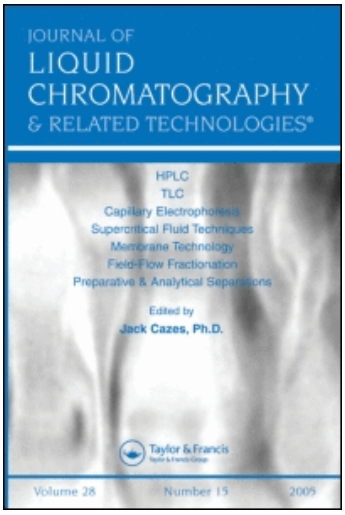

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THE USE OF REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE STRUCTURAL MAPPING OF POLYPEPTIDES AND PROTEINS*

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ABSTRACT

The application of reversed phase high performance liquid chromatogarphy (RP-HPLC) in analytical and preparative mapping of polypeptides and proteins is reviewed. Compared to conventional techniques, superior peak resolution for both hydrophilic and hydrophobic peptides can be obtained with a variety of convenient RP-HPLC methods which also offer the advantages of short elution times and good recoveries.

It is now a common experience for researchers in biochemistry and related biomedical sciences to require a knowledge of the primary structure of a particular naturally occurring peptide or protein as a necessary prelude to unravelling its biological significance. It is also desirable to have similar information for large synthetic polypeptides in order to validate the synthesis. In many cases the molecule of interest will be available in only minute amounts and this limitation necessitates high sensitivity methods for sequence analysis. With few exceptions, large

* High performance liquid chromatography of amino acids, peptides and proteins XXVI. For part XXV see ref. [1].

polypeptides and proteins, as isolated from natural sources, are poorly suited to direct application of sequencing methods. This partly arises due to post-translational processing or the formation of complex internal or multi-subunit structures, e.g. those arising from intra- or inter-chain disulphide bonds, glycosylation or polypeptide chain associations. In addition, limitations in the yield of repetitive stepwise degradations experienced with sequencing techniques result in accumulated memory effects and these can make interpretation at each additional step increasingly difficult. Once individual polypeptide chains, which generally can be generated from multi-subunit structures by appropriate changes in pH or salt concentration or, when necessary, by disulphide bond reduction followed by a suitable alkylation reaction, are obtained in the requisite state of purity, sequence determination from the N- or C- terminal residues can be initiated.

When the polypeptide chain is large (ie. greater than 30 residues) cleavage of the original molecule in a known manner into a number of short peptides often considerably simplifies the sequence determination. When correctly aligned, the primary structure of these fragments completely characterises the total sequence. Since no single fragmentation procedure is ideally suited for this task, the protein chemist generally resorts to a combination of enzymatic, or enzymatic and chemical, fragmentation techniques. Besides the direct value to sequence determinations, the analysis of the peptide fragments can be used to compare proteins, detect distinctive sequence homologies or identify characteristic protein variants such as in certain genetic disorders like haemaglobinopathies.

Fragmentation studies with natural peptides, polypeptides and proteins usually involve treatment with cyanogen bromide, with proteolytic enzymes or both, followed by separation of the peptide mixture by chromatographic or electrophoretic techniques. Peptide maps (or 'fingerprints') of a parent protein have been traditionally generated using two dimensional paper or thin-layer chromatographic methods or, alternatively, electrophoresis in one direction followed by chromatography in a perpendicular direction[2,3]. As usually employed, conventional methods suffer from limitations in sensitivity and resolving power and for preparative separatives exhibit generally low yields of recovered purified peptides (typically 20% from paper [4]). Although the detection sensitivity can be increased by the use of chromogenic or fluorogenic derivatisation reagents [5], radio-iodination [6], or incorporation of a radio-actively labelled amino acid into the protein or polypeptide [7], the application of peptide mapping methods to large proteins is limited by the lack of resolving power of conventional techniques for a large number of small, often similar, peptides. Over the past few years high performance liquid chromatography (HPLC) has become of increasing importance for the rapid, high resolution separation of amino acids, peptides and proteins. It is the purpose of this article to illustrate the potential of HPLC, particularly when operated in the reversed phase mode, for the analytical and preparative separation of peptide mixtures generated during the structural mapping of natural polypeptides and proteins. Clearly, similar mapping criteria can be applied to synthetic polypeptides to confirm the accuracy of a particular synthesis.

