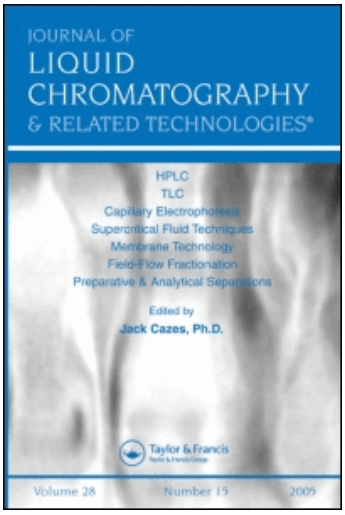

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THE ANALYSIS OF INSULIN-RELATED PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY†

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† High Performance Liquid Chromatography of Peptides and Proteins XVIII. For the previous paper in this series see Ref. (1).

Abstract

This paper describes the use of high performance liquid chromatography (HPLC) for the rapid analysis and purification of insulin-related peptides prepared by solid-phase synthetic procedures. Examples include the bovine insulin C-peptide (34-45), the porcine insulin C-peptide (41-53) and the insulin B-chain fragment (22-27). Chromatographic elution systems containing reducing reagents like ß-mercaptoethanol allow the direct analysis of insulin reduction products. Similar systems should allow the rapid analysis of disulphide bond pairing patterns in appropriate polypeptides and proteins either directly or following proteolytic digestion.

Reversed-phase high performance liquid chromatography (RP-HPLC) is a versatile and rapid technique useful for the analysis and purification of biological substances. In a series of recent publications[1-4] we have described methods for the analysis of underivatised amino acids, peptides and proteins on reversed-phase packings using ion-pairing or stationary phase modifying reagents as components of the mobile phase. These studies demonstrated that excellent resolution of closely related peptides can be achieved under a variety of elution conditions. The addition of low levels of phosphoric acid, inorganic or organic phosphates to a mobile phase (generally water-organic solvent mixtures), in particular, allows rapid and reproducible analysis of peptidic compounds with high sensitivity detection at wavelengths down to 190nm[5,6]. It is the purpose of this report to show that these chromatographic conditions allow the facile analysis, and purification, of a variety of insulin-related peptides.

Materials and Methods

Chemicals and Peptides: AnalaR methanol (Mallinckrodt) and ACS acetonitrile (Fischer Chem. Co.) were purified as described previously[2]. Orthophosphoric acid was obtained from May and Baker. Water was glass distilled and deionised. The insulin related peptides were prepared by solid-phase methods using standard procedures. The bovine C-peptide (34-45) and porcine C-peptides (41-53) were purified using Sephadex G-25 (fine) gel filtration, CM-Sephadex c-50 ion-exchange chromatography and Sephadex G-25 (fine) partition chromatography by the procedure of Hurrell et. al.[7] The bovine insulin, obtained from Sigma Chemical Co. was rechromatographed on DEAE-Sephadex using the gradient elution procedure of Nolan et.al.[8] Peptides were cleaved from the resin and chemically characterised from their amino acid compositions, or sequences using reported methods [7,9].

Apparatus: A Waters high performance liquid chromatography system was used which included two M6000A solvent delivery units, a M660 solvent programmer and U6K universal liquid chromatographic injector, coupled to a M450 variable wavelength UV monitor and a Rikadenki dual channel chart recorder. The reversed-phase columns were obtained commercially. Sample injections were made with a Pressure-Lok liquid syringe, Series B110 from Precision Sampling (Baton Rouge, La.). Filtration of solvents and solutions of the peptide samples was carried out using Millipore AP2500 filters (Millipore Corp., Bedford, Mass.).

