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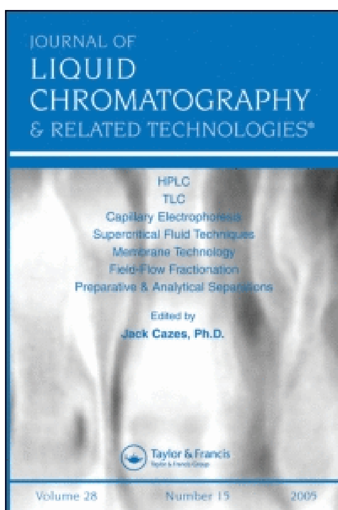

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The Semi-Preparative Separation of Peptides on Reversed Phase Silica Packed into Radially Compressed Flexible-Walled Columns

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THE SEMI-PREPARATIVE SEPARATION OF PEPTIDES ON REVERSED PHASE SILICA PACKED INTO RADIALLY COMPRESSED FLEXIBLE-WALLED COLUMNS.†

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ABSTRACT

The semi-preparative separation of underivatised peptide mixtures from the tryptic digestion of thyroid and pituitary proteins has been accomplished on a 10dp spherical octadecyl-silica stationary phase, Radial Pak A/C_{18}, packed into flexible-walled polyethylene cartridges (10 x 0.8cm). With volatile ionic modifiers, such as ammonium bicarbonate, excellent resolution and peptide recoveries were obtained with this support

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under gradient elution conditions. Compared to the 'capped' alkylsilicas of high carbon loading per gram packing, this octadecylsilica support exhibits significant differences in selectivity consistent with the greater involvement of silanophilic interactions between the ionised peptides and the stationary phase. With the available flexible-walled cartridges sample loadings equivalent to 10-500nmole peptide(s) per injection can be routinely used. Good recoveries of hydrophobic polypeptides and small proteins were also achieved using shallow gradients of the organic solvent modifier.

INTRODUCTION

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Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity over the past several years for the structural mapping of polypeptides and proteins and, in particular, for the analysis and micro-preparative separation of enzymatic digests [1-5]. Compared to conventional open column ion exchange and gel permeation chromatographic techniques, RP-HPLC methods exhibits superior peak resolution for complex mixtures of hydrophilic and hydrophobic peptides. These features are also associated with short analysis times and, usually, good recoveries. Most micropreparative procedures have been based on conventional stainless steel analytical columns, e.g. 25 x 0.4cm I.D., packed with 5- or 10-μm dp octyl- or octadecyl-silicas. Depending on the complexity of the separation, the sample capacity of these analytical columns is frequently less than 1mg. In previous studies we have described [6,7] the use of radially compressed polyethylene cartridges (30 x 5.7cm I.D.) containing a 75μm dp octadecyl-silica support in the purification of 1-10gm amounts of unprotected peptides. In the present report a similar approach has been employed for the semi-preparative separation of the tryptic peptides of several proteins, including thyroglobulins and human growth hormone, using a related 10μm dp octadecyl-silica sorbent, packed into flexible-walled polyethylene cartridges of dimensions (10 x 0.8cm, I.D.) and volatile mobile

phases. Application of these procedures to the separation of native and radio-iodinated peptide and polypeptide hormones is also described.

MATERIALS AND METHODS

Chemicals and Reagents.

Phosphoric acid, formic acid, ammonium bicarbonate and ammonium sulphate were either ARISTAR or AnalaR grade reagents from B.D.H. (Poole, U.K.). Triethylamine, also from B.D.H., water and acetonitrile were purified using established procedures [8-10].

Human thyroglobulin 19S iodoprotein was extracted from human thyroids by the method of Salvatore et al. [11] and fractionated on Sepharose CL-4B using a 150mM NaCl-10mM Tris-HCl, pH 8.0, buffer followed by affinity chromatography using established procedures [12,13]. Guinea pig thyroglobulin 19S iodoprotein was isolated from an albino inbred strain using the procedure of Haeberli et al. [14]. Human growth hormone was prepared by the method of Chapman et al. [15] based on the procedure of Lumley Jones et al. [16]. The ovine thyrotrophin was isolated from an ovine pituitary extract by salt fractionation, lectin affinity chromatography, ion exchange chromatography and gel permeation HPLC as described elsewhere [17]. Lysozyme (grade 1) was a commercial sample from Sigma Chem. Co. (St Louis, Mo. U.S.A.). The source of some of the peptides and polypeptides has been given previously [18], the remainder were commercial samples, purified in this laboratory, from Sigma (St Louis, Mo., U.S.A.), Bachem (Torrance, Ca., U.S.A.), Calbiochem (La Jolla, Ca., U.S.A.) or Vega Biochem (Tuscon, Ariz., U.S.A.).

