Journal of Chromatography, 206 (1981) 59–70
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,300

THE USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH RADIAL COMPRESSION FOR THE ANALYSIS OF PEPTIDE AND PROTEIN MIXTURES

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(Received August 26th, 1980)

SUMMARY

This report describes the separation of peptide and protein mixtures on a C_{18}-microparticulate support which was packed in a polyethylene cartridge and subjected to radial compression of *ca.* 2600 p.s.i. The purpose of the radial compression was to minimise inhomogeneities in the column packing, in particular in the region of the column wall and end fittings. The effectiveness of this new chromatographic system was demonstrated by the efficient separation of the following mixtures: the C-apolipoproteins isolated from human very low density lipoproteins; the polymorphs of apolipoprotein A-I; the tryptic fragments of apolipoprotein C-II; the complex mixture generated by partial proteolysis of apolipoprotein B; the tryptic fragments of ^{3}H- and ^{14}C-labelled β-chain of murine IA alloantigen and the tryptic fragments of carboxymethylated lambda chain isolated from human immunoglobulin G. The separated peaks were identified by amino acid analysis, radioactivity counting and in the last example by amino acid sequence determination. The mobile phase consisted of 1% aqueous solution of triethylammonium phosphate, pH 3.2 with acetonitrile or isopropanol as the organic modifier.

INTRODUCTION

Recently reversed-phase high-performance liquid chromatography (HPLC) has allowed the separation of a variety of peptide and protein samples[1–11]. An important factor in the resolution of such a diverse range of samples, by a single chromatographic system, was the introduction of a number of ionic materials which can be added to

0021-9673/81/0000–0000/$02.50

the mobile phase[1,4,11–14]. Such materials probably influence the chromatographic separation by a mixture of ion pairing effects[12–15], solvophobic interactions[16] and column modification[11,17]. Despite these important applications, the separations achieved by reversed-phase HPLC still require additional improvements, particularly for complex mixtures present in partial protein hydrolysates and in mixtures of closely related proteins.

Recent reports have described the purification of peptides on a support which consisted of 70-μm C_{18}-silica particles, packed in large polyethylene cartridges which can be radially compressed[18,19]. These reports have demonstrated that radial compression does greatly improve the efficiency of the column, so that rapid preparative separations can be achieved. The purpose of this publication is to demonstrate that the corresponding analytical system, namely 10-μm C_{18}-silica particles packed in a 10 × 0.8 cm polyethylene cartridge and subjected to a radial compression, can be used to generate high efficiency separations of peptide and protein mixtures.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used for the analytical separations, which 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 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 purchased from Waters Assoc.

Chemicals

Orthophosphoric acid (analytical-reagent grade) was obtained from May & Baker (Dagenham, Great Britain). Water and glass distilled, acetonitrile (UV grade) was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Triethylamine was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. The A-I and C-apolipoprotein mixtures were obtained using established procedures[20,21]. Typical conditions for the preparation of a tryptic digest of a protein sample are described in the publication on the determination of the amino acid sequence of apolipoprotein C-II[22].

Methods

The triethylammonium phosphate (TEAP) was prepared from a 1% aqueous solution of phosphoric acid, to which triethylamine was added until a pH of 3.2 was reached. All pH values quoted refer to the aqueous phase before addition of the organic component in the gradient analysis.

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. Other chromatographic conditions were the same as has been described previously[9].

RESULTS

The separation of a mixture of C-apolipoproteins isolated from human very low density lipoproteins (VLDL) is shown in Fig. 1. This mixture represents a partially purified serum fraction which was isolated by ultracentrifugal flotation, delipidation and Sephacryl S-200 fractionation[9]. The order of elution of the apolipoproteins on the radially compressed C_{18} (RC-C_{18}) column is apolipoprotein C-I, C-$III_{1,2}$ and C-II respectively. Apolipoprotein C-III is a glycoprotein and exists in three major isomeric forms which contain two, one or no sialic acid residues (C-III_2, C-III_1 and C-III_0 respectively). The RC-C_{18} column does not separate the major isomers C-III_2 and C-III_1, while the small amount of C-III_0 present in this sample (as shown by polyacrylamide gel electrophoresis) probably elutes at the trailing edge of the C-III peak. As is shown in Table I amino acid analysis data allowed identification of these peaks. The additional minor peaks in the chromatogram probably represent other apolipoproteins which are coisolated with the C-apolipoproteins. The amounts present in these minor peaks were insufficient for identification by amino acid analysis.