Two important developments underlie the emergence of reversed phase HPLC as a powerful analytical and preparative technique in peptide chemistry. The first development relates to the availability of an increasing array of reliable chemically bonded, pressure stable packing materials in a variety of particle diameter and pore size ranges. The second development has evolved from the recent appreciation that problems of poor resolution, reproducibility and recovery can be circumvented by the rational choice of one of a large variety of mobile phase systems, many of these being new to the field of peptide separation. The extreme sensitivity and flexibility of these reversed phase HPLC procedures are precisely the features required for analytical peptide mapping, detection of cleavage intermediates and following the time course of the cleavage reaction as well as related preparative studies involving the separation of the cleavage products of proteins or polypeptides.

Choice of Stationary Phase.

The characteristics of many of the commercially available reversed phase silicas have recently been reviewed [8,9]. Currently, most interest for analytical (10ng-100µg) and semipreparative (10µg-1mg) separations of peptides, centres on the irregular or spherical macroporous alkylsilicas, nominally of 10- or 5-µm dp. These are routinely used in standard stainless steel columns (eg. 30cm x 3.9mm). In some instances, larger sample loadings (5-10mg) can be achieved with these columns, whilst, with the recently introduced radial compression columns [10], sample loadings as high as 50mg may be contemplated. Larger fully porous reversed phase packings (dp ranging 30-50µm) are also

available and their use in preparative separations (1gm and up) of peptides has been reported [11,31]. Pore size is usually not a major consideration for peptide separation in the reversed phase mode (i.e. a globular protein on the range 40-50,000 daltons is theoretically capable [12] of diffusing through a silica support with a pore diameter of 100Å) but the chain length and extent of coating of the bonded ligand can have a significant influence on selectivity. For example, not all the free silanols may necessarily react during the bonding process (alkyl loadings for reversed phases are usually in the range 10-20%, i.e. 0.5-1.5mmol/g, with about 50% of the total silanols coated) and this can lead to mixed polar and non-polar adsorption mechanisms. Similarly, the short chain alkylsilicas, like Lichrosorb RP-2, can also exhibit mixed adsorption mechanisms with peptides, resulting in poor resolution and reduced recoveries. The high coverage C_8- and C_{18}- hydrocarbonaceous phases have proved particularly reliable and give excellent peak shapes for peptides under suitable conditions, eg. typically 25,000 theoretical plates per metre at a flow rate of 1ml/min. Although, a uniform, relatively-ordered monolayer of alkyl bristles was originally envisaged [13], for the ligand coat, there is still some debate [14] whether the bonded ligand actually forms solvophobic aggregates of non-polar liquid-droplet clusters. With either structure, the reduction of the total solvophobic surface area will be the driving force for the chromatographic distribution of peptide solutes between the mobile and stationary phases.

Choice of Mobile Phase Conditions.

