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USE OF MIXED-MODE, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF PEPTIDE AND PROTEIN MIXTURES

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SUMMARY

The packing material recently introduced for use in a radial compression chamber, with a partially flexible cartridge, has a low C_{18}-coating (5% w/w) combined with an absence of secondary capping. This report demonstrates that such a support, which contains significant concentrations of both free silanol and hydrocarbon groups, can allow a mixed-mode separation to occur via adsorption and reversed-phase separation mechanisms. In any given separation, the predominant mechanism depends both on the nature of the sample and the mobile phase. For efficient peptide and protein separations, it was necessary to suppress most silanol group interactions by the use of a mobile phase which contained a high concentration of an amine phosphate, *e.g.*, 0.17 *M* triethylammonium phosphate, pH 3.2. In addition, it was necessary to deactivate further the silanol groups by an initial column wash of at least 20 column volumes of methanol. Samples which contained strongly basic groups, for example the guanidino group of arginine, can still exhibit poor separation efficiencies on such a support. These problems were largely overcome, however, with the use of isopropanol as an organic modifier. If these precautions were followed, the packing material gave excellent selectivities in the separation of closely related materials, as well as allowing increased sample capacities.

These observations will be supported by an examination of the chromatographic properties of a range of small peptides, the C-apolipoprotein mixture present in human very-low-density lipoproteins and the purification of an 8-mg sample of a synthetic pentadecapeptide in a single chromatographic run.

INTRODUCTION

We described earlier[1] the use of the Radial Compression Separation System[2] for the high-efficiency separation of complex peptide and protein mixtures. This

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system uses flexibly walled cartridges packed with spherical 10-μm silica particles which were derivatised with a relatively light loading (*ca.* 5% w/w) of a C_{18}-silane[5]. Since the unreacted silanol groups are not blocked by "end-capping" with trimethylchlorosilane, this packing material contains a significant concentration of free silanol groups which can interact with the solute molecules as well as with the mobile phase. Several authors[4–7] have suggested that such a packing material would give mixed retention mechanisms. Also Rabel and co-workers[7,8] found that the use of a support with low organic loadings gave greater selectivities in the separation of polar, low-molecular-weight solutes. The relatively high column efficiencies obtained with non-polar stationary phases with low surface coverages can be attributed to a higher concentration of accessible silanol groups. Such groups, which are likely to be hydrated, may reduce mass transfer resistance for a polar solute from the aqueous eluent to the surface of the stationary phase. In addition silanol groups, in the presence of low pH mobile phases, may introduce specific hydrogen bonding interactions with polar solutes.

This report, therefore, examines the chromatography of small basic peptides on this column, and demonstrates that a strong interaction between basic solutes and silanol groups present in the packing material gives irreproducible results. However, the use of hydroxylic organic solvents and amine phosphate buffers minimises the effect of silanol groups, so that satisfactory peak shapes and sample recoveries can be obtained. The resulting mixed-mode chromatographic system was shown to allow the efficient separation of a range of peptide and protein samples.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatographic (HPLC) system was used for the analytical separations. This consisted of two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to an M450 variable wavelength UV spectrophotometer (Waters Assoc.) and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Sample injections were made using a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.). The Radial Compression Module and the Radial-PAK A(C_{18}) cartridge were also purchased from Waters Assoc. For convenience the radially compressed cartridges will be referred to as RC-C_{18}.

Chemicals

The solvents and chemicals used in this study are identical to those described in a previous paper[1]. The synthetic peptides were prepared by the solid phase method[8] and the details of the synthesis will be described elsewhere. The C-apolipoprotein mixture from very-low-density lipoproteins (VLDL), and the partial tryptic digest of apolipoprotein B were prepared as described[1].