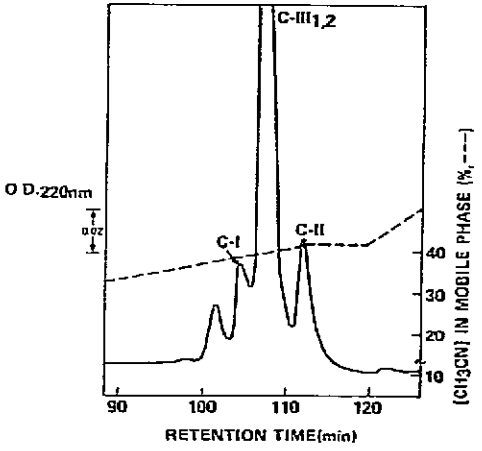

Fig. 1. The elution profile of a mixture of C-apolipoproteins (0.2 mg) on a radially compressed C_{18} cartridge (RC-C_{18}). A flow-rate of 1.5 ml/min was used with a mobile phase consisting of 1% TEAP. After elution from the column, the fractions containing the apolipoproteins were pooled separately, freeze dried, hydrolysed with acid and identified by amino acid compositional data. The identity of the protein is indicated by lettering.

The analysis of a highly purified sample of apolipoprotein A-I on the RC-C_{18} column is shown in Fig. 2. The three protein containing peaks (3, 4 and 5 respectively) were examined by amino acid analysis. Peaks 4 and 5 gave compositional data which agreed closely with the expected values for apolipoprotein A-I, while the amino acid content of peak 3, although present in amounts too small for quantitation, was also suggestive of this apolipoprotein.

Fig. 3 shows the elution profile for the tryptic digest of apolipoprotein C-II on a RC-C_{18} column. Each peak was collected and identified by amino acid analysis (see Table III). Some large optical density peaks contained no significant amounts of amino acids, such as peak 11, while a few small peaks accounted for some of the

TABLE I

AMINO ACID COMPOSITIONAL DATA OBTAINED IN THE SEPARATION OF A C-APOLIPOPROTEIN MIXTURE ON A RC-C_{18} COLUMN

Other peaks did not give significant amino acid analyses. Trp and Cys were not measured. Values expressed as moles per 100,000 g protein; expected values from A. M. Scanu, *Biochemistry of Atherosclerosis*, Academic Press, New York, 1979, p. 3.

Amino acid	*C-I*		*C-II*		*C-III*$_{1,2}$	
	Obtained	*Expected*	*Obtained*	*Expected*	*Obtained*	*Expected*
Asp	79	81	72	64	91	80
Ser*	52	45	82	101	57	57
Thr*	96	106	81	99	120	125
Pro	—	15	29	26	24	23
Glu	140	136	125	104	118	114
Gly	26	15	36	28	28	34
Ala	42	45	75	75	120	114
Val	21	30	49	45	74	68
Met	9	15	20	21	11	23
Ile	47	45	13	10	0	0
Leu	102	91	91	92	98	57
Tyr	8	0	55	53	19	23
Phe	47	45	25	24	48	46
His	0	0	0	0	12	11
Lys	131	136	83	70	75	68
Arg	—	45	10	13	24	23

* Uncorrected for losses during acid hydrolysis.

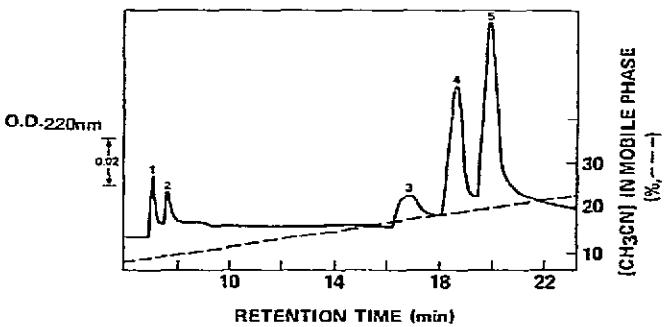

Fig. 2. The elution profile obtained for 100 μg purified apolipoprotein A-I when analysed on the RC-C_{18} column. A flow-rate of 2 ml/min was used with a mobile phase of 1% TEAP. Peaks: 1 and 2 = artifacts caused by the solvent and salts present in the sample; 3, 4 and 5 = collected, freeze-dried, hydrolysed and subjected to amino acid analysis.

tryptic fragments (peaks 6 and 7). These fragments were small peptides which did not contain any significant UV chromophores.