As a result of accumulated experiences gained over the last few years we now have a fairly adequate understanding

on how selectivities of a group of peptides, when chromatographed on C_8- or C_{18}- alkylsilicas, can be manipulated by rational changes in the mobile phase. Provided size exclusion and ion exchange effects are minimal, the most polar peptide in a mixture will have the shortest chromatographic retention on reversed phase columns with the remainder eluting roughly in order of their relative hydrophobicities. Molecular size *per se* would not be expected to be the dominant parameter but rather the hydrophobic contact area of the molecular with the stationary phase. For a series of peptides this property can be expressed quantitatively in terms of the relative hydrophobicities of the amino acid side chains substituted in an ordered manner into a polyglycinyl oligomer. With small related peptides hydrophobicity parameter summations have proved useful [23,26] for the prediction of elution orders under a given mobile phase condition. Reduced predictive discrimination is obtained with larger polypeptides due to conformational effects in solution which reduce the number of exposed, complimentary hydrophobic residues. Because of the inherent ionogenic and hydrophobic properties of all unprotected peptides, the polarity differences expressed by a group of peptidic substances in solution will be very dependent on the involvement of a number of solution equilibria notably protonic, solvation, ion-pairing and dynamic liquid-liquid ion exchange interactions. The success of a particular reversed phase separation will thus depend on the ability of the mobile phase to take advantage of electrostatic, hydrogen bonding and hydrophobic equilibrium interactions between the solute molecules and components in the mobile or stationary phases.

Recent studies [15-25] from this and other laboratories have shown that the retention characteristics of peptides, and related amphoteric molecules, on reversed phase HPLC supports can be profoundly influenced by variation in pH, the addition of suitable counter-ionic reagents to the mobile phase (usually 5mM or less) and the choice of the organic solvent modifier (for introductory compendum on the theoretical and experimental aspects of reversed phase HPLC and its role in protein chemistry see [13,26-28]). Since these mobile phase parameters can be readily varied experimentally, reversed phase HPLC has the necessary versatility to not only distinguish peptides with major differences in composition and sequence but also those with minor changes, e.g. an amino acid replacement or deletion, partial de-amination, phosphorylation, racemisation, etc. In addition, these chromatographic conditions are generally compatible with high sensitivity variable or fixed wavelength, (eg. 210nm), UV or, alternatively, fluorometric detection of the separated peptides. Many of methods are also amenable to the use of specific post-column chemical, enzymatic, radioimmuno- or radioligand reaction detectors and the application of these detection methods will undoubtably become increasingly evident in the near future.

Although mixtures of polar peptides can be eluted from hydrocarbonaceous phases with neat aqueous buffers at low pHs, these conditions are generally not suitable for polypeptide or protein digests. In most cases digests will contain peptides varying widely in terms of their hydrophobicities and consequently a broad spectrum of mobile phase elution strengths is required. This can be simply achieved by varying

the water content, ie. by either isocratic, or, preferably, gradient elution with an organic solvent modifier, so that adequate peak shape, resolution and chromatographic retention are obtained. With the commonly used solvents acetonitrile, methanol, ethanol, 1- and 2-propanol the retention of peptidic solutes is generally inversely related to the elutropic value of the organic solvent. Thus, 1-propanol gradients, despite the high viscosity of this solvent, are well suited for the elution of very hydrophobic peptides from octyl- or octadecyl-silicas. A slow rate of change of the secondary solvent e.g. 0.2-0.5% per min at flow rates 0.5-2.0ml/min, should preferably be used with gradient elution of protein digests. The gradient shape can also have a significant effect on resolution. Linear gradients for acetonitrile systems and shallow exponential gradients for methanol and ethanol are recommended for exploratory investigations on the separation of enzymatic digests.

At this stage, both solvent flow programming and temperature variation, as means of controlling separations of complex peptide mixtures, present in protein or polypeptide digests, have been poorly investigated. Knowledge [18,23,26] gain with less difficult separations, generally with synthetic peptides, clearly indicate that under isocratic conditions, increases in flow rate can significantly reduce the retention times of peptides although the respective elution volumes remain essentially constant. Similarly, flow rate changes under gradient elution conditions make only small differences to the elution volume provided the rate of change of the secondary solvent is low. Peptides appear to show temperature-dependent, entropy dominated selectivities on reversed phase supports. In general, retention of peptides is reduced on raising the

temperature and column efficiencies enhanced due to the more rapid mass transfer. These advantages have to be balanced against the possibility of solute degradation.