The triethylammonium phosphate (TEAP) buffer (1%, pH 3.2) was prepared by the addition of phosphoric acid (1%, v/v) to purified water and adjusting the pH with triethylamine (unpurified; Aldrich, Milwaukee, WI, U.S.A.). The buffer was purified by passage through a C_{18}-radially compressed cylinder (30 × 5.7 cm) in a Waters Prep-500 preparative liquid chromatograph. Before use the cartridge was

washed successively with 10 l each of isopropanol, isopropanol–water (1:1) and water, to remove any unbonded organosilane polymer. The amine phosphate buffer (20 l) was then pumped through the column at a flow-rate of 100 ml/min and collected in dark glass bottles. The use of plastic tubing was avoided whenever possible, because the strong solvent properties of this buffer made further contamination inevitable. The first 5 l of buffer were discarded. Each bottle was stored under nitrogen at 4°C in the dark. The buffer had satisfactory stability under these conditions, for at least 1 month.

All chromatography was carried out at room temperature (*ca.* 22°C). The samples were dissolved in a solvent which corresponded to the initial component of the mobile phase.

RESULTS

Fig. 1 shows the elution profile obtained for the tripeptide Gly-Gly-Tyr on a new radially compressed (RC-C_{18}) column, with a mobile phase of acetonitrile–1% TEAP (20:80). Each successive injection (B to F respectively) represents 50 μg of the peptide chromatographed under identical conditions. Although a reproducible elution

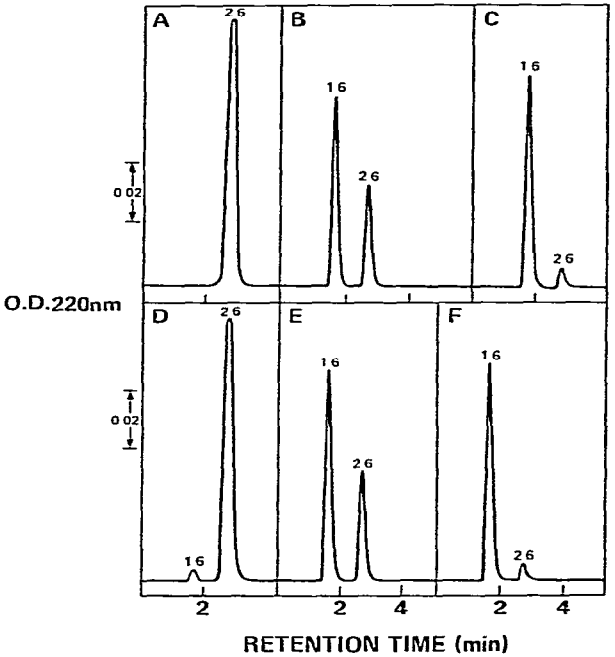

Fig. 1. The elution profile for the peptide Gly-Gly-Tyr on a RC-C_{18} column. The mobile phase consisted of 1% TEAP–acetonitrile (80:20) at a flow-rate of 2 ml/min. The elution profiles shown in parts B to F were obtained with a RC-C_{18} column which had not been subjected to a pre-wash with 200 ml of methanol at a flow-rate of 2 ml/min. The elution profile shown in A was obtained with a column which had a methanol pre-wash before equilibration with the mobile phase. All injections consisted of 50 μg of the peptide dissolved in 50 μl of the mobile phase. All chromatographic parameters were identical for elution profiles A–F.

profile could not be obtained, a pattern of two optical density peaks with constant retention times was always observed. A different result was obtained if the RC-C_{18} column was washed with at least 200 ml of methanol at a flow-rate of 2 ml/min. After equilibration of the column with the same mobile phase as before, chromatography of the tripeptide now only gave one peak with a retention time of 2.6 min. This effect was quite reproducible and could be demonstrated with a fresh column.

Other peptides, when chromatographed on the column without a methanol pre-wash, exhibited either two peaks, an extremely broad peak, or were retained indefinitely on the column even in the presence of high levels of acetonitrile. In most cases, the use of a methanol pre-wash improved the elution profile of the peptide so that a reasonably sharp peak was observed.