In Fig. 4 the gradient map for a partial digest of apolipoprotein B is described. The apolipoprotein was solubilised using the copper(II) procedure of Huang and Lee[23]. Due to the presence of multiple basic residues in this protein, a partial digestion yielded an extremely complex mixture of peptides. The presence of overlap fragments

TABLE II

AMINO ACID COMPOSITIONAL DATA OBTAINED IN THE ANALYSIS OF APOLIPOPROTEIN A-I ON A RC-C_{18} COLUMN

Amino acid	*Peak number**			
	3	*4*	*5*	*apo A-I***
Asp	+ +***	25	26	21
Ser	+	15	11	10
Thr	+ +	15	17	15
Pro	+	7.0	7.9	10
Glu	+ + +	44	46	46
Gly	+	13	24	10
Ala	+ +	16	13	19
Val	+	15	11	13
Met	—	3	2.5	3
Ile	—	2.5	—	0
Leu	+ + +	28	32	37
Tyr	+	8.0	7.1	7
Phe	+	6.5	6.2	6
His	+	3.8	4.1	5
Lys	+ +	24	19	21
Arg	+ +	14	14	16

* Other data same as for Table I.

** Theoretical values obtained from ref. 21.

*** Not sufficient material to allow quantitation of the amounts of each amino acid, but the relative amounts are indicated by the notation +, + + and + + +.

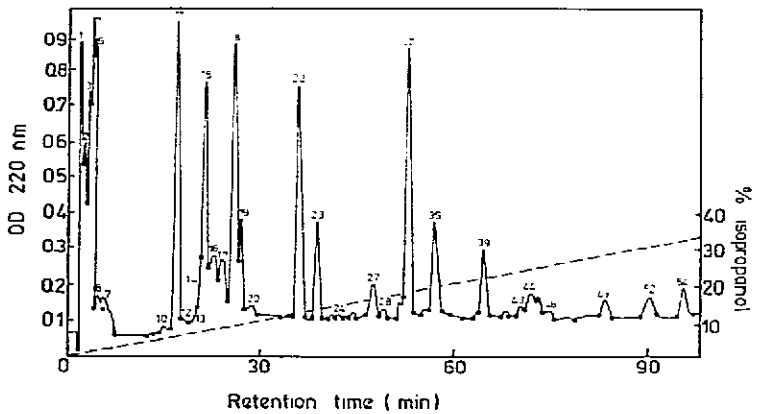

Fig. 3. The elution profile obtained by chromatography of the peptide mixture obtained from the digestion of apolipoprotein C-II with trypsin. The amount of peptides loaded on to the column was 0.7 mg. The mobile phase consisted of 1% TEAP and a flow-rate of 1.5 ml/min was used; solvent B was isopropanol. A 0.5-ml sample was taken from the reaction mixture of the tryptic digestion which contained 0.2 *M* ammonium bicarbonate and 0.5 g of guanidine hydrochloride. To this aliquot, 0.5 ml of 1% TEAP, which contained 6 *M* guanidine hydrochloride, was added and the total sample was injected. A 2-h linear gradient from 0 to 40% isopropanol was run. No significant peaks were observed after 100 min of the gradient run and thus this section of the elution profile is not shown. Each peak was collected and subjected to amino acid analysis after acid hydrolysis.

TABLE III

PEAK ASSIGNMENT FOR THE TRYPTIC PEPTIDES OF APOLIPOPROTEIN C-II SEPARATED ON A RC-C_{18} COLUMN

Mobile phase: 1% TEAP; flow-rate, 1.5 ml/min with a gradient of 0–40% isopropanol. Assignments based on amino acid analytical data and the amino acid sequence data of Jackson *et al.*[22].