Tryptic digestion of the proteins was carried out using TPCK-treated trypsin following the procedure of Chernoff and Liu [19]. Radio-iodination of the proteins was performed using

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a modified lactoperoxidase procedure based on the method of Thorell and Johansson [20]. Guinea pig thyroglobulin 19S-iodoprotein was labelled *in vivo* with ^{125}I and isolated by established procedures [14].

Apparatus.

A Waters Assoc. (Milford, Mass., U.S.A.) high performance liquid chromatography system was used which included a M660 solvent programmer, two M6000A solvent delivery systems, a U6K universal injector, coupled via a RCM-module, to a M450 variable wavelength UV detector and a Rikadenki dual channel recorder. The Radial-Pak A (C_{18}) cartridges (10 x 0.8cm and 10 x 0.5cm) were obtained from Waters Assoc. and washed extensively with methanol (*ca* 500ml) prior to use. Aliquots of column effluents containing radio-active peptides were counted in a Packard Tri Carb Gamma Scintillation Counter. Sample injections were made with Pressure Lok liquid syringes, series B110 from Precision Sampling (Baton Rouge, La., U.S.A.). The pH measurements were performed with a Radiometer PHM64 Research pH meter, equipped with a combination glass electrode.

Experimental.

All chromatograms were carried out at ambient temperature (*ca* 18^{0}). Bulk solvents and mobile phases were degassed by sonication. Following a change in mobile phase conditions, all columns were equilibrated to initial or new elution conditions for at least 30min. Flow rates were maintained between 1.0ml/mm and 4.0ml/min. Detection of the peptides was at 210nm or at higher wavelengths depending on the optical transparency of the mobile phase. The triethylammonium phosphate and formate buffers were prepared at different concentrations over the range 15-150mM by titrating the appropriate acid with freshly distilled triethylamine until pH 3.5 was obtained. All peptides and tryptic digests were

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made up in the mobile phase corresponding to initial equilibration conditions.

RESULTS AND DISCUSSION

The composite effects due to non-polar and polar group selectivities in peptide separation on alkylsilicas have been much discussed of late [2,4,21-23]. Although the predominant mechanism of retention under these RP-HPLC conditions is the hydrophobic expulsion of the peptidic solutes from the polar mobile phase to the less polar sorbent, polar effects due to unreactedand accessible silanol groups can also influence retention behaviour. For example, several studies [21-24] have demonstrated that both solvophobic and silanophilic processes are involved in the retention of peptides with most commercially available types of reversed phase silicas, even supports of high surface coverage and carbon loading. Under inappropriate elution conditions, these competing distribution processes can result in low solute recoveries and poor resolution due, in part, to undesirable peak broadening. Other column and extra-column effects are also known to influence chromatographic efficiencies. Even in well packed columns, 'wall' and associated non-uniform flow phenomena can lead to significant zone dispersion effects for peptidic solutes. Theory predicts [25-28] that radial compression of flexible walled cartridges can create an efficient and homogeneous chromatographic bed in which these dispersion effects are largely circumvented. Due to their favourable column characteristics, e.g. wide diameter, short columns, relatively high flow rates and sample loadings are also possible with these cartridges without a significant loss of resolution. In recent studies [6,7], we have employed this approach in the purification of multi-gram amounts of synthetic peptides using preparative reversed phase columns with sample capacities greater than

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lgm/injection. As is evident from the investigations reported in the present and other recent studies [29], the corresponding analytical system, now available commercially in the form of Radial Pak A/C_{18} columns, is suited to high efficiency semi-preparative separations of complex mixtures of peptides.