For small peptides, which contained the very basic guanidino group of arginine, the methanol pre-wash was not effective, however, and Fig. 2B and D show broad peak shapes for the peptides Leu-Trp-Met-Arg and Met-Arg-Phe respectively with a mobile phase of 1 % TEAP–acetonitrile (80:20). The replacement of acetonitrile with the hydroxylic solvent isopropanol caused a dramatic improvement in the peak shapes as is shown in Fig. 2A and C, respectively. A similar observation was made for other peptides which exhibited poor peak shapes with acetonitrile as the organic modifier.

Fig. 3 shows the successful chromatography of a mixture of C-apolipoproteins from human VLDL using two different gradient programmes. As was described[1],

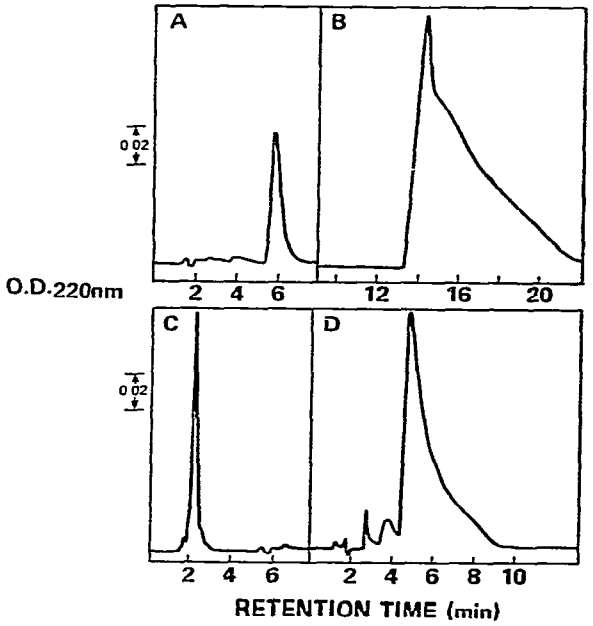

Fig. 2. The chromatography of the peptides Leu-Trp-Met-Arg (A and B) and Met-Arg-Phe (C and D) on a RC-C_{18} column. The column had been subjected to the methanol pre-wash. Each analysis was carried out on 50 μg of the peptide dissolved in 50 μl of the mobile phase. The O.D. sensitivity was increased two-fold in B and D to allow for the broader peak shape. The flow-rate was 2 ml/min. In A and C the mobile phase was 1 % TEAP–isopropanol (80:20) while B and D used a mobile phase of 1 % TEAP–acetonitrile (80:20).

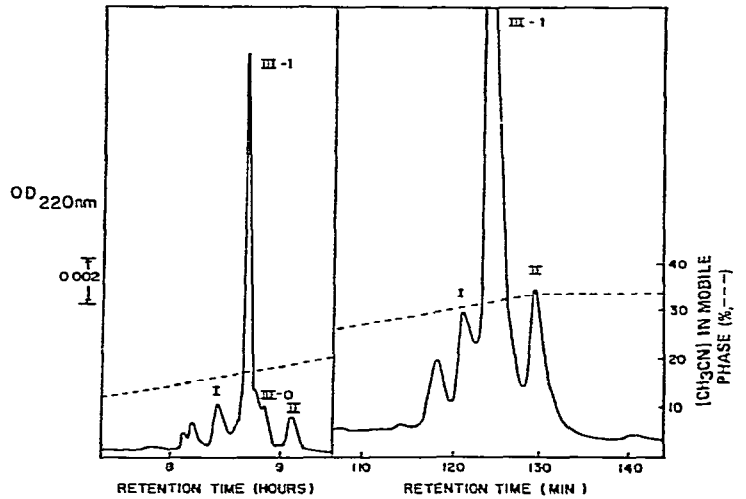

Fig. 3. The elution profiles of a mixture of C-apolipoproteins obtained from human VLDL when chromatographed on a RC-C_{18} column and with different acetonitrile gradients (see dashed lines). A 0.1-mg sample of the protein mixture was dissolved in 0.1 ml of 1% TEAP, 6 *M* guanidine hydrochloride and then chromatographed on the RC-C_{18} column with an initial mobile phase of 1% TEAP, pH 3.2. The TEAP used in the mobile phase was unpurified.