Peak number	*Assignment*
6	T-8
7	T-5
15	T-6
18	T-3
19	T-5,6
22	T-4
27	T-1 (deamidated?)
32	T-2
35	T-1
43–52	Fragments of partial tryptic digests
54	T-7,8

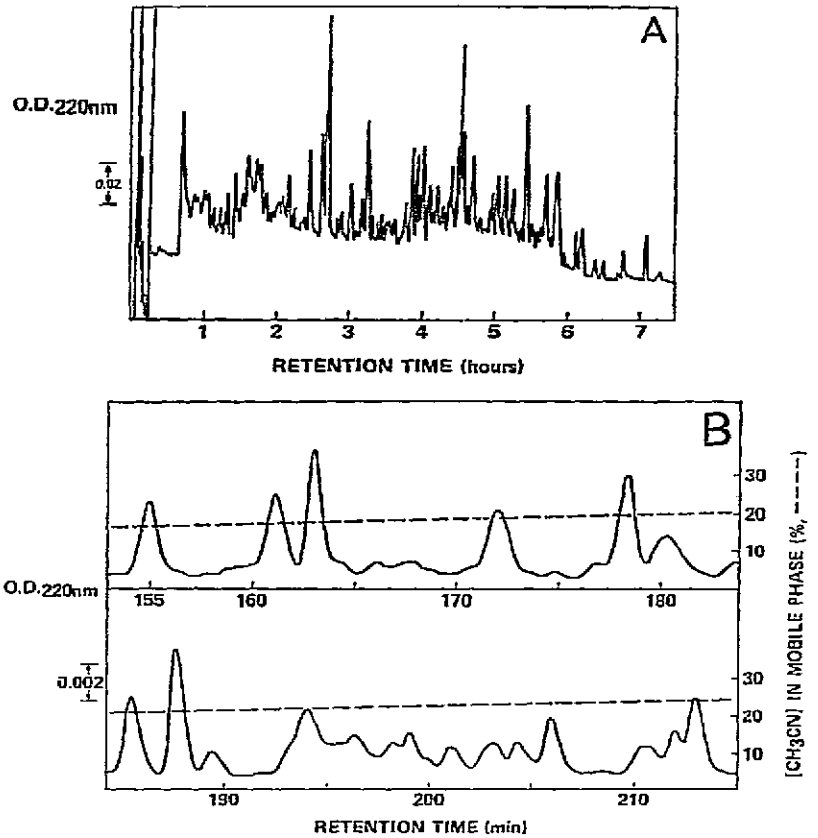

Fig. 4. The elution profile of the tryptic digest of apolipoprotein B on the RC-C_{18} column: A, the full gradient; B, an expansion of a 1-h segment of the full analysis. A 10-mg sample of the tryptic digest was dissolved in 0.5 ml of 1% TEAP, 6 *M* guanidine hydrochloride, pH 3.2 and applied to the column. The mobile phase was 1% TEAP, pH 3.2 at a flow-rate of 1 ml/min. A linear gradient of 0–35% acetonitrile over 10 h was run, 1-ml fractions were collected and the major peaks were pooled, hydrolysed and examined by amino acid analysis. The apolipoprotein B was solubilised using the 6 *M* guanidine–20 m*M* NH_4Cl–NH_4OH procedure of Huang and Lee[23].

in this mixture was confirmed by amino acid analysis studies which demonstrated that many of the peptides still contained more than one arginine or lysine residue. Conditions which give complete digestion of the protein, yielded a much less complex mixture. Over 200 peptides were separated in a single gradient run and Fig. 4B shows an expansion of a 1-h segment of the gradient to illustrate more clearly the complex nature of the elution profile.