Acidic amine phosphate buffers have recently gained popularity in analytical RP-HPLC of peptides and proteins. Low concentrations, usually in the region of 5-20mM, of these buffers have been added to mobile phases of high-intermediate water content in order to mask polar group interactions at the surface of the chemically bonded alkylsilica stationary phase. Compared to the corresponding eluents which lack these buffer components, mobile phases which contain amine phosphates, formates or trifluoroacetates generally give rise [2,4,21-23,30] to reduced retention times and improved peak shapes for small peptides when chromatographed on such conventional reversed phase silica supports as μBondapak C_{18} or LiChrosorb RP-18. With the Radial Pak A/C_{18} support, it was found necessary to use considerably higher concentrations of these buffer combinations to avoid excessive band spreading. Although satisfactory column efficiencies could be achieved with, for example, 150mM triethylammonium phosphate incorporated into the mobile phase (Fig. 1a), mobile phases containing lower concentrations of the acidic triethylamine buffers resulted in inferior chromatographic performance. The decreased resolution observed with eluents containing low concentrations of the acidic amine buffers was most noticable with basic peptides and particularly with peptides containing a N-terminal arginine residue. These observations are consistent with the reported [21] surface characteristics of this totally porous, spherical, reversed phase support and parallel earlier experiences [2,21,22] on convenient procedures to mask silanophilic interactions. In order to obtain optimal chromatographic reproducibility, it was

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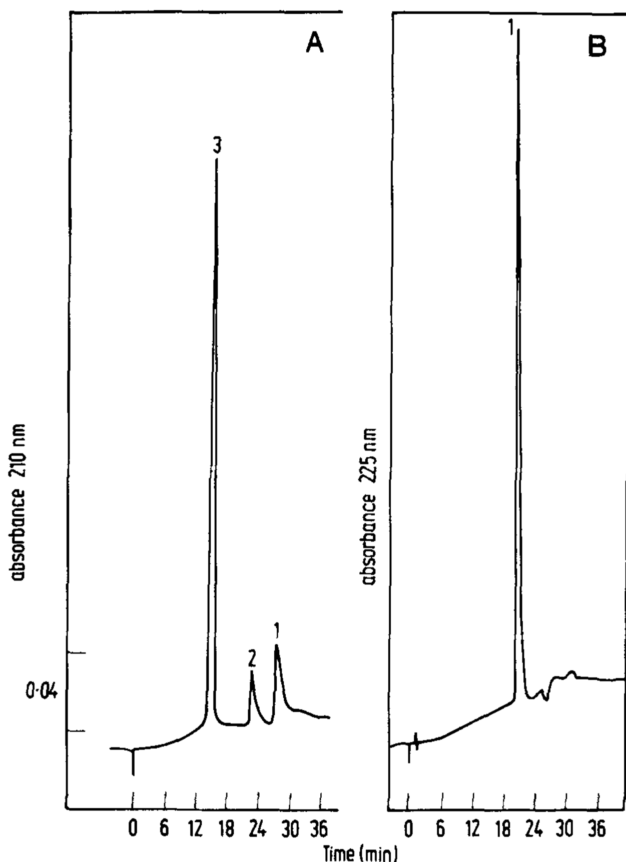

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Figure 1. (A) Gradient elution profile of a mixture of bovine insulin (1), angiotensin I (2) and trityrosine (3) (= *ca* 20μg each). Chromatographic conditions: column, Radial Pak A/C_{18}; flow rate, 2ml/min; gradient elution beginning with 150mM triethylammonium phosphate, pH 2.6 with a linear 30min. increase of the modifying mobile phase 50% acetonitrile-50%-water-150mM triethylammonium phosphate. (B) Chromatogram of bovine insulin (1) obtained under 30min. linear gradient elution conditions on the same Radial Pak A/C_{18} column. Flow rate, 2ml/min; mobile phase, aqueous 100mM ammonium sulphate (pH3.0) tp 50% acetonitrile-50% water-100mM ammonium sulphate (pH3.0).

also found necessary to condition the sorbent by means of an extensive wash with either methanol or another alcohol followed by elution with the appropriate mobile phase containing the acidic amine buffer for *ca* 200 column volumes. As most commercial organic amines are either of low purity or low volatility, triethylamine has proved to be the reagent of choice. However, even with triethylamine it is essential for semipreparative and analytical separations to ensure that associated contaminants, which interfere with high sensitivity UV or fluorometric detection, are removed by distillation procedures, followed by percolation of the freshly prepared mobile phase containing the appropriate concentration of the acidic triethylammonium buffer through an octadecylsilica support prior to use.

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Ammonium sulphate, at concentrations up to 100mM, was also found effective as an ionic modifier with the Radial Pak A/C_{18} support. With mobile phases of similar pH and organic solvent content, peptides show different selectivities with this reagent compared to triethylammonium phosphate buffer systems. Typical of these selectivity differences are the elution profiles for bovine insulin compared in Figs. 1a and 1b.