Theory predicts that the retention of non-polar peptides to reversed phases will progressively increase as the pH is lowered below their pI values. Due to the chemical instability of most alkylsilicas it is necessary to use mobile phases with pHs below pH 7.5. Usually separations are carried out at low pHs, i.e. pH 2-3, since overall decreases in column selectivities can occur with peptides as the pH is increased from pH 3.0 to 7.0 [15, 18,19]. Depending on the nature of the digest, pH optimisation can be a simple method to achieve selectivity changes for a limited region of the chromatogram.

Manipulation of the elution strength of a mobile phase by the addition of organic solvent modifiers or changes in the pH are frequently insufficient to ensure adequate resolution of a complex peptide mixture. Large selectivity differences can, however, be achieved in such peptide separations by the addition, at a suitable pH, a low concentrations, eg. 5mM, of counterionic reagents which either interact with the peptides, eg. via ion-pair formation or modification of the hydrophobic characteristics of the stationary phase to a dynamic ion exchanger. A large number of anionic and cationic reagents have been applied [15,16,17, 18,20,21,23,26,27,29-31] to analytical separations whilst, for obvious reasons, the phosphate, acetate, formate and trifluoroacetate hydrophilic anions are the most commonly used for preparative studies. Elution under two mobile phase conditions of similar composition, differing only with regard to the nature of the counterionic reagent present in low

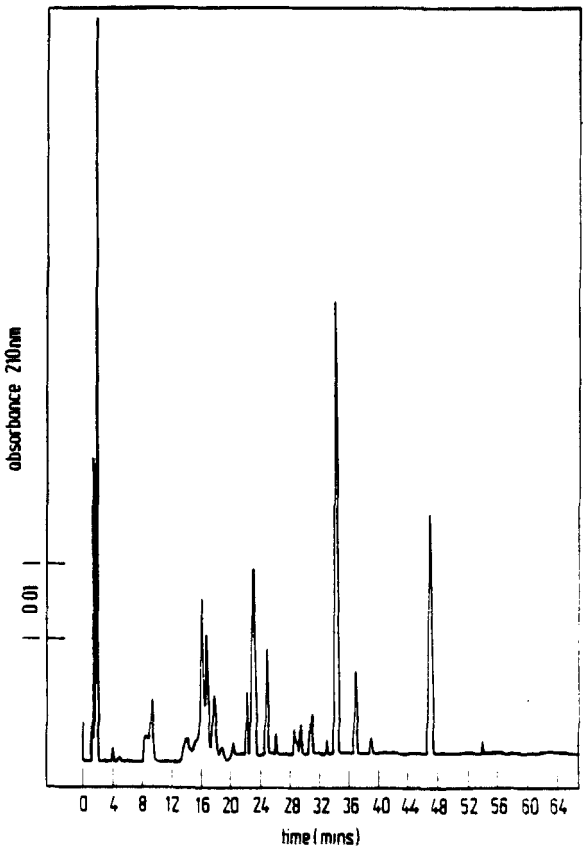


Figure 1. Gradient elution profile for the tryptic digest of human growth hormone (hGH). Column, μBondapak alkylphenyl [illegible]; flow rate 2.0ml/min; temperature 20°; elution conditions, a 60-min linear gradient was generated from water - 0.1% H_3PO_4 to 50% acetonitrile-water-0.1% H_3PO_4, detection, 210nm, 0.2AUFS, sample size [illegible] μl. In [34].

concentration, can be used [15] to provide pairs of chromatographic peptide maps formally analogous to those obtained with two dimensional electrophoretic techniques.