The elution profile from a RC-C_{18} analysis of the murine IA alloantigen beta chain tryptic digest is shown in Fig. 5. This membrane glycoprotein was labelled *in vitro* with [^{14}C]- and [^{3}H]-Leu, -Phe, -Arg and -Lys. The eluted peaks were monitored by a flow through radioactive cell. Fig. 6 shows the elution profile for a tryptic digest

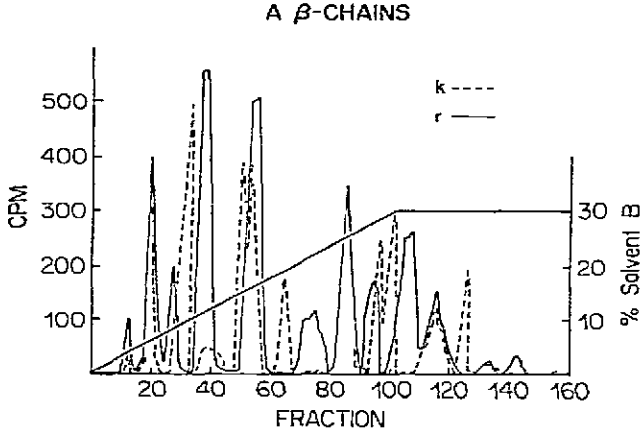

Fig. 5. The elution profile for the separation of the tryptic fragments of the murine IA alloantigen beta chains. The tryptic digestion was carried out for 4 h. The mobile phase was 1% ammonium bicarbonate and the acetonitrile gradient used is shown by a solid line. The flow-rate was 1 ml/min.

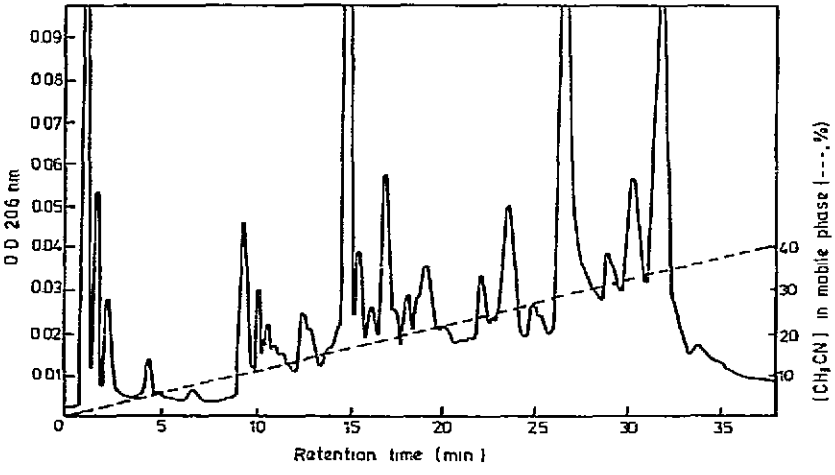

Fig. 6. The separation of the tryptic fragments from the digestion of a carboxymethylated derivative of a human lambda chain. A 10-mg sample was dissolved in 0.17 ml of 1% ammonium bicarbonate and injected onto the column. The mobile phase was 1% ammonium bicarbonate and the flow-rate was 2 ml/min. A linear gradient of 0–40% acetonitrile over 40 min was used.

of a carboxymethylated sample of a human immunoglobulin lambda chain. The major peptides isolated from this separation were subjected to an automated Edman sequence analysis[24]. The results of the sequence analysis are shown in Table IV.

TABLE IV

SEQUENCE DATA FOR THE MAJOR ELUTED PEAKS FROM THE SEPARATION OF THE TRYPTIC FRAGMENTS OF A CARBOXYMETHYLATED LAMBDA CHAIN OF HUMAN IMMUNOGLOBULIN G

The one letter code for amino acids was used[25]: C = Cys; H = His; I = Ile; M = Met; S = Ser; V = Val; A = Ala; G = Gly; L = Leu; P = Pro; T = Thr; F = Phe; R = Arg; Y = Tyr; W = Trp; D = Asp; N = Asn; E = Glu; Q = Gln; K = Lys.

Peak number	*Sequence*
1	AEK
2	QSNNK
3	SHK
4	AAPSVTLFPPSSEELQANK
5	SYAGR
6	TVAPTECS
7	LIIYGVWK
8	FIILLK
9	ATLVCLISDFYPGAVTVAWK

DISCUSSION

Even in well packed columns, a "wall region" is present where the dispersion of solutes is much greater in the packed region close to the walls than in the core of the column. In certain cases the "wall region" can be very significant, for example it is stated that, for a column of 5 mm bore containing 20-μm particles, the fraction of the column cross-section taken up by the annular wall layer is about 40%[27]. In an effort to minimise this region, Waters Assoc. have introduced a Radial Compression Separation System, in which flexibly walled cartridges are compressed radially into high efficiency columns[26]. The use of a 30 × 5.7 cm polyethylene cartridge with radial compression has allowed the preparative separation of multigram amounts of crude materials[28].