The major limitation of acidic amine phosphate or sulphate buffers in preparative RP-HPLC is the necessity to subsequently remove the buffer components. Although this desalting step can be achieved by classical open column gel permeation, high performance gel permeation and RP-HPLC techniques using volatile eluents, these subsequent steps may be undesirable and could well lead to lower recoveries. Volatile amine formate, acetate and triflouroacetic buffers have been employed [1,2,6,7,30] in preparative and semi-preparative RP-HPLC separations of peptides. Prior purification of the buffer reagents is invariably a prudent precaution. Ammonium bicarbonate offers a convenient alternative despite its limitations as an ionic modifier of peptide selectivity due to the restriction this reagent places

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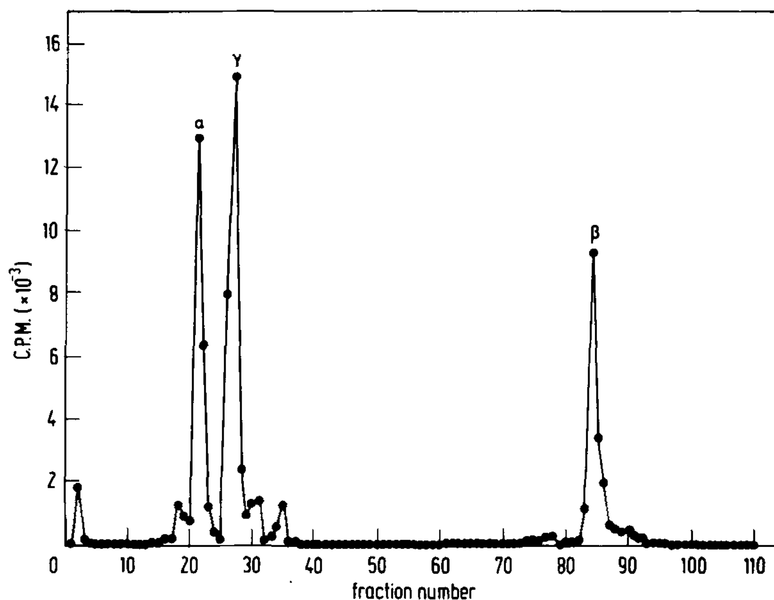

Figure 2. Separation of [^{125}I]-labelled α-, β- and γ-endorphin on a Radial Pak A/C_{18} column under gradient elution conditions. Chromatographic conditions: flow rate, 4ml/min; mobile phase A, aqueous 100mM ammonium bicarbonate; mobile phase B, 65% acetonitrile-35% water-100mM ammonium bicarbonate. The gradient elution commenced with 20% B with a 30min. linear increase up to 100% B.

on the operational pH range of the mobile phase. Desirably, peptides can be recovered directly by lyophilisation from hydro-organic solvent eluents containing this reagent. Micropreparative separations of peptides on conventional octadecylsilica supports have been described [1,2,4,32] using ammonium bicarbonate-based mobile phases. As is evident from the studies illustrated in Figs.2-7, similar eluents can be employed for the separation of peptides, including radio-iodinated polypeptides and hydrophobic tryptic peptides, on the Radial Pak A/C_{18} support.

Figure 2 shows the separation of [^{125}I]-labelled α-, β- and γ-endorphin. The recovery of radioactivity for each of

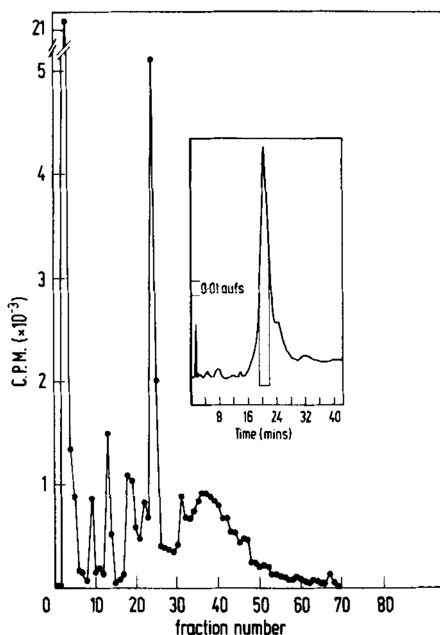

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Figure 3. Gradient elution profile of a partially purified [^{125}I]-labelled ovine thyrotrophin preparation chromatographed on a Radial Pak A/C_{18} column. Chromatographic conditions: flow rate, 2ml/min; a 30min. linear gradient was generated from aqueous 100mM ammonium bicarbonate to 50% acetonitrile-50% water-100mM ammonium bicarbonate. Also shown is the elution profile and recovery of concanavalin A-purified ovine thyrotrophin (500μg) chromatographed under the same conditions.

