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Note

Facile, semi-preparative, high-performance liquid chromatographic separation of synthetic peptides using ammonium bicarbonate buffers

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An important goal of our high-performance liquid chromatography (HPLC) studies[1,2] has been the development of a simple procedure for the purification of synthetic peptides prepared by the solid phase method[3] Frequently a small scale synthesis will yield 25–100 mg of crude peptide which is contaminated by aberrant peptides produced by side-reactions. A recent study[4,5] demonstrated that the Radial-Pak C_{18} columns that are used in the Waters Radial Compression Module are particularly suitable for the separation of peptides[5] The purpose of this report is to demonstrate that this separation system can be used for surprisingly large sample loadings (typically 25 mg) with a volatile mobile phase (containing ammonium bicarbonate) that allows the rapid isolation of the purified peptide by lyophilisation

EXPERIMENTAL

Apparatus

A Waters Assoc (Milford, MA, U S A) HPLC gradient system was used for the separations (see Refs 1 and 2 for details) Sample injections were made using a Microliter 802 syringe or a 10-ml Gastight 1010 W syringe (Hamilton, Reno, NV, U S A.) The Radial-Pak CN and C_{18} cartridges (8 mm I D) were also purchased from Waters Assoc. For optimal column life the column was protected with a guard column and an in-line prefilter (Waters Assoc.)

UV spectra were determined using a Shimadzu MPS-5000 instrument

Chemicals and separation conditions

The solvents and chemicals used in this study are identical to those described in a previous paper[5] The peptide solutions were prepared as described previously[2], except that all solutions contained 6 *M* urea or 3 *M* guanidine–HCl to prevent sample aggregation.

The ammonium bicarbonate buffer (0 1 *M*, pH 7.7) was prepared freshly each day, and was used as the initial solvent (A) in the gradient separations The second solvent (B) consisted of isopropanol–acetonitrile–solvent A (30·30 40) (Figs 1 and 2) or isopropanol–solvent A (80 20) (Fig. 3). A linear gradient from 0 to 100% B over 60 min was used throughout this study. The separation was achieved at ambient

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temperatures and a flow-rate of 1 ml/min was used. The eluted peptides were detected by UV absorbance (generally at 280 nm).

RESULTS

Fig. 1. shows the purification of 60 μg of the synthetic peptide Leu–Glu–Ser–Phe–Lys–Val–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Glu–Tyr–Thr–Lys–Ala (I) using the chromatographic system described in the Experimental Section Although this peptide had been previously purified by ion-exchange chromatography, the sample still contained two deletion peptides which were readily removed by HPLC. The large O.D.