the RC-C_{18} column clearly separated apolipoprotein C-I from C-$III_{1,2}$ and C-II. While a 2-h gradient of 0 to 40% acetonitrile gave adequate resolution of the protein mixture, a much longer gradient of 0 to 40% over 10 h did further improve the resolution (see Fig. 3). A distinct advantage of the longer gradients was that the C-apolipoproteins were eluted at a significantly lower concentration of organic modifier, for example C-$III_{1,2}$ was eluted at 33% acetonitrile with the 10-h gradient, while the 2-h gradient required 38% acetonitrile for elution of the protein. In a research situation where repetitive assays are not required, the long separation time is not necessarily a disadvantage, particularly as the separation can be run overnight, and the separated proteins collected with a fraction collector. A key feature was the use of a high concentration of TEAP (0.17 *M*) in the mobile phase. If a ten-fold lower concentration of the salt was used, a much lower recovery of the C-apoproteins was observed (41% compared to 84% with the higher concentration of the ion-pairing reagent). In addition poor peak shapes were observed with significant tailing.

For preparative studies, it was found necessary to purify the TEAP as it contained a non-polar impurity which co-eluted with apolipoprotein C-II. The salt was purified as a 1% (v/v) solution by preparative HPLC on a C_{18}-cartridge using the Waters Prep-500 liquid chromatograph. The purification procedure resulted in the removal of impurities with a range of polarities. Fig. 4 shows the considerable improvement in the low wavelength UV transparency of the 1% TEAP solution after passage through the preparative C_{18}-column. A blank gradient analysis (see Fig. 5) also showed considerable improvement in the number of optical density peaks eluting during an acetonitrile gradient. In the analysis of both crude and purified TEAP mobile phases a 30-min column equilibration with just the aqueous mobile phase was used to allow any impurities to accumulate on the column, and thus increase the

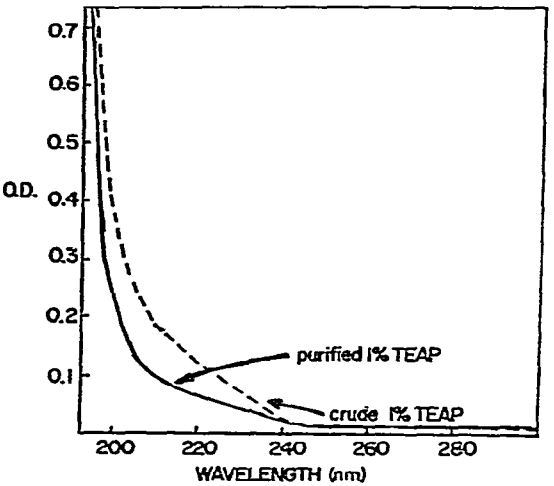

Fig. 4. The UV absorption spectra of a sample of crude 1% TEAP (dashed line) and purified 1% TEAP (solid line). The purification procedure was described in Experimental.

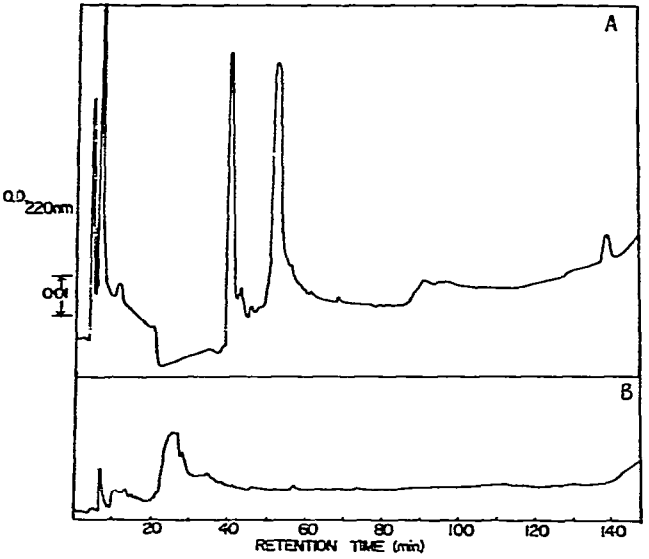

Fig. 5. A blank gradient run for the analysis of the purity of the 1% TEAP aqueous component of the mobile phase. A and B show the elution profile for crude and purified 1% TEAP respectively. In both analyses the column was equilibrated for 30 min at a flow-rate of 1.5 ml/min with the 1% TEAP solution. A 250-μl sample of each mobile phase was injected and then gradient analysis of 0 to 80% acetonitrile was carried out over 2 h.