these labelled peptides was greater than 97% using a linear 30min acetonitrile gradient at a flow rate of 4ml/min. Similarly, high recoveries were obtained for a crude [^{125}I]-labelled ovine thyrotrophin preparation (Fig.3) (99% recovery) and for an *in vivo* labelled guinea pig thyroglobulin 19S iodoprotein complete tryptic digest (85% recovery). Also shown in Fig. 3 is the elution profile and recovery of a 500μg loading of Concanavalin A purified ovine thyrotrophin (*ca* 4μU/μg bioactivity based on a U.S.P. bovine thyrotrophin standard) chromatographed under the same elution conditions.

The high recovery of this glycoprotein hormone from the Radial Pak A/C_{18} column parallels similar results [17] obtained with a 500Å pore diameter octadecylsilica support and suggest that relatively hydrophobic proteins with molecular weights at least up to 30,000 may be successful separated on a semi-preparative basis using these radially compressed columns and mobile phases of suitable elutropicity. Because of their favourable solvent characteristics and peptide solubility parameter dependencies [2,23], acetonitrile and 2-propanol appear to be the two common organic modifiers best suited to polypeptide/protein separation on Radial Pak A/C_{18} supports.

The application of analytical RP-HPLC to peptide mapping of proteins has generated much valuable information on the primary structure of a large variety of proteins of current interest in molecular biology. Following our earlier reports on the development of elution strategies [1,2,18,33,34] for the micropreparative RP-HPLC separations of enzymatic digests of proteins, we have extended this micromethodology to haemoglobin variants [35], protein hormones [1,36] and other proteins with intrinsic biological activity [37,38] using in several cases as little as 100pmole of protein digest. In several instances where the parent protein was available on the nanomole scale, semi-preparative RP-HPLC would have been most useful.

Fig. 4 shows the elution profiles obtained after application of the 18h and 20h tryptic digest of chick lysozyme to a Radial Pak A/C_{18} column and elution with linear water-acetonitrile-100mM ammonium bicarbonate gradient. Under these chromatographic conditions excellent resolution and peptide recoveries were obtained. Consistent with the earlier results, the corresponding low pH phosphate based eluent gave significantly inferior resolution. As can be seen from Fig. 5, a 0-50% 2-propanol gradient with 100mM ammonium bicarbonate

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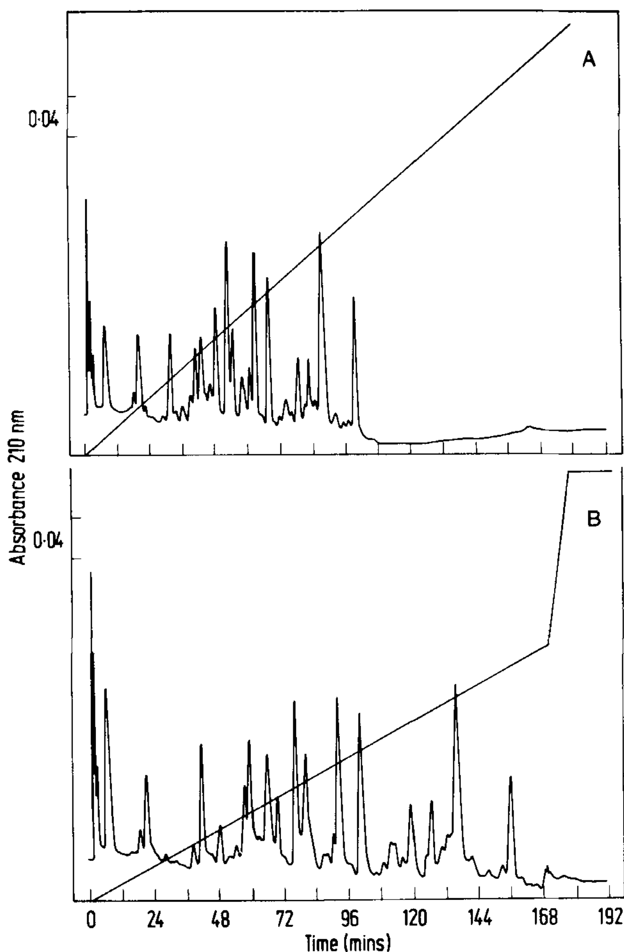