The advantages of phosphate-mediated elution conditions at pH 2-3 for the reversed phase separation of peptides have

been recognised [18,20,30] for several years. In an initial study, we reported [32] the analysis of peptides generated by tryptic or thermolysis digestion of a number of globular proteins including acyl carrier protein and the thiol protease, actinidin, using aquo-acetonitrile gradients (0.2-1.0% per min at flow rates 0.5-2.0ml/min) containing 0.1% orthophosphoric acid. Under these conditions high sensitivity UV detection at 200nm permits the peptide mapping of 10nanomolar, or smaller, quantities of proteins. Following recovery of the eluted peaks, compositional analyses can be readily carried out at a later stage. Similar elution conditions based on this simple phosphate system have subsequently been applied to the separation of the tryptic peptides of haemoglobin variants [16,17,54], the enzyme phosphofructokinase [17], human and sheep thyroglobulins [15,33], pituitary protein hormones [15,34,35], ribonuclease S peptide [20], peptidic prohormones [1,17], bovine and chick intestinal calcium-binding proteins [36] and immunoglobulin G and M heavy chains [35,37]. Although UV detection of the peptides can be routinely carried out in the range 200-220nm with this elution system, monitoring at 254 or 280nm also provides useful information regarding the location of aromatic residues. Typical of the excellent resolution which can be achieved with the 0.1% orthophosphate systems is the acetonitrile gradient elution profile for the tryptic digest of human growth hormone (Fig.1) where the separation of the digest peptides (maximum of 21 expected) is achieved in less than 45min.

Related methods have been applied to the separation of radiolabelled tryptic peptides from a number of ^{3}H-, ^{14}C- or ^{125}I-labelled proteins, includihg rat caseins [38] (Fig.2),