sensitivity of the analysis. A very different elution profile was observed with the purified buffer as the C-apolipoproteins exhibited a much stronger interaction with the column. With the purified buffer, approximately twice as much acetonitrile was required to elute the protein sample and poor peak shapes with tailing were observed. As was found with small basic peptides, the use of isopropanol instead of acetonitrile

overcame these problems, and allowed the satisfactory analysis of the C-apolipoproteins in the presence of purified TEAP in the mobile phase. Fig. 6 shows the elution profile of the C-apolipoprotein mixture with a mobile phase containing the purified buffer and a isopropanol gradient. Again two different gradient programmes were used to illustrate that a very shallow organic solvent gradient gave a better separation of the protein mixture. The similarity in the elution profile obtained for the protein mixture with the two different mobile phases was confirmed by amino acid analysis of an 24-h, 6 *M* HCl hydrolysate of the pooled peaks (see ref. 1 for representative values).

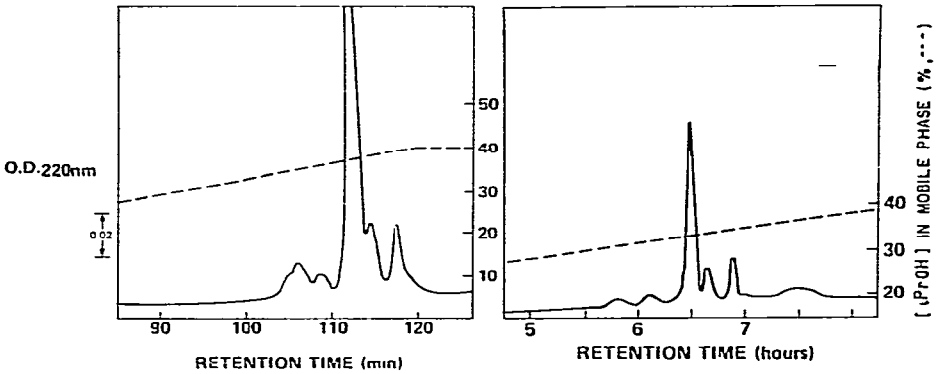

Fig. 6. The elution profiles of a mixture of C-apolipoproteins with different isopropanol gradients (see dashed lines). The TEAP used in the mobile phase was purified as described in Experimental. All other chromatographic parameters were as described in Fig. 3. The identity of the eluted peaks was the same as shown in Fig. 3.

The successful purification by the column of a synthetic pentadecapeptide is shown in Fig. 7. This peptide represents an analogue of the amino terminal region of apolipoprotein C-I (1–15), in which phenylalanine at residue 14 is replaced with *p*-iodophenylalanine. The peptide was purified by successively larger injections of material (0.5, 2 and 8 mg shown in Parts A to C respectively) without any diminution of the separation efficiency. A shallower gradient was used, however, at the higher loadings as a slight decrease in retention times was observed with these concentrations. In this example injections of up to 8 mg of crude synthetic product could be separated without any decrease in resolution (Fig. 7). The material from peak 3 in the different runs was pooled, and analysed by analytical HPLC (Fig. 7D). The isolated material was shown to be homogeneous by the single peak in the elution profile and by amino acid analysis of a hydrolysate. Peaks 1 and 2 consisted of small amount of deletion products (less than 10%) formed by incomplete coupling reactions near the end of the synthesis. The early eluting large O.D. peaks were either due to the guanidine hydrochloride added to the injection mixture or to non-peptidic contaminants.