Figure 4. Separation of the tryptic peptides of chick lysozyme on a Radial Pak A/C_{18} column. In (A) is shown the chromatogram for the 18h. digest eluted at a flow rate of 1.5ml/min with a linear 3h. gradient generated from aqueous-100mM ammonium bicarbonate to 50% acetonitrile-50% water-100mM ammonium bicarbonate, sample size, 1400μg. In (B) is shown the chromatogram for the 20h. digest (sample size 2100μg) eluted under similar conditions except that a biphasic 5h. gradient was employed.

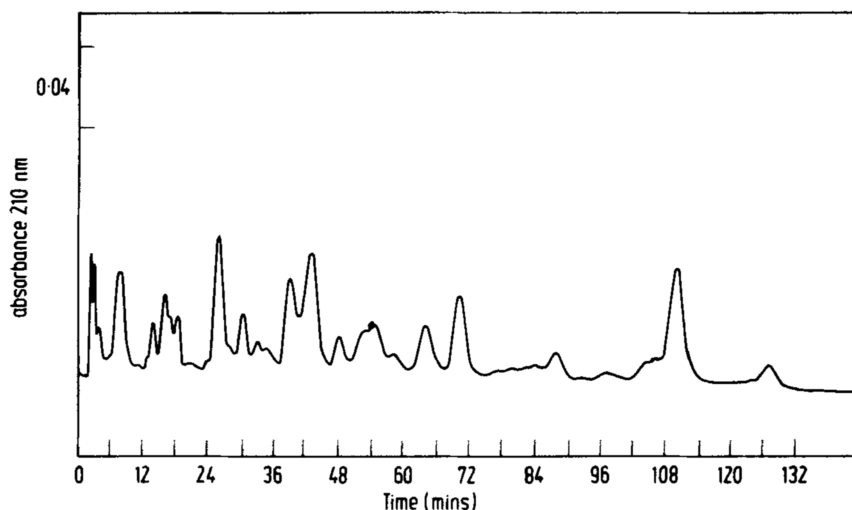

Figure 5. Gradient elution profile for the tryptic peptides of chick lysozyme chromatographed on a Radial Pak A/C_{18} column using a 5h. linear gradient generated from aqueous 100mM ammonium bicarbonate to 50% propan-2-ol-50% water-100mM ammonium bicarbonate at a flow rate of 1.5ml/min. Sample size 2100μg in 150μl.

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present resulted in more rapid elution of the less polar lysozyme tryptic peptides but reduced efficiencies compared to acetonitrile gradients. These results are typical for these two solvent modifiers and reflect relative differences in their elutropicities and solute diffusion rates [23].

Fig. 6a illustrates the elution profile obtained for the 6h tryptic digest of human growth hormone eluted under ammonium bicarbonate-acetonitrile gradient conditions. In Fig. 6b is shown the corresponding chromatogram obtained for the same tryptic digest on an analytical μBondapak C_{18} column eluted with a low pH phosphate-based mobile phase. As anticipated the Radial Pak A/C_{18} column gave better column performances with larger sampling loadings for the human growth hormone enzymatic digests. Application of these methods has