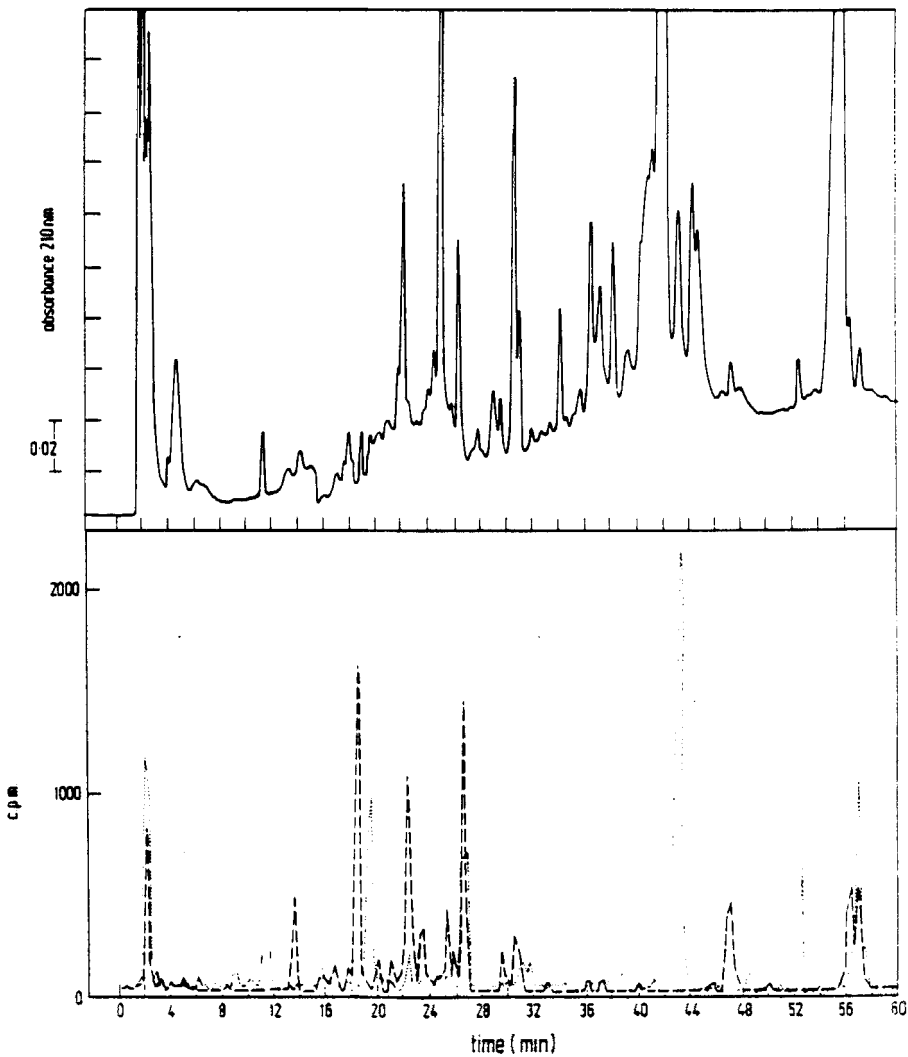


Figure 2. Gradient elution profiles for the tryptic maps of rat casein type I, native (-) specifically labelled with ^{3}H-Met(- - -) and specifically labelled with ^{3}H-Leu (• • •). The chromatographic conditions were: column, μBondapak alkylphenyl (30 x 0.4cm); flow rate 2.0ml/min; temperature 20^{0}; mobile phase, 60-min linear gradient from water-0.1% H_3PO_4 to 50% acetonitrile-50% water-0.1% H_3PO_4. In [38].

thyroglobulins [33] and influenza-related glycoproteins [39]. McMillan et al. [40] have employed this phosphate approach with an elution gradient of acetone for the separation of the tryptic peptides of the α- and β- polypeptides of Ia antigens from the I-E subregion on a Zorbax-CN column. Rivier has reported [18] the use of the trialkylammonium phosphate buffers for the separation of peptides and applied the triethylammonium phosphate system to the analysis of the tryptic digest of myelin basic protein (Fig.3).

Because of their volatility, acetate, formate and trifluoroacetate, usually as the ammonium or pyridinium salts,

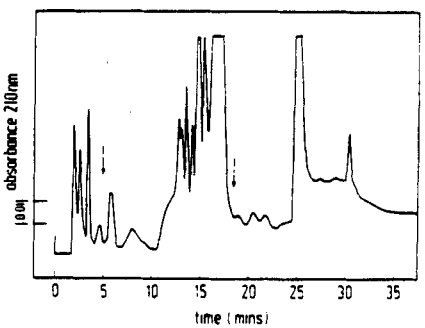
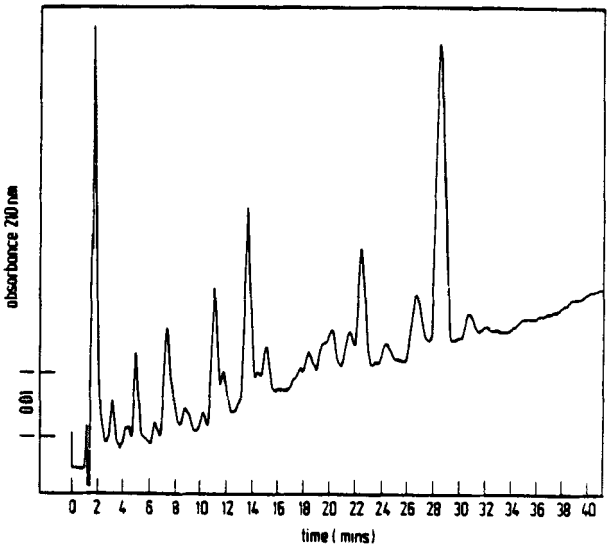
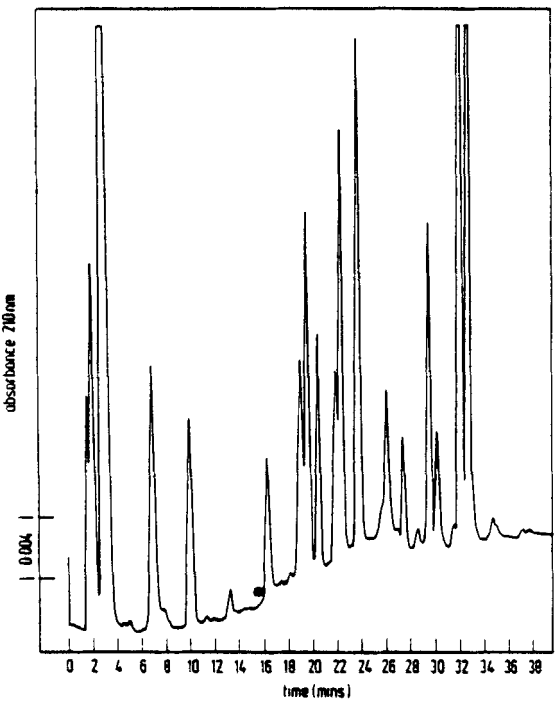