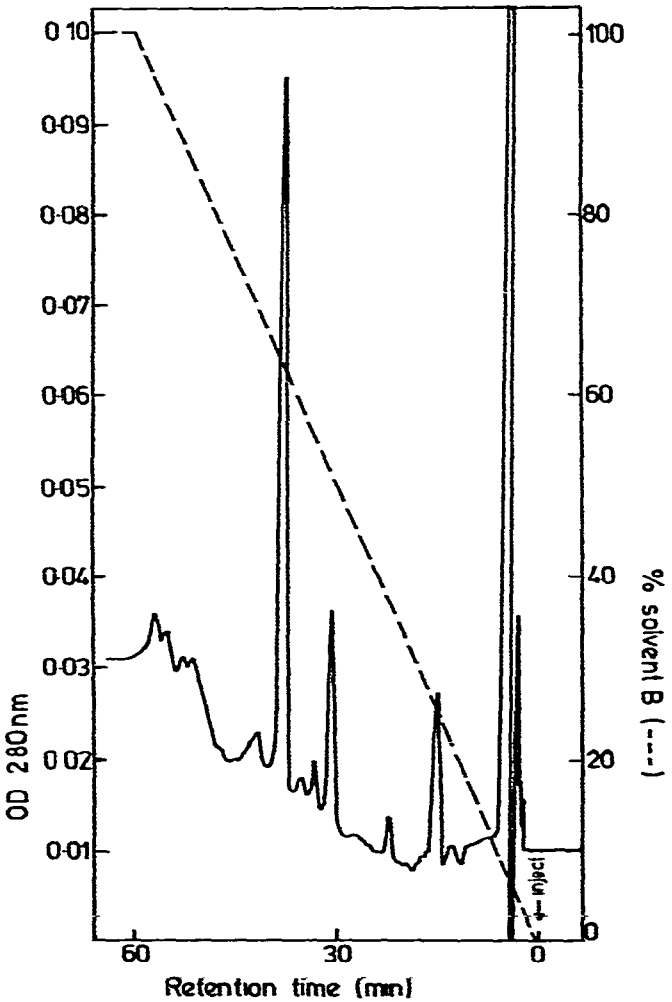

Fig. 1. The purification of 60 μg of the synthetic peptide Leu–Glu–Ser–Phe–Lys–Val–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Glu–Tyr–Thr–Lys–Ala on a Radial-Pak CN column was achieved with a linear gradient from 0.1 *M* ammonium bicarbonate to isopropanol–acetonitrile–0 1 *M* ammonium bicarbonate (3.3.4) at a flow-rate of 1.0 ml/min.

peak at the start of the gradient was due to guanidine–HCl which was added to the sample to prevent aggregation

Fig. 2A shows the purification of 350 μg of the synthetic peptide Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala using the conditions described in Fig 1. Prior to the HPLC separation, this peptide had been partially purified by gel filtration and ion-exchange chromatography The sample was loaded in 5 ml of 6 *M* urea as three approximately equal volumes through the U6K sample injector. The large peak was collected (see bar in Fig 2A) and re-chromatographed as shown in Fig. 2B. Before rechromatography the trapped peak (2 ml) was diluted to 6 ml with 0.1 *M* ammonium bicarbonate and loaded as three approximately equal volumes using identical conditions as those in Fig 2A