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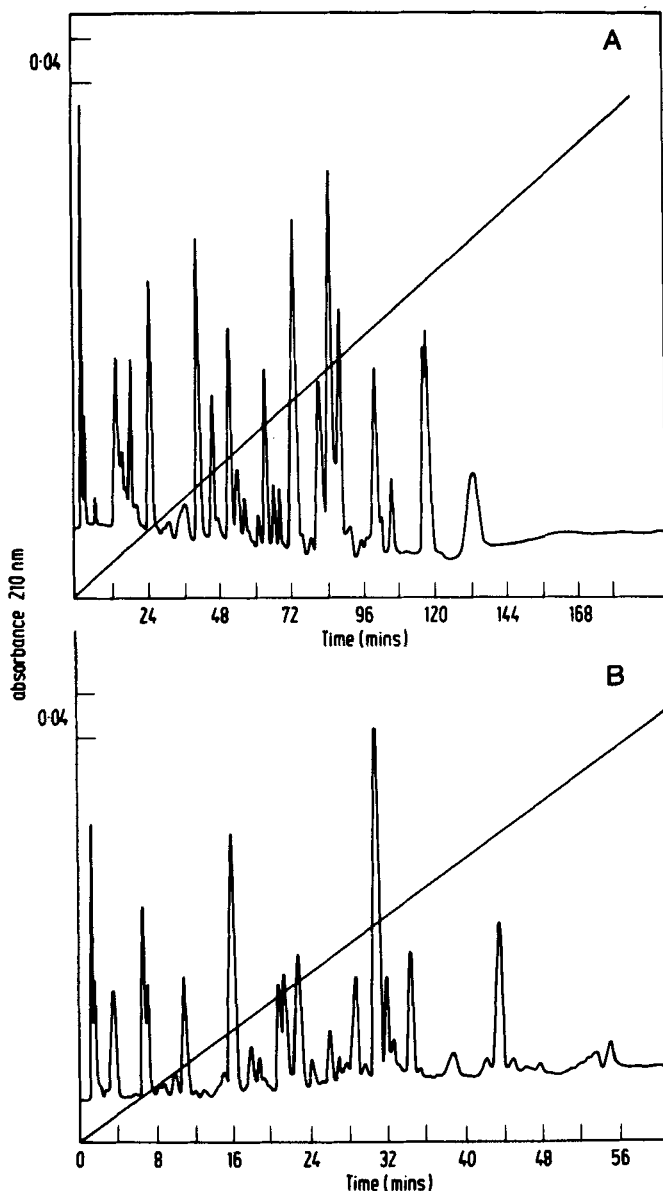

Figure 6. Separation of the tryptic peptides of human growth hormone on (A) the Radial Pak A/C_{18} column with the elution conditions given in the legend to Fig. 4A, and (B) on the μBondapak C_{18} analytical column with a 60min. linear gradient generated from water-15mM orthophosphoric acid to 50% acetonitrile-50% water-15mM orthophosphoric acid, flow rate, 2ml/min.

permitted the amino acid sequence of the 20K human growth hormone variant to be confirmed in this laboratory [36].

Fig. 7 demonstrates further the general applicability of the Radial Pak A/C_{18} column to resolve particularly complex peptide mixtures on a semi-preparative scale. In this figure, the elution profiles of the 20h tryptic digests of human (A) and guinea pig (B) thyroglobulin 19S iodoproteins are shown. Compared to previous peptide mapping techniques used in the study of these two macroglobulins [39] these rapid, high resolution RP-HPLC separations are clearly superior. Using similar chromatographic techniques, we have been able to resolve [38] milligram quantities of [^{125}I]-labelled peptides from _in vivo_ or _in vitro_ iodinated thyroglobulins from several species. Despite the reported [14,40] similarities in amino acid composition for human and guinea pig thyroglobulin 19S iodoproteins, significant sequence and cleavage site differences are evident from a comparison of the chromatograms shown in Fig.7. At this stage, little information is available on the amino acid sequence of the subunits of these microheterogeneous macroglobulins due, in part, to the poor resolution of the enzymatic or the CNBr-fragments of these proteins obtained with classical open column gel permeation or ion-exchange techniques. For example, on Sephadex G75 columns, the same tryptic digest as used for the RP-HPLC separation shown in Fig. 7A is resolved into only nine discrete fractions [38].

In summary, high efficiency separations of complex peptide and polypeptide mixtures can be achieved using the Radial Pak A/C_{18} support packed into flexible-walled polyethylene cartridges. From the available chromatographic data, this non-polar sorbent exhibits different selectivities for peptides including the angiotensins, insulins and endorphins when compared to conventional octadecylsilica supports such as μBondapak C_{18}, LiChrosorb RP-18 or Hypersil ODS. A further