Chromatographic Procedures: All chromatograms were carried out at room temperature (ca. 20°). All peptides were made up in the eluting solvent. Bulk solvents were degassed separately and the appropriate mobile phase prepared and left to equilibrate for

ca.30mins. Other chromatographic conditions were similar to those reported by Hearn et. al.[2] for the elution of angiotensin analogues. Sample size varied between 0.1µg and 5mg of peptide injected in 20-200µl volumes. For semipreparative purifications on reversed-phase columns appropriate fractions were pooled, the pH adjusted to ca. pH7, concentrated and desalted on Sephadex G-25 (fine). The purified peptides were then re-examined by analytical HPLC procedures and amino acid analysis.

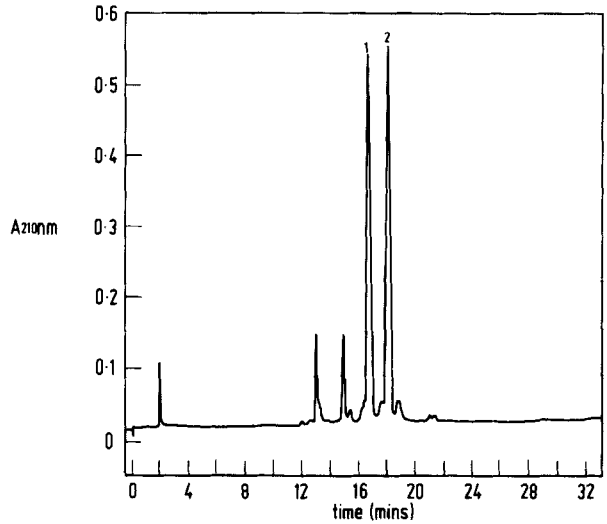
Results and Discussion

The application of reversed-phase high performance chromatography to the analysis and purification of synthetic hormonal peptides, related to the angiotensins and the enkephalins, has recently been reported[2]. In that study, HPLC conditions were developed which permitted very rapid recognition of deletion or partial deprotection products generated during peptide synthesis. Furthermore, the elution order was found to follow the order of relative hydrophobicities for a series of closely related peptides. As is evident from the present study with insulin-related peptides, these general methods allow the homogeneity of crude synthetic mixtures or partially purified peptides to be rapidly assessed and also provide highly purified peptides.

A typical elution profile for the crude bovine insulin C-peptide (34-45) (H-Val-Glu-Gly-Pro-Glu-Val-Gly-Ala-Leu-Glu-Leu-Ala-OH) obtained by solid phase synthesis, following HF cleavage and concommitant deprotection, is shown in Figure 1a, Extensive gel , ion-exchange and partition chromatographic purification failed[7] to satisfactorily resolve the bovine insulin C-peptide (34-45) from the des-Pro^{9}-peptide, which constitued the major synthetic deletion product. However, as

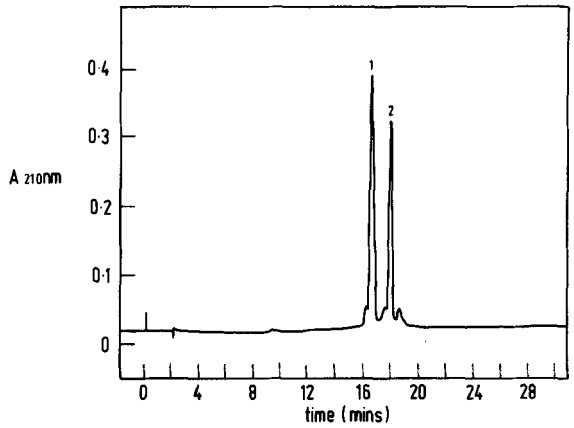
can be seen from Figures 1b, c these two peptides are completely resolved with standard reverse-phase columns, operating at moderate pressure using either isocratic or gradient elution conditions with chromatographic times less than 30 mins. In a similar manner the crude and partially purified crude porcine C-peptide (41-53) (H-Glu-Leu-Gly-Gly-Gly-Leu-Gly-Gly-Leu-Glu-Ala-OH) was resolved on a porous 10μm reversed phase and the open column ion-exchange purification readily followed (Figs. 2a,b). Furthermore, these methods, using either conventional analytical columns, e.g. 30cm x 4mm I.D. containing 5-or 10μm reversed phase packings, or larger preparative columns, e.g. 60cm x 7mm, I.D. containing 37-75μm reversed phase packings, permit semi-preparative separations of these peptides to be carried out. Illustrative of these applications is the semi-preparative purification of the human insulin B-chain fragment (22-27) (H-Arg-Gly-Phe-Phe-Tyr-Thr-OH). Figures 3a,b show the analytical gradient elution profiles of this hexapeptide before and after purification on μ-Bondapak/C_{18} reversed phase columns. With the above group of synthetic peptides, semi-preparative purifications were routinely carried out with loadings of ca 5mg with recoveries of 70-85% on commercially available 5- or 10-μm reversed-phase columns. Considerably larger peptide loadings (100mg-10g) are possible using fully preparative systems, with radial-compression reversed-phase columns[10].