DISCUSSION

The development of a packing with a significant concentration of free silanol groups does run counter to recent progress in liquid chromatography for the produc-

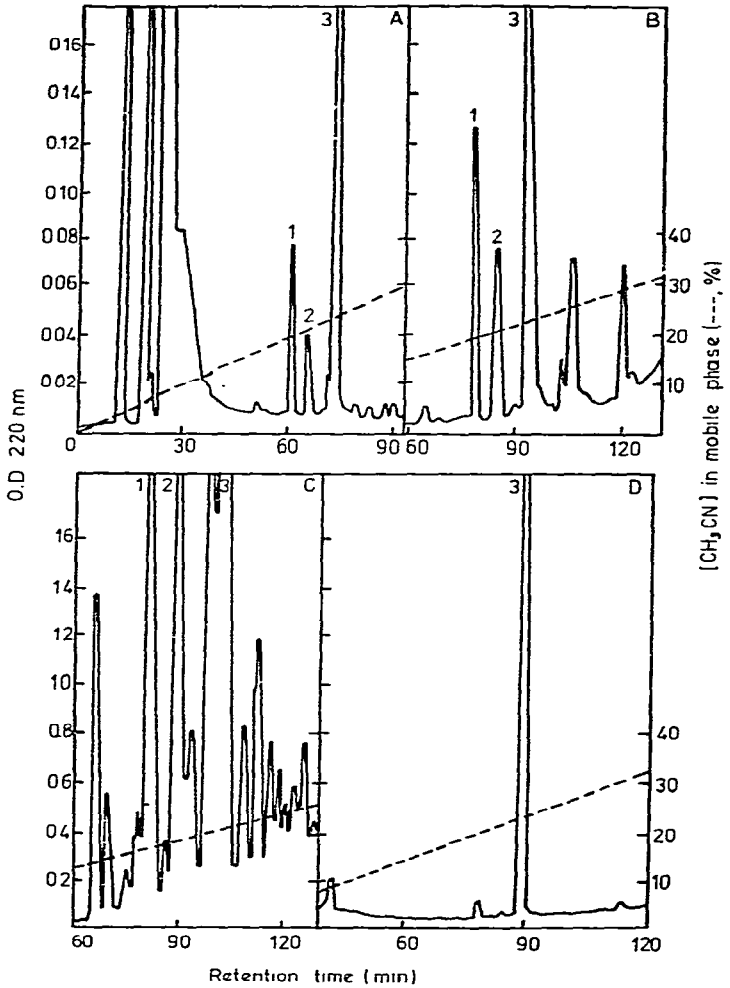

Fig. 7. The purification of a synthetic analog of the 1–15 segment of human apolipoprotein C-I, in which Phe-14 is replaced with *p*-iodophenylalanine in the chemical synthesis. The mobile phase was purified 1% TEAP with a linear gradient of acetonitrile (see the dashed lines). The flow-rate was 1.5 ml/min. The loadings in A to C were 0.5, 2 and 8 mg of crude peptide dissolved in 1% TEAP, 3 *M* guanidine hydrochloride at a concentration of 10 mg/ml. Peak 3 corresponded to the desired peptide, and this fraction from each run was pooled. The analysis of an aliquot of this pool is shown in D.

tion of hydrocarbonaceous supports with very high carbon bondings and minimal silanol content[9,10]. While such supports are undoubtedly successful for the separation of low-molecular-weight solutes such as phenylthiohydantoin (PTH)-amino acids or aromatic compounds, the high carbon bondings can cause difficulties in the chromatography of high-molecular-weight samples such as proteins. For example both C- and A-apolipoproteins are retained indefinitely on C_{18}-reversed-phase packings which have a surface loadings of greater than 20% w/w. This strong interaction between the non-polar stationary phase and the apolipoproteins cannot be overcome by high

levels of organic solvents, as the proteins precipitate and denature irreversibly under these conditions.

