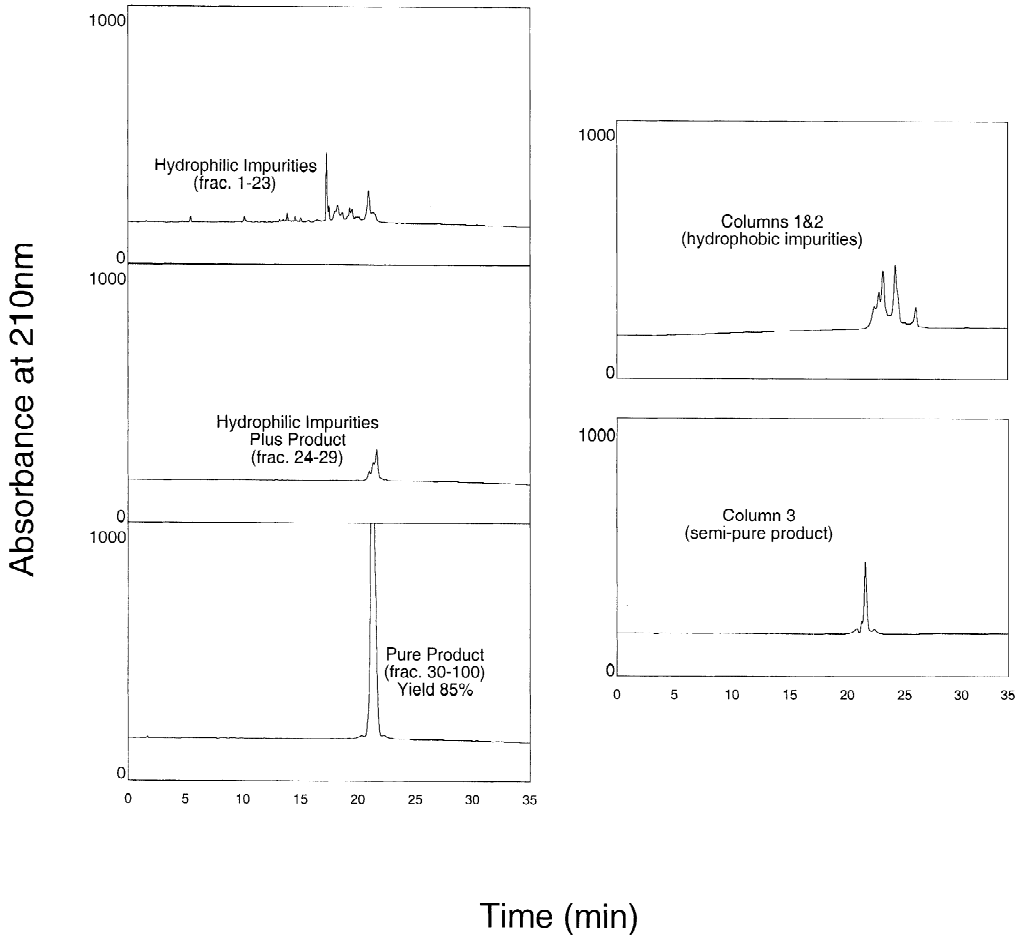

Fig. 10. Two-step isocratic RP-HPLC purification of peptide B-10238. Sample (157 mg at 7 mg/ml in 0.05% aqueous TFA) was loaded onto the three-column system (Fig. 2) at 0.5 ml/min. The columns were then eluted at 1 ml/min with two isocratic washes: (1) 16% aqueous acetonitrile containing 0.05% TFA for 30 min, followed by (2) 21% aqueous acetonitrile containing 0.05% TFA for 70 min. Fractions were collected every minute during isocratic elution with aqueous acetonitrile. The columns were then separated and individually eluted with 5 ml of 60% aqueous acetonitrile containing 0.05% TFA. Analytical RP-HPLC column and conditions for individual fraction analysis and analysis of pooled fractions shown in Section 2; injection volume, 10 μl.

could readily be enhanced still further by increasing the volume of the second isocratic wash.

From the preparative separations shown in Figs. 6–10, certain principles of isocratic elution/SDC emerge as advantageous to obtaining high product purity and yield. Thus, in each separation, product is distributed over a significant number of fractions, i.e. over a relatively long time period during these 100-min runs. This allows straightforward location of cut points for product pooling, i.e. which fractions represent the start and finish points for product of required purity. For instance, the separations shown in Figs. 6 and 7 only had one transition fraction each, i.e. only a single fraction had product cross-contaminated with impurities. By carrying out isocratic elution, product is well separated from hydrophilic and hydrophobic impurities, often eluted close to the peak of interest during linear gradient elution. Thus, the sample displacement effect under overload conditions is present but the two isocratic elution steps

help to ''spread'' the components of the crude peptide mixtures, making successful separations at high yield of purified product readily achievable.

4. Conclusions

The present study extended novel reversed-phase sample displacement methodology to carry out laboratory scale preparative separations on existing analytical columns and equipment where the researcher has ≤200 mg of crude peptide to purify. A two-step isocratic SDC protocol, utilizing a three-column arrangement, was developed and applied to the purification of important biologically active peptides, i.e. bradykinin antagonists of 10 and 11 residues. By allowing the sample displacement effect under sample overload conditions to take place during isocratic elution with aqueous acetonitrile at two different concentrations, purified product was obtained at >99% purity and at least 85% yields for crude peptide mixtures ranging from 67 to 200 mg sample loads on column dimensions totaling just 150×4.6 mm I.D. This flexible two-step SDC approach shows great promise as a universal method for purification of peptide mixtures of different hydrophobicities and with loads varying by as much as a factor of three on a single 15 cm analytical column.

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