Various studies with model peptides have previously shown[3,5,11] that the generation of hydrophilic ion-pairing conditions with anionic species, such as dihydrogen phosphate or perchlorate, present in the mobile phase results in highly efficient separations In the absence of these reagents, poor peak shapes and low recoveries are frequently observed. In view of the high



(a)

sensitivity U-V detection that is possible with phosphate-based elution buffers, ie. at wavelengths down to 190nm, these conditions are particularly suitable for the separation on reversed-phase columns of peptides available from enzymatic or CNBr digests of proteins[4,15]. A logical extension of these methods is the direct analysis of poly-peptides following chemical reduction eg. with reducing reagents such as β-mercaptoethanol as a component in the eluant. Illustrative of this approach are the two chromatograms obtained with bovine insulin. Figure 4a shows the elution profile of a bovine insulin sample, purified by standard DEAE-Sephadex procedures, on a μ-Bondapak fatty acid column using a 0→50% acetonitrile-water-0.1% phosphoric acid gradient. Following the addition of 0.5% β-mercaptoethanol to the mobile phase, the reduction products of insulin[16,17] can be readily analysed (Figure 4b). In Figure 4c, the elution profile of partially

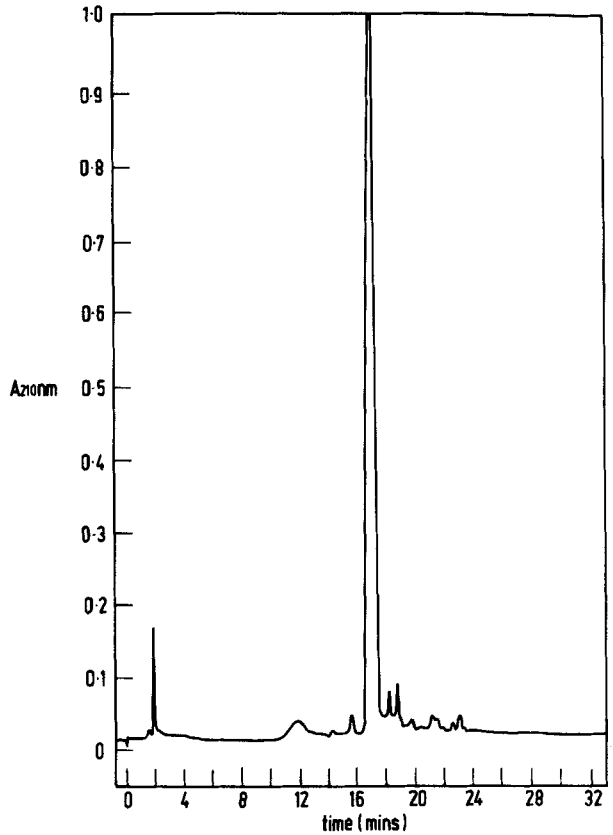


(b)