In recent studies[18,19] this chromatographic system was used successfully to purify a variety of synthetic peptides. It was observed[18] that despite the relatively large size particles (70 μm) packed in the polyethylene cartridge, high efficiency separations were observed. Such results suggested that radial compression did indeed improve the separation efficiencies obtainable for a given silica-based support.

Recently, Waters Associates introduced the corresponding analytical system. The flexibly walled columns consisted of a 10 × 0.8 cm polyethylene cartridge which was packed with 10-μm octadecyl-silica particles (RC-C_{18}). Fig. 7 shows scanning electron micrographs of the interior of one of these columns before and after radial compression. These photographs indicate that such a column should have a smaller dispersion region near the column wall than for columns with inflexible walls. Because of the successful use of this technology for preparative isolations, it was decided to

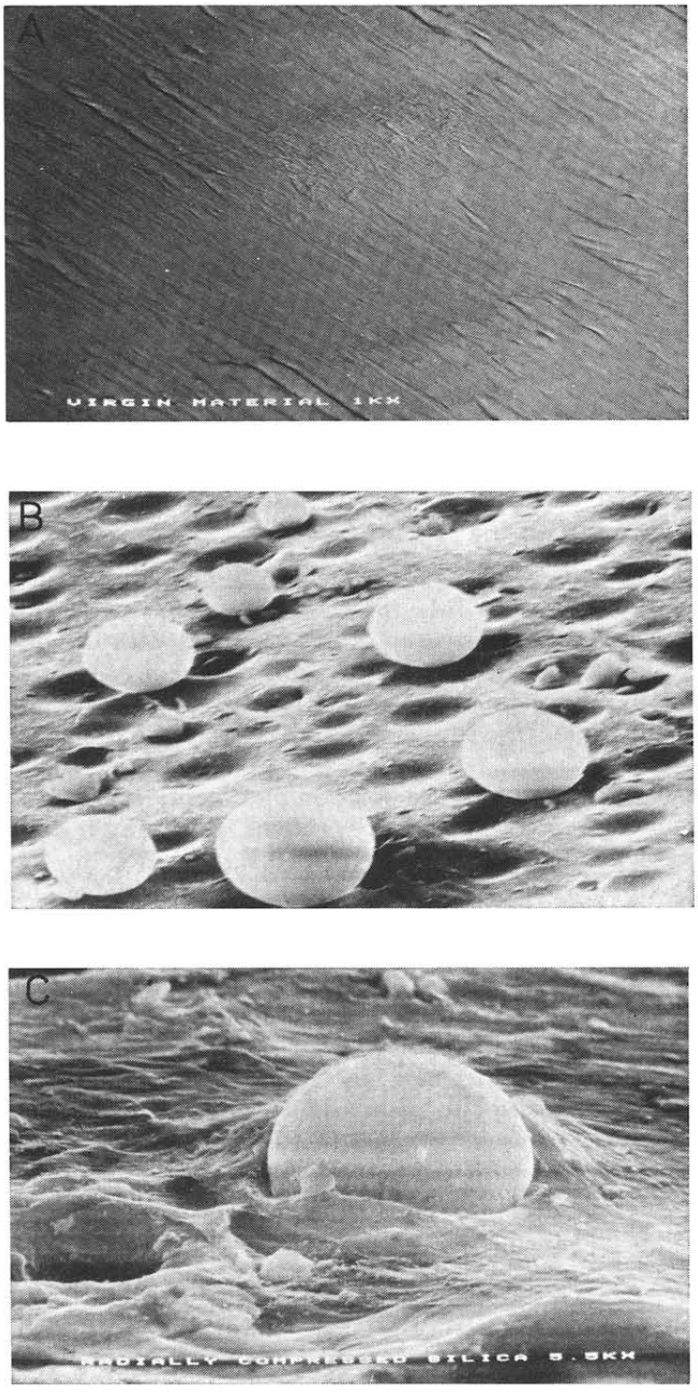

Fig. 7. Scanning electron micrographs of the interior of a polyethylene cartridge before compression (A) and after compression (B and C). In the latter two examples some of the radially compressed silica particles can be seen adhering to the column walls after the column had been emptied.

examine the potential of the analytical system for the separation of complex peptide and protein mixtures.

