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USE OF SEP-PAK CARTRIDGES FOR ON-LINE PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Sep-Pak C_{18} cartridges have found wide application to the extraction, concentration, and fractionation of peptides. Their use has hitherto been limited to step-wise loading, washing, and eluting procedures. By the use of commercially available plastic column end-fittings it was possible to connect steel tubing to both ends of the cartridges and allow low-pressure operation in a high-performance liquid chromatography (HPLC) instrument. The cartridge was then able to function as a disposable, reversed-phase (RP) preparative column. Operation in a gradient mode gave several advantages over step-wise elution, including the direct UV absorption monitoring of the eluant. Gradient conditions could be optimised for sample recovery. The cartridge was compared with standard analytical RP-HPLC columns for the separation of both proteins and peptides. A test protein gave a single peak with good recovery. Experiments with peptide mixtures showed that the cartridge could resolve some components sufficiently to allow fractions of pure peptides to be collected.

INTRODUCTION

The use of small, disposable cartridges containing chromatographic adsorbents has found wide application for the enrichment and/or separation of a variety of compounds from crude extracts. The Sep-Pak C_{18} cartridge is an example of such an adsorbent. These cartridges are used to separate retained from non-retained compounds in a fashion analogous to reversed-phase high-performance liquid chromatography (RP-HPLC), although with a cartridge, pre-equilibration, loading, and elution are usually performed in a series of steps by forcing the required solvent through with a syringe[1]. Although step-wise elution procedures are well suited to processing multiple samples, they suffer from a number of inherent disadvantages. The loading and elution procedure has to be specifically developed for the compound of interest, including quantitation or recovery after elution. Although the behaviour of the compounds of interest on analytical RP-HPLC columns can be used as a guide, the

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retention characteristics of the cartridge packing may be significantly different. Step-elution, in contrast to gradient elution, may well lead to incomplete recoveries of the desorbed compounds.

This work describes the simple conversion of a Sep-Pak cartridge for on-line operation in an HPLC instrument. The cartridge can then be used as a mini-preparative HPLC column with the attendant advantages of gradient elution and on-line UV detection.

EXPERIMENTAL

Cartridge conversion

The Sep-Pack C_{18} cartridge (Millipore/Waters, Harrow, U.K.) was converted for on-line operation as shown in Fig. 1. The long end of the cartridge was cut back with a scalpel to match the length of a Kel-F fingertight male nut (Field Instruments, Weybridge, U.K.). Two of these Kel-F fittings were carefully screwed into each end of the cartridge until the soft fritted disks were slightly compressed. Lengths of standard 1/16 in. O.D., 0.1 mm I.D. stainless-steel HPLC tubing were then inserted into the end-fittings. The assembly was fitted into the HPLC instrument in the usual way, although before connection to the UV monitor, the cartridge was flushed, using the HPLC pumps, with approximately ten volumes of eluent to remove any loose packing material.

HPLC equipment

The RP-HPLC analytical column used for protein separation was a 25 cm × 4.6 mm I.D. Bakerbond wide-pore C_4 5-μm column (HPLC Technology, Macclesfield, U.K.). For analytical peptide separations, a 12 cm × 4.6 mm I.D. Hypersil ODS 3-μm (Shandon Southern, Runcorn, U.K.) was used. The HPLC equipment was from Spectra-Physics (St. Albans, U.K.) and consisted of a SP8700 solvent delivery system and a LC871 UV–VIS detector with a SP4270 computing integrator.

Buffers

Two different systems were used. For protein separations, solvent A was 0.1% trifluoroacetic acid (TFA) (BDH Spectrosol, Poole, U.K.) in water, and solvent B was 0.1% TFA in acetonitrile (BDH Hypersolve). For peptide separations, a solvent

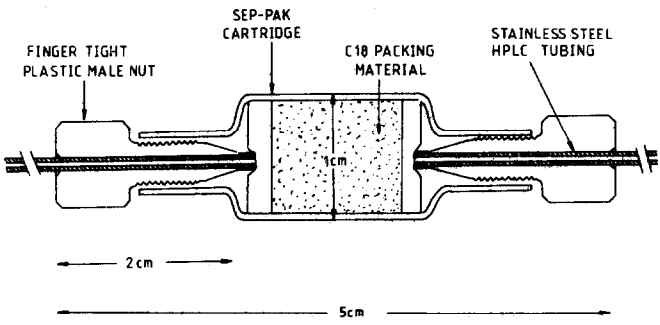

Fig. 1. A diagram showing the main features of the adapted Sep-Pak cartridge.

system based on 25 m*M* ammonium acetate was used according to Kehl *et al.*[2]. This was made up from a 50 m*M* stock solution brought to pH 6.0 with dilute phosphoric acid. For solvent A the stock solution was diluted with an equal volume of water, and for solvent B with an equal volume of acetonitrile.