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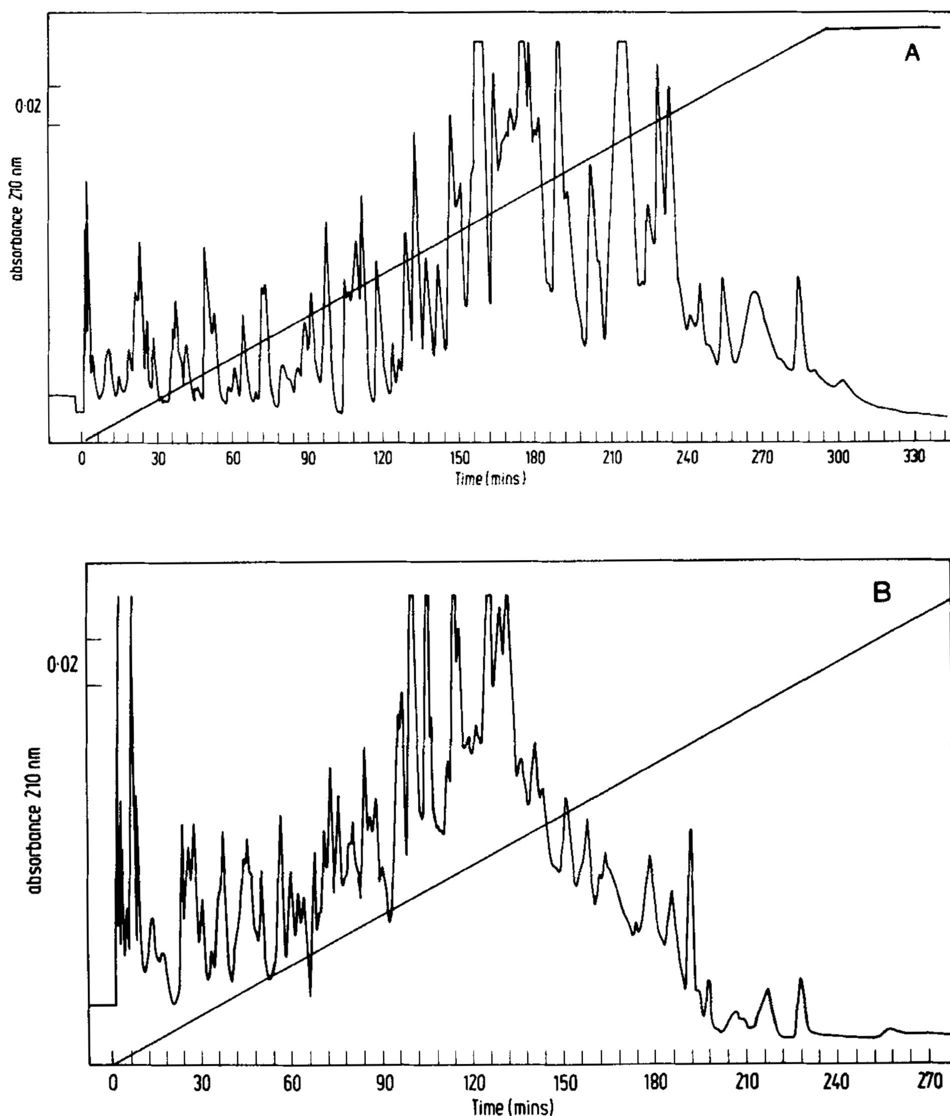

Figure 7. Gradient elution profiles for the tryptic peptides of human thyroglobulin 19S iodoprotein (A) and guinea pig thyroglobulin 19S iodoprotein (B) chromatographed on Radial Pak A/C_{18} columns at a flow rate of 1.5ml/min. A linear 5h. gradient was employed in each case using the mobile phase conditions given in the legend to Fig. 4.

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distinction can be made with these other types of bonded reversed phase silicas in so far that hydrophobic peptides tend to be eluted from the Radial Pak A/C_{18} support with mobile phases of lower organic solvent content. With mobile phases containing volatile buffers such as triethylammonium formate or ammonium bicarbonate, good selectivity and recoveries were obtained. Shallow gradients were preferred for the separation of larger polypeptides or complex mixtures derived from the enzymatic digestion of proteins. Because higher flow rates can be used with these flexible-walled columns than is the case with stainless steel columns, re-equilibration times were significantly shorter. No significant loss of resolution was noted with the Radial Pak A/C_{18} support, packed into 10 x 0.8cm cartridges, for sample loadings between one and two orders of magnitude greater than those typical for standard (25 x 0.4cm) stainless steel HPLC columns. For example, the semi-preparative capability of the radially-compressed chromatographic system permitted 10-100nmoles per injection of the thyroglobulin tryptic digests to be separated with excellent resolution and recovery in ca 6h.

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