Figure 1 (a) Gradient elution profile of the crude bovine insulin C-peptide (34-45) preparation obtained from solid-phase synthesis on a μ-Bondapak-fatty acid column at a flow rate of 2mL/min. A 30 minute linear gradient was generated from water 0.1% phosphoric acid to acetonitrile-water-phosphoric acid (50:50:0.1%) using a 50μg peptide loading.
(b) Gradient elution profile of the same preparation after Sephadex G-25 (fine) gel filtration, CM-Sephadex C-50 ion-exchange and Sephadex G-25 (fine) partition chromatography. Peak 1 corresponds to the des-Pro^{9}-undecapeptide and Peak 2 to the (34-45) C-peptide fragment. The same elution conditions to (a) above were used.

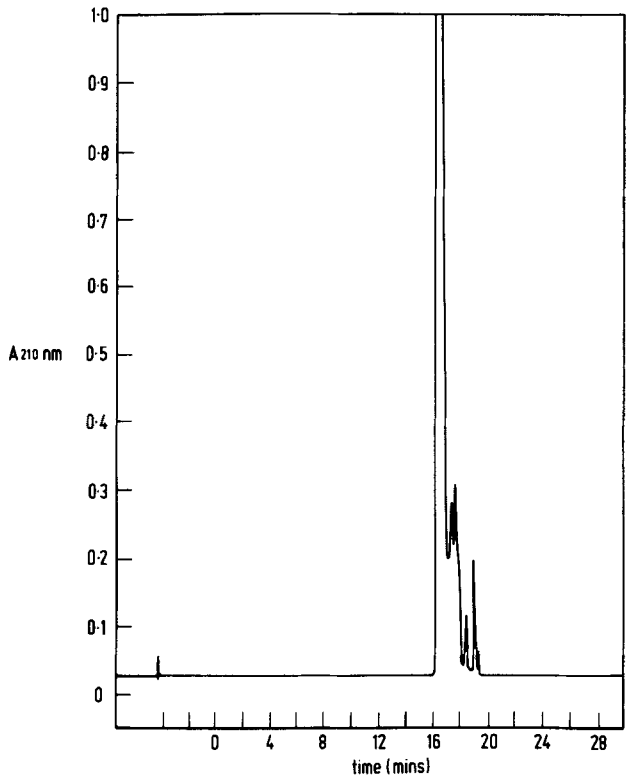
oxidised bovine insulin B-chain is shown run under the same chromatographic conditions with 0.5% β-mercaptoethanol.

The examples described in this paper demonstrate the value of RP-HPLC for the analysis and purification of peptides related to insulin. Similar methods have been successfully used with hypothalamic and pituitary polypeptides[2,6,18,19].



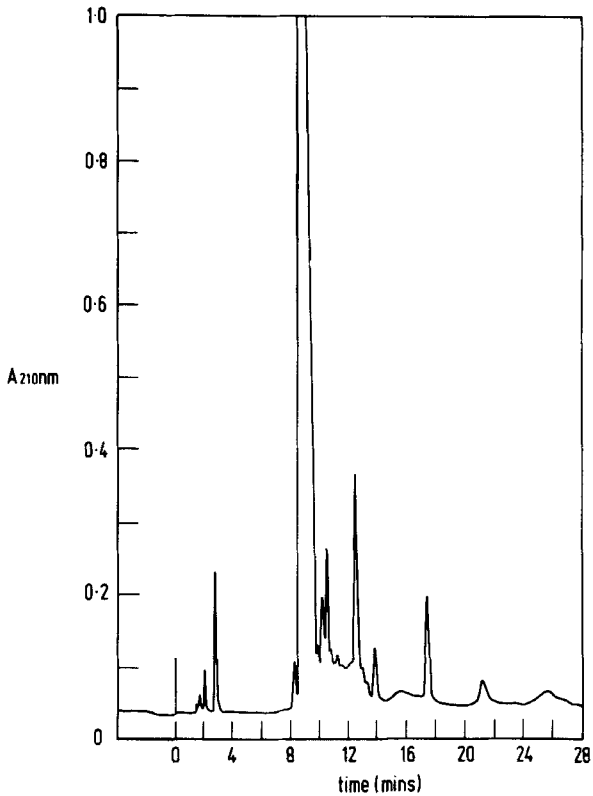
(a)