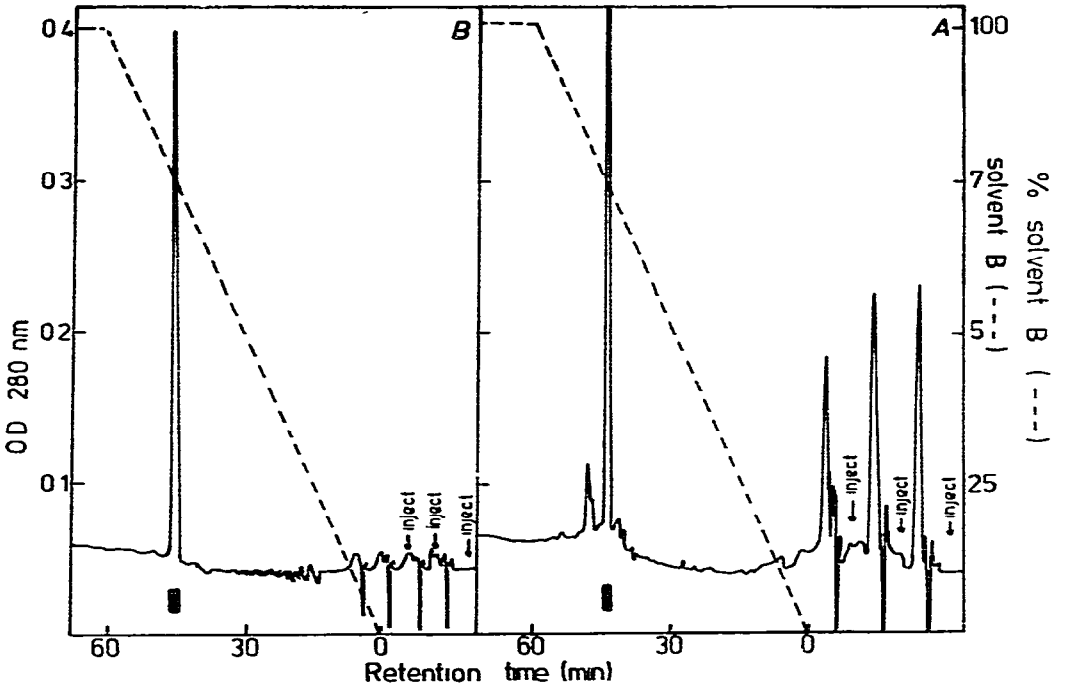

Fig 2. The purification of 350 μg of the peptide Leu–Glu–Ser–Phe–Leu–Lys–Ser–Trp–Leu–Ser–Ala–Leu–Glu–Gln–Ala–Leu–Lys–Ala was accomplished using the conditions described in the legend in Fig 1. The sample was loaded in three injections from a solution in 5 ml of 6 *M* urea Part A shows the elution profile for the crude peptide mixture. The area defined by the solid bar was pooled (2 ml), diluted to 6 ml with 0.1 *M* ammonium bicarbonate, and re-chromatographed using the same conditions as in Part A

Fig. 3A shows the separation of another synthetic peptide Val–Ser–Ser–Leu–Leu–Ser–Ser–Leu–Lys–Glu–Tyr–Trp(CHO)–Ser–Ser–Leu–Lys–Glu–Ser–Phe–Ser (2 5 mg) on the Radial-Pak C_{18} column The separation was achieved with a linear gradient of 0.1 *M* ammonium bicarbonate to 0 1 *M* ammonium bicarbonate–isopropanol (1.4). The separation shown in Fig. 3B was achieved under identical conditions as part A, except that the sample loading was only 0.37 mg and 0.1 *M* ammonium formate (pH 7.5) replaced the 0 1 *M* ammonium bicarbonate in both solvent A and B.

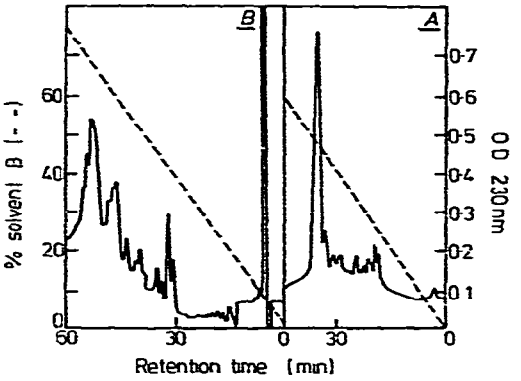

Fig. 3 The purification of 2.5 mg of the peptide Val–Ser–Ser–Leu–Leu–Ser–Ser–Leu–Lys–Glu–Tyr–Trp(CHO)–Ser–Ser–Leu–Lys–Glu–Ser–Phe–Ser on a Radial-Pak C_{18} column was achieved in part A with a linear gradient from 0.1 *M* ammonium bicarbonate to isopropanol–0.1 *M* ammonium bicarbonate (4:1). The absorbance was monitored at 300 nm with 2.0 a.u.f.s. Run B was achieved using identical conditions except that ammonium formate was used instead of ammonium bicarbonate. In this analysis the absorbance was monitored at 230 nm with 0.2 a.u.f.s. and 0.370 mg of peptide was loaded. A guard column filled with Porasil B C_{18} was used in both chromatograms.

DISCUSSION

We[4] and others[6] have reported the use of ammonium bicarbonate as a suitable mobile phase for the Radial-Pak C_{18} flexible-walled columns. The high apparent pH of this mobile phase (7.7–8) precludes its use with siliconaceous supports packed in inflexible columns, due to the generation of column voids caused by dissolution of the silica. The radial compression used with the flexible-walled columns circumvents this problem as any voids that may be generated are removed under compression. Provided the column is washed with water and then isopropanol each evening, we have found that an extended life-time of at least 6 months can be achieved with Radial-Pak C_{18} or CN columns and the mobile phases used in this study. As a further precaution we have recently introduced the use of a guard column filled with Porasil B C_{18} packing material. The elution profiles shown in Fig. 3 were achieved with the guard column in line with the prefilter. We have shown[7] that the guard column does not degrade the separation, but does allow for a significant increase in column lifetime due both to removal of contaminants from the sample and mobile phase and to dissolution of silica in the guard column (thus partially presaturating the mobile phase with silica).