Sample preparation

The test protein used was the S-carboxymethylated (Scm) Bβ-chain of human fibrinogen. Scm fibrinogen was prepared according to Henschen *et al.*[3] and purified by preparative HPLC on a 25 cm × 8 mm Vydac TPRP10 column (HPLC Technology), using the conditions described by Kehl *et al.*[4]. The collected peak was concentrated to 20 mg/ml by partial freeze-drying. To prepare the fibrinopeptide concentrate a 15-mg/ml fibrinogen solution (Grade L, Kabi Diagnostica, London, U.K.) was made up with distilled water. Highly purified human thrombin, 3000 NIH units/ml (a gift from J. W. Fenton, New York State Department of Health, New Albany, NY, U.S.A.), was added to give a final concentration of 20 units/ml and clotting was allowed to proceed for 10 min at room temperature. After boiling for 3 min, the denatured clot was compressed with a glass rod and the removed supernatant was forced through a 0.22-μm filter (Gelman Sciences, Northampton, U.K.) with a syringe.

HPLC

All separations were carried out at room temperature at a flow-rate of 1.5 ml/min. For the analytical separation of the Scm Bβ-chain, 8 μl of the solution was injected into the Bakerbond C_4 column and a gradient of 0–60% B in 25 min was run at 0.32 absorbance units full scale (a.u.f.s.) at 280 nm. For the Sep-Pak separation 100 μl was injected under the same conditions. For the peptide analysis, 10 μl of the fibrinopeptide solution was injected into the Shandon 3-μm ODS column. The gradient was run at 10–30% B in 10 min at a.u.f.s. of 0.16 at 210 nm. For the Sep-Pak preparative analysis, 800 μl of fibrinopeptide solution was injected and eluted with a gradient of 12–30% B in 30 min at 0.32 a.u.f.s. at 210 nm. For the rechromatography experiment, 1 ml from the central section of each main peak was diluted 5-fold with solvent A, and 200 μl was injected into the analytical column.

RESULTS AND DISCUSSION

Using the simple conversion procedure as described, it was possible to adapt the C_{18} Sep-Pak cartridge for operation in an HPLC instrument, the only requirement being the capability of the pumps to operate at low pressures. Leak-free operation was possible up to approximately 2 ml/min.

The performance of the cartridge was compared with that of standard analytical RP-HPLC columns. For trial protein separations the widely used 0.1% TFA in acetonitrile solvent system was used. A "cleaning gradient" was required to remove residual UV-absorbing substances from the cartridge. For a test polypeptide the Scm Bβ-chain of human fibrinogen (M_r 65 000) was used[2]. The results are shown in Fig. 2. With the C_4 wide-pore column (*i.e.* a nominal pore size distributed around 30 nm) this material gave a symmetrical main peak with a loading of 160 μg (Fig. 2B). A small amount of contaminating Aα-chain was eluted as a minor peak just before the