Figure 3. Gradient elution profile for the tryptic digest of myelin basic protein. Column, μBondapak C_{18} column (3 x 0.4cm); flow rate, 1.5ml/min; elution conditions, isocratic elution for 5 min using 3% acetonitrile-100% 1.25N H_3PO_4, adjusted to pH 3.0 with triethylamine (TEAP buffer) then 5 min linear gradient to 18% acetonitrile followed by 8 min isocratic elution and finally by a 5 min linear gradient to 48% acetonitrile; detection, 210nm, 0.1 AUFS, sample size 50μg. Gradient changes indicated by arrows. In [18].





have also found application in the separation of tryptic or CNBr peptides by reversed phase HPLC. Compared to the phosphate-mediated systems, these anions may give poorer peak shapes and reduced resolution but these limitations must be weighed against the advantage of direct recovery of the peptides by lyophilisation of the eluant. Again gradient elution with an organic solvent modifier is usually required.

The direct use of low concentrations (0.1-5%) of the organic acids with acetonitrile gradients has been reported. For example, the tryptic peptides of lysozyme can be resolved on a μBondapak C_{18} column using 0.1% acetic acid and a 0.5%/min acetonitrile gradient [37]. Similar methods have been employed [41] to separate the tryptic peptides of doubly (^{3}H- and ^{14}C-) labelled α and β subunits of murine I-A alloantigens. The CNBr-peptides of avian virus P-27 structural protein have been resolved [37] on a μBondapak C_{18} column using 0.1% acetic

Figure 4. (a) Gradient elution profile of the tryptic digest of the β-chain of HβA; column μBondapak C_{18}; flow rate, 2.0ml/min; temperature, 20°; elution conditions, a 60min linear gradient was generated from 10mM NaOAc-HOAc pH 6.5 to 50% acetonitrile-10mM NaOAc-HOAc pH 6.5; detection 230nm, 0.1 AUFS, sample size 120μg/45μl.

(b) Gradient elution profile of tryptic digest of the β-chain of HβA. Column, μBondapak alkylphenyl; flow rate, 2.0ml/min; temperature, 20°, elution conditions, a 60min linear gradient was generated from water-0.1% H_3PO_4 to 50% acetonitrile-50% water-0.1% H_3PO_4; detection 210nm, 0.4 AUFS, sample size 810μg/100μl.

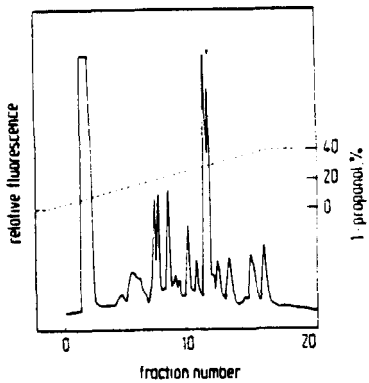


Figure 5. Trypsin treated camel pituitary pro-opiocortin chromatographed on a LiChrosorb RP18 column (25 x 0.46cm), flow rate 15ml/hr; temperature 25°; mobile phase, 0.5M formic acid-0.35M pyridine, pH 4.1 and a linear gradient of 1-propanol from 0 to 20% for 120 min. The elution position of the β-lipotropin (61-89) fragment is noted. In [33].

acid isocratic elution. The CNBr-peptides of the C-1 chymotryptic fragment of bacteriorhodopsin have been separated [34] on a μBondapak C_{18} using 5% formic acid and gradient elution from 40-80% ethanol. A similar elution system has been used [37] for the CNBr peptides of the influenza virus M protein.