The use of ammonium bicarbonate, with its excellent volatility, allows the semi-preparative separation of synthetic peptides as the separated material can be simply isolated by freeze-drying. The organic solvent can be also removed at this stage, provided the sample is diluted with water to allow freezing of the sample. Alternatively the organic solvent can be removed by reduced pressure evaporation before the freeze-drying step. The perfluoroalkanoic acids, which also have excellent volatility and can be used in peptide separations[8], are not suitable for use with the Radial-Pak C_{18} or CN columns due to the limited stabilities of these packing materials at low pH values[9]. Amine salts, which can be adjusted to suitable pH values, are not sufficiently volatile to allow facile preparative separations[10].

The only significant problem with ammonium bicarbonate buffers is the possibility of bubble formation with the use of either high concentrations of ammonium bicarbonate (0.5 *M*) or organic solvent (above 80%) Therefore, this mobile phase may not be suitable for the chromatography of extremely basic peptides which require high salt concentrations to suppress silanophilic interactions We are presently investigating[11] suitable volatile buffers for use in this circumstance

Figs. 1–3 show that a range of synthetic peptides can be separated with high efficiency by the ammonium bicarbonate system The eluted peptides were identified by amino acid analysis of an acid hydrolysate (see Table I for a representative example) Also it should be noted that an increase in sample load (0 06 to 0 35 to 2 5 mg in Figs. 1–3, respectively) was achieved with no loss of separation efficiency. Current studies with this system show that sample loads as high as 25 mg can be used without significant loss of separation efficiency This result, when combined with the excellent volatility of the mobile phase, demonstrates the suitability of this system for the semi-preparative separation of synthetic peptides

TABLE I

REPRESENTATIVE RECOVERIES AND AMINO ACID COMPOSITIONAL DATA* OBTAINED IN THE SEPARATION OF THE SYNTHETIC PEPTIDES

Amino acid	*Expected*	*Obtained*
Ser**	3	3 0
Glu + Gln	3	3 1
Ala	3	2 9
Leu	5	5 0
Phe	1	1 0
Lys	2	2 0
Trp***	1	1 0
Recovery	87%***	

* Expressed as number of residues per molecule
** Corrected for destruction of serine during acid hydrolyses
*** Obtained by UV spectra determination (λ_{max} = 280 nm, ε_{280} = 5200 l mol^{-1} cm^{-1})

One final feature that is crucial for preparative separations is the ability to achieve high recoveries of the purified material. Recoveries were determined either by amino acid analysis or UV spectra determination (using the characteristic tryptophan absorption) In all cases the recovery of the purified material was in excess of 85% and a typical example is shown in Table I.

Much of the selectivity that is achieved in reversed-phase HPLC of peptides has been attributed to ion-pairing[2,12] and silanophilic interactions[5]. As is shown by a comparison of the elution profiles shown in Fig. 3A and B ammonium bicarbonate allows excellent peak shapes which may, in some cases, be superior to those obtained with other buffers In Fig 3B, although the sample loading was significantly lower than in part A (0 37 *vs* 2 5 mg), the use of ammonium formate gave broader peak shapes and lower recoveries than did the use of ammonium bicarbonate (see part A) The success of ammonium bicarbonate as a mobile phase additive can be related to

the presence of ammonium ions which will suppress unwanted silanol-solute interactions, while bicarbonate may have similar ion-pair effects to that of phosphate[2].

In conclusion this report demonstrates that ammonium bicarbonate solutions when combined with suitable organic modifiers allows the facile semi-preparative HPLC separation of synthetic peptides. This system gives excellent peak shapes in a separation, as well as yielding purified peptides with excellent recoveries after a simple freeze-drying step.

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