The combination of the high selectivity found with porous non-polar hydrocarbonaceous stationary phases and elution conditions, containing reducing reagents like β-mercaptoethanol, similar to those reported above, should permit the facile analysis of the reduction products from a wide variety of disulphide bridged cyclic or multisubunit polypeptides, particularly when



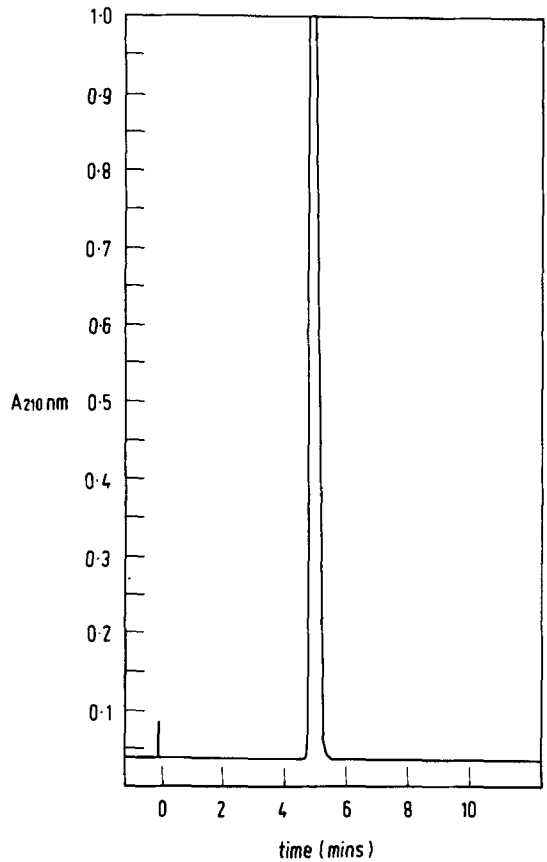
(b)

Figure 2. Analytical chromatograms of (a) crude porcine insulin C-peptide (41-53) fragment obtained by solid-phase synthesis and (b) after CM-Sephadex purification (sensitivity x 10). Chromatographic conditions were identical to those given for Figure 1a.



(a)

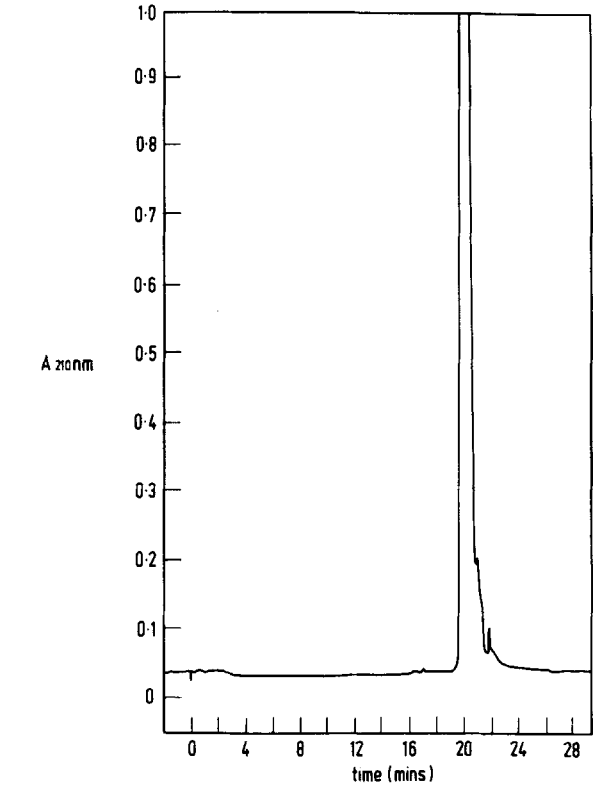
associated with fluorometric detection methods. An analogous approach should also be useful for the HPLC analysis of the proteolytic digests of proteins containing disulphide bonds. In preliminary studies with the tryptic digests of several thyroid proteins, as well as a recent report[20] on the analysis of the pepsin digest of lysozyme, our and other groups have found that rapid HPLC methods are complementary to existing paper and diagonal paper electrophoretic techniques[21,22] for the analysis of disulphide bond pairing patterns in proteins.