For these reasons, the development of a reversed-phase column packing which had a higher polarity due to the presence of free silanol groups, could provide a useful medium for the chromatography of relatively hydrophobic proteins. In addition the silanol groups would be expected to increase the hydration of the pores in the microparticulate silica, thereby facilitating diffusion of the protein molecules. A serious problem with the use of such packings, however, is the propensity of proteins to bind irreversibly to silanol groups[11]. It was decided, therefore, to investigate the use of the RC-C_{18} column with small basic peptides and with protein samples known to be sensitive to silanol groups.

Microparticulate silica presents a heterogeneous surface with silanol groups of varying accessibility and acidity, with a pK_a range of 5–6 (refs. 10, 12). The presence of intramolecular hydrogen bonding is probably the cause of this variation in pK_a values. In adsorption chromatography the more reactive silanol groups are deactivated by treatment of the silica with polar solvents such as water and methanol[10]. Fig. 1 shows the importance of a methanol pre-wash of the RC-C_{18} column before chromatography of peptide samples. In the absence of this pre-wash, the tripeptide Gly-Gly-Tyr exhibited irreproducible elution profiles. The peptide had been previously shown to be homogeneous by a variety of chromatographic studies[13], so that the observation of multiple peaks could be attributed to the packing material and not the sample. After deactivation of silanol groups by the methanol wash, a single elution peak was observed for all subsequent injections. All other studies were performed on a RC-C_{18} column which had been subjected to the methanol wash.

The addition of salts to the mobile phase is also an important procedure for minimising interactions between the silanol groups of the column and ammonium groups present in the solute. The salt can either act as a general electrolyte thereby suppressing ionic interactions, or in certain cases a more specific interaction may occur. It was shown[13,14] that phosphate is capable of forming an ion pair with suitable solutes, as described in the following equation:

$$R\text{–}NH_3^+ + H_2PO_4^- \rightleftharpoons [RNH_3^+\ H_2PO_4^-]$$

Such a reaction would be expected to also decrease interactions between ammonium groups of the sample and silanol groups in the column packing.

Recently amine phosphate buffers have become popular in the HPLC of peptides and proteins[15–18]. Part of the success of these buffers can be attributed to the deactivation of silanol groups by ionic and/or hydrogen bonding interactions with amines added to the mobile phase. Although a mobile phase of 0.1% H_3PO_4 was adequate for the successful chromatography of Gly-Gly-Tyr, other peptides such as Gly-Leu-Tyr required a mobile phase which contained an amine phosphate such as triethylammonium phosphate (TEAP).

The presence of arginine in a peptide presents an additional strongly basic site due to the guanidino side chain. It is not surprising, therefore, that the peptides Met-Arg-Phe, and Leu-Trp-Met-Arg exhibit poor peak shapes on the RC-C_{18} column even in the presence of 1% TEAP in the mobile phase (see Fig. 2B and D). The replacement of acetonitrile with isopropanol as the organic modifier, however,

dramatically improves the elution profile for these basic peptides (see Fig. 2A and C). Methanol does not have this effect when it is used instead of acetonitrile. A possible explanation is that isopropanol, which has a significant non-polar region, is particularly suitable for penetrating the reversed-phase support and further deactivating the silanol groups by hydrogen bonding.

In addition, the following peptides were also examined on the RC-C_{18} column with the different mobile phases described above: Leu-Trp-Met-Arg-Phe, Gly-Phe, Phe-Ser-Lys-Gly-Asp-Gly, Gly-Leu-Tyr, Ala-Lys and Ser-Lys. The elution profiles observed with these peptides demonstrated again that the methanol pre-wash of the column was essential. The only peptides which could be satisfactorily chromatographed in the absence of an amine salt in the mobile phase, were simple peptides which did not contain strongly basic and/or hydrophobic groups, *e.g.*, Gly-Gly-Tyr or Gly-Phe. The other peptides required an amine salt in the mobile phase to suppress the major proportion of silanol group effects, while possibly retaining sufficient polar interactions to allow useful selectivities to still be achieved. These studies suggested that a mobile phase which contained 1% TEAP, pH 3.2, with isopropanol should allow the chromatography of most peptides and proteins on the RC-C_{18} column.