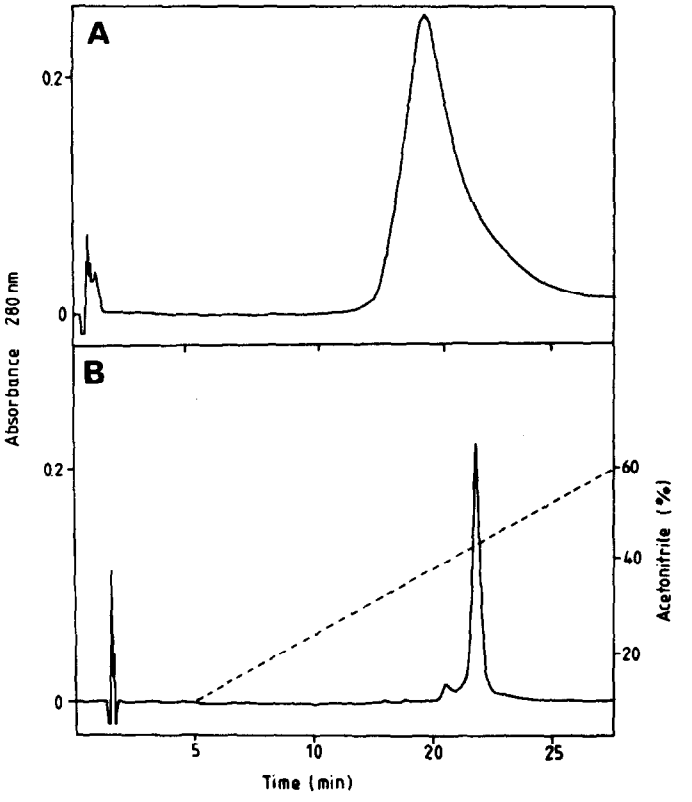

Fig. 2. Comparison of the Sep-Pak cartridge (A) with a Bakerbond C_4 wide-pore column (B) for the elution of fibrinogen Scm Bβ-chain. Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. Using these solvents a gradient of 0–60% acetonitrile in 25 min was run at 0.32 a.u.f.s.

Bβ-chain. With the Sep-Pak cartridge, at a sample load of 2 mg, the Bβ-chain was eluted at a slightly lower acetonitrile concentration as a single, broad peak, although some tailing was evident (Fig. 2A). Subsequent blank gradient runs did not elute significant amounts of material, thus indicating comparable recoveries for the cartridge and analytical column.

A different solvent system was used to evaluate peptide separations on the cartridge. Fibrinopeptides were used as a test mixture, because with the solvent system as described this group of four main peptides shows a consistent separation pattern for a range of different C_{18} column packings[5]. The results of comparing the cartridge with a 3-μm analytical column are shown in Fig. 3. The analytical column (Fig. 3B) is capable of clearly resolving the two major peptides, fibrinopeptide A (FPA), with sixteen amino acids, and fibrinopeptide B (FPB), with fourteen amino acids. The early eluted minor peak is FPA, phosphorylated at Ser 3 (FPAP). Another minor peak, FPA lacking the N-terminal Ala residue (FPAY), is incompletely separated from FPA. The separation of the same peptide mixture on the Sep-Pak column, Fig. 3A, is poor in spite of the use of a more extended gradient, although a partial separation between the two main peaks has been obtained. To demonstrate the utility of the cartridge as a preparative column, fractions were collected from the

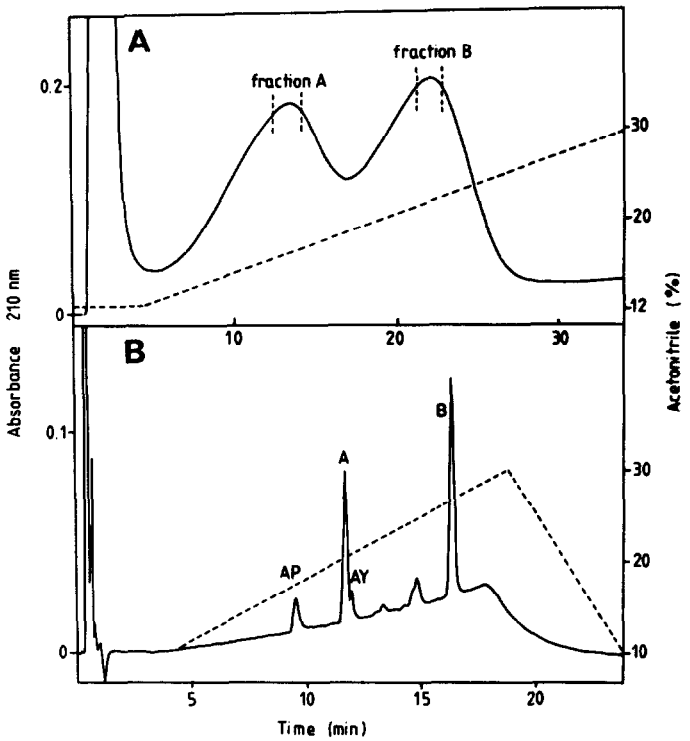

Fig. 3. Comparison of the Sep-Pak cartridge (A) with a Shandon ODS 3-μm column (B) for the elution of fibrinopeptides. Solvent A was 25 m*M* ammonium acetate pH 6.0, and solvent B the same solution but containing 50% acetonitrile. For the chromatogram (A) the gradient was 6–15% acetonitrile in 30 min at 0.32 a.u.f.s. and for (B) 5–15% acetonitrile in 10 min at 0.16 a.u.f.s. The peptides were identified by their elution positions and labeled according to Kehl *et al.*[2].

maxima of two main peaks in Fig. 3A for testing by rechromatography on the analytical column. The results of rechromatography are shown in Fig. 4B and 4C, along the starting mixture for comparison in Fig. 4A. Both fractions from Fig. 3 contained essentially pure peptides, although FPA and AY are not resolvable under these conditions.