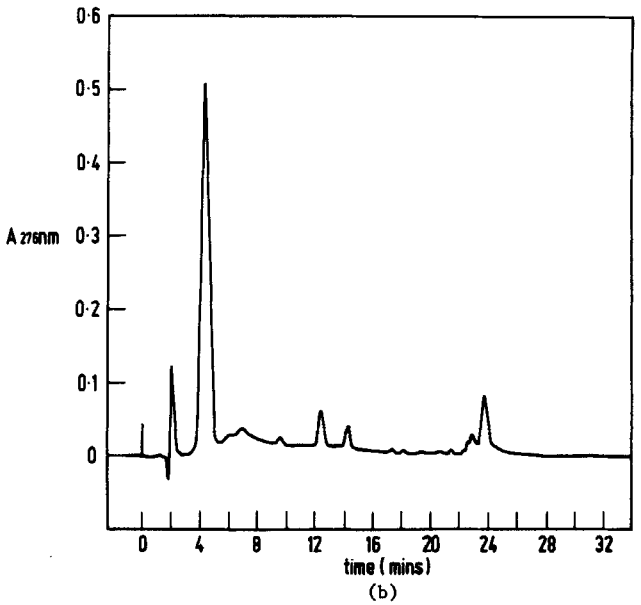
(b)

Figure 3 (a) Elution profile of a solid-phase synthetic preparation of human insulin B-chain (22-27) peptide on a μ-Bondapak-C_{18} column at a flow rate of 2mL/min. A 20 minute linear gradient from acetonitrile-water-phosphoric acid (10:90:0.1%) to acetonitrile-water-phosphoric acid (75:25:0.1%) was used to generate the chromatogram.

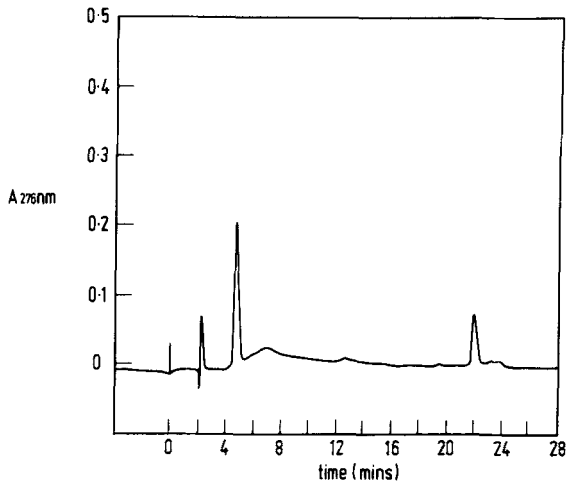
(b) Analytical chromatogram of the B-chain hexapeptide, following purification on 2 x μ-Bondapak-C_{18} columns. The chromatographic conditions were the same as given for Figure 1a.



(a)



(b)



(c)

Figure 4. Elution profile of bovine insulin on a μ-Bondapak-fatty acid column using as elution conditions:
(a) A linear 30 minute gradient of water-0.1% phosphoric acid to acetonitrile-water-phosphoric acid (50:50:0.1%) and
(b) Same gradient system with 0.5% β-mercaptoethanol added to the eluants with pre-reduced bovine insulin (50μg loading). Shown in (c) is the chromatogram of partially purified bovine insulin B-chain run under the same chromatographic conditions in the presence of 0.5% β-mercaptoethanol.

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