Fig. 1 shows that the major C-apolipoproteins isolated from human very low density lipoproteins (VLDL) can be separated on a RC-C_{18} column. The elution order and separation is similar to that obtained on a μBondapak-alkylphenyl column[9]. Both columns give much better separation of the C-apolipoproteins than the μBondapak C_{18} column. The lower surface loadings of the organosilane present in the former column packings (5 and 10% *versus* 15% respectively) is suggested as a possible reason for the differences in selectivity between the columns[29]. Although the RC-C_{18} can be used at high flow-rates (up to 10 ml/min) the separation of proteins was more efficient at lower flow-rates (0.5–1.5 ml/min), because of the lower diffusion rates of the high-molecular-weight solutes.

The molecular dimensions of the C-apolipoproteins (which range from 57 to 79 residues) is probably comparable to the pore size of the RC-C_{18} packing material (*ca.* 90 Å). Apolipoprotein A-I, which contains 245 residues, is unlikely to be able to penetrate most pores in this packing material and thus interaction of this protein with the support probably occurs mainly on the exterior surface of the silica particles. Despite this limitation, the separation of at least 1-mg amounts of apolipoprotein A-I into three components was readily achieved on the RC-C_{18} column. Amino acid analysis indicated that the composition of each of the three peaks was consistent with the values expected for the apolipoprotein. This result is in agreement with the observation of Herbert *et al.*[32] that highly purified samples of apolipoprotein A-I could be resolved into three species on polyacrylamide electrophoretic analysis. It is not known if these multiple forms of the protein are caused by aggregation, deamidination or some other process.

Figs. 3–6 show a number of elution profiles for complex peptide mixtures produced by tryptic digestions of high-molecular-weight proteins. A common difficulty with apolipoprotein proteolytic digestions is the partial aggregation of the protein during the cleavage reaction, which results in incomplete digestion of the protein. In Figs. 3 and 4 a number of additional fragments could be detected in the complex elution profile for the digestion products from apolipoprotein C-II and B respectively. In addition the yield of purified peptides based on the amount of protein digested was lower for the apolipoproteins (20–60%) than for other proteins (often quantitative). In each example the RC-C_{18} column gave high efficiency separations which were characterised by excellent selectivities and high sample capacities (up to 20 mg). These separations were clearly superior to comparable analyses carried out on the corresponding 10-μm analytical columns, packed in stainless steel columns without radial compression. The improved performance of the RC-C_{18} column can probably be attributed both to radial compression of the polyethylene cartridge and to a mixed mode separation mechanism. Evidence is presented in a companion publication that the packing material present in the RC-C_{18} cartridge can give rise to both a silanol and a reversed phase interaction with the solute[33]. Such a mechanism can be attributed to significant levels of free silanol groups present in the packing material. For this reason, the mobile phase used in this study contained an amine salt at relatively high ionic strength, which minimises the interaction of basic peptides with silanol groups.

Other chromatographic parameters were chosen to be suitable for the analysis

of peptides and proteins. For example, organic modifiers were chosen which exhibited good solvent properties for the solutes, and slow, shallow solvent gradients were used to avoid the precipitation and/or denaturation of the samples[31].

In conclusion, this publication has demonstrated that the RC-C_{18} system is capable of high efficiency separations of complex peptide and protein mixtures. The application of this new separation system to the analysis and purification of a number of protein samples is currently under investigation.

ACKNOWLEDGEMENTS

The authors wish to acknowledge support from the National Heart Foundation and Medical Research Council of New Zealand. This material was developed by the Atherosclerosis, Lipids and Lipoprotein Section of the National Heart and Blood Vessel Research and Demonstration Center, Baylor College of Medicine, a grant supported project of the National Heart, Lung, and Blood Institute, National Institutes of Health Grant No. 17269. J.T.S. is an Established Investigator of the American Heart Association. The authors would also like to thank Mr. C. Pidacks of Waters Assoc. for information on the Radial Compression Separation System and for supplying the photographs shown in Fig. 7.

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