Notable examples of the application of ammonium acetate buffers, generally 10mM in the pH range 2.0-6.5, with methanol, acetonitrile or propanol gradient elution, include the separation of the tryptic peptides from isolated normal and variant human haemoglobin chains [43,53], the tryptic peptides from apohaemoglobin E [44], the tryptic peptides of $^{3}_{H}$-endorphin [45], the CNBr peptides of vasoactive intestinal polypeptide and human pancreatic polypeptide [45], the CNBr cleavage products from the β-galactosidase-insulin A or B

chain hydrid proteins [46], the CNBr peptides of the A-γ and G-γ chains of human haemoglobin F [47]. Illustrative of the use of these acetate systems (and in comparison with the corresponding phosphate elution conditions) are the chromatograms, shown in Figure 4, of the tryptic peptides of the isolated normal Hb β-chain.

Ammonium formate and trifluoroacetate have also found application in this area. For example, isocratic elution with 10mM ammonium formate, pH4.0 and pH6.0, and 20% acetonitrile has been used to resolve the tryptic, chymotryptic and carboxypeptidase A digests of α-melanocyte-stimulating hormone (α-MSH) and N,O-diacetylserine, α-MSH [48]. Similar methods have also been found applicable to the adrenocorticotropic family [35].

As indicated above, post column fluorometric derivatisation and detection can be used to improve the sensitivity levels. Fluorescence monitoring of enzymatic or CNBr digests also permits the use of UV opaque buffers such as pyridine acetate or formate and avoids the base line changes often experienced with UV detection at low wavelengths during gradient elution. Generally buffers of higher molarity have been used under these conditions. Most of the tryptic peptides of ovalbumin can be resolved [24] on a LiChrosorb RP-18 column with a 1M pyridine-0.5M acetic acid buffer and a linear gradient of 0-20% n-propanol in the same buffer. Similar methods have been used to confirm the identity of rat β-endorphin with camel β-endorphin via their common tryptic

peptides [24], to separate the tryptic peptides of myoglobin [49], pro-opiocortin [50] (Fig. 5) and the putative enkaphalin precursors found in bovine adrenal medulla [51], as well as to resolve the CNBr peptides of the MOPC-315 mouse immunoglobulin heavy chain [52].

Conclusions

The advantages of short elution times, excellent resolution with sample loadings in the pico- to micro- molar range and high recoveries are the hallmarks of reversed phase HPLC separations of peptide mixtures. The examples discussed in this paper indicate that these techniques permit the reliable and sensitive mapping of polypeptides and proteins. In those cases where a large number of cleavage products are expected, eg. h-thyroglobulin has ca 470 possible tryptic peptides, a primary separation by a conventional gel fractionation technique may be required. A further benefit of these methods is the ease of sample preparation and, in many cases, direct recovery of the eluted peptides without the need for desalting or removal of buffer components. The problem of overlapping peptides in an otherwise straight forward analytical or preparative application, can usually be remedied in one of a number of simple ways, eg. by rechromatographing the collected coverlapping peaks under conditions which take advantage of pH-dependent resolution optima or changes in the polarity of the added counterion. Finally there is considerable scope in these reversed phase HPLC methods for automation which should ultimately permit the direct, on-line structural mapping of polypeptides and proteins via dedicated compositional analysers.

Acknowledgements

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