Sep-Pak cartridges contain silica particles of 60 μm size distribution. The C_{18} loading is equivalent to that of the μBondapak (Waters) 5-μm column packing and is fully end-capped[6]. As might be predicted, the chromatographic performance is inferior to the analytical columns tested, presumably due to the large particle size, loose packing, and sample-broadening effects at both ends of the cartridge. Nevertheless, the performance was adequate for the assembly to be used as a preparative column for certain applications. The main advantage is the relatively low cost, which makes its short life-span and/or disposability acceptable. Pre-packed conventional preparative columns cost at least 100 times more. Desalting of proteins is a possible application, especially where reagents, such as detergents or strong alkali, are present, which could damage RP-HPLC column packings. Preliminary fractionation of peptides could be performed, starting directly from very crude extracts which might be

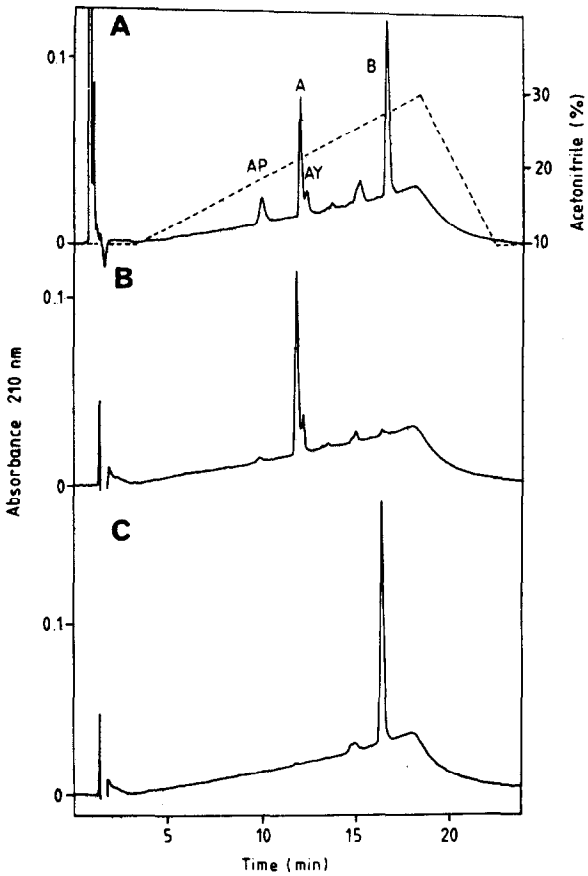

Fig. 4. Rechromatography experiment. This was carried out using a Shandon ODS 3-μm column using conditions identical to those already described for chromatogram B in Fig. 3. The collected fractions, from the Sep-Pak separation in Fig. 3A, labelled A and B, after dilution, were each rechromatographed on the Shandon ODS 3-μm column. The rechromatography of fraction A is shown in chromatogram B and the rechromatography of fraction B, in chromatogram C. The uppermost chromatogram, A, is identical to Fig. 3B, and shows a separation of the complete fibrinopeptide mixture that was applied to the Sep-Pak cartridge.

unsuitable even for pre-column concentration. The option of on-line HPLC could also be used after substances of interest had been adsorbed from very large volumes by syringe loading a cartridge in the usual way.

REFERENCES

1 J. R. Walsh and H. D. Nial, *Endocrinology (Amsterdam)*, 107 (1980) 1258.
2 M. Kehl, F. Lottspeich and A. Henschen, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 1661.
3 A. Henschen and P. Edman, *Biochim. Biophys, Acta*, 263 (1972) 351.
4 M. Kehl, F. Lottspeich and A. Henschen, *Hoppe-Seyler's Z. Physiol. Chem.*, 363 (1982) 1501.
5 C. Southan, unpublished results.
6 D. Collis, Waters U.K., 1986, personal communication.