The chromatography of the C-apolipoprotein mixture has been shown to be extremely sensitive to silanol group effects[16]. For example, it was recently shown that the separation of this protein mixture on a μBondapak–alkylphenyl column with a mobile phase of 0.1% H_3PO_4–acetonitrile (20:80) was predominantely a normal rather than reversed-phase separation[16]. In this system the most polar apolipoprotein C-I was eluted last, rather than first as in the reversed-phase separation shown in Fig. 3. Also an increase in concentration of organic modifier resulted in an increased retention time, an observation which is consistent with the proposed normal phase separation mechanism. Not surprisingly this separation was characterised by low recoveries of the C-apoliproteins[16]. If the mobile phase was modified from 0.1% H_3PO_4 to 1% TEAP then a reversed-phase separation was observed similar to the elution profiles shown in Fig. 3.

In a similar manner the separation of the C-apolipoproteins on the RC-C_{18} column could be shown to be extremely sensitive to the nature and concentration of the amine phosphate used in the mobile phase. Triethylamine was shown to be most effective of a number of amines, *e.g.*, ammonia, triethylamine, hexylamine and ethanolamine. In the comparative study each amine was converted to the phosphate salt as described for TEAP in Experimental. Also a high ionic strength was required, as a decrease from 1% to 0.1% in the concentration of TEAP caused a two-fold reduction in recoveries of the apoproteins, as well as resulting in poor peak shapes with excessive tailing.

When purified TEAP was used for the aqueous mobile phase, a distinct difference was observed in the analysis of the C-apolipoprotein mixture. As was described in Results, the use of the purified amine phosphate buffer apparently allowed a stronger interaction to occur between the apolipoproteins and the column. If acetonitrile was used as the organic modifier, the proteins could not be eluted in a satisfactory manner from the column. In fact the results obtained parallel those obtained with the small basic peptides, in that a change in organic modifier from acetonitrile to isopropanol allowed the convenient elution of the C-apolipoproteins. At this stage it is not clear what impurities are present in the crude solution of TEAP

that can account for the differences in chromatographic behaviour of the apolipoproteins between the crude and purified mobile phases. Since the apolipoproteins are known to be extremely sensitive to silanol group effects, it is possible that a basic impurity present in the crude triethylamine was able to complex with the silanol groups still accessible in the RC-C_{18} packing after methanol deactivation.

The important feature of these studies, however, is that operating conditions have been described, which allow the separation of a range of peptide and protein samples by a mixed-mode separation mechanism. Apparently the mixed retention mechanism, which involves both reversed-phase and adsorption interactions, allows for a greater discrimination to be made in a mixture of closely related polar materials. The separation of the extremely complex mixture of tryptic fragments from a partial digestion of apolipoprotein B on a RC-C_{18} column was described previously[1]. An extra-ordinary degree of selectivity was obtained in this separation with over 200 distinct peptides separated in a single gradient run. The maximum number of peptides separated on a μBondapak C_{18} or μBondapak–alkylphenyl column was 50–60 in a gradient run. The major reason for the poorer performance of the fully silanized analytical columns relative to the RC-C_{18} system was not in terms of column efficiencies, but in the lack of selectivity of the former system where a large number of peptides coeluted at *ca.* 20% acetonitrile in the gradient analysis. In the separation shown on the RC-C_{18} column[1] these peptides were spread throughout the gradient, suggesting that greater selectivity is an important factor in the success of the Radial Compression Separation System in the analysis of complex peptides mixture.

Another important feature of this system is that higher sample capacities are available, despite the amount of packing material which is comparable to conventional analytical columns. Often 10–20-mg loadings of complex peptide mixtures can be chromatographed on the RC-C_{18} column without any decrease in separation efficiency, for example Fig. 7 shows the purification of 8 mg of a crude synthetic peptide (the 1–15 amino terminal fragment of apolipoprotein C-I). The corresponding maximum loading on a μBondapak C_{18} column is usually up to 0.2 mg.

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