CHREV 190

WEAK ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES

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CONTENTS

1. INTRODUCTION

During the past decade, high-performance liquid chromatography (HPLC) has become a powerful tool for peptide separations. The reversed-phase mode of HPLC (RP-HPLC) has been the most popular and broadly used technique for this purpose (for reviews, see refs. 1 and 2). Ion-exchange HPLC has also been employed for peptide separations, although to a lesser extent (for reviews, see refs. 3 and 4).

Recently, a novel method was introduced for the separation of underivatized dipeptides, including resolution of sequence isomeric and diastereomeric dipeptides by weak anion-exchange HPLC (WAE-HPLC)[5]. For this purpose, a commercially available column, *i.e.* MicroPak AX-10 (Varian, Walnut Creek, CA, U.S.A.), was used. The MicroPak AX-10 is a weak anion-exchange bonded stationary phase carrying the functional group $-CH_2-CH_2-CH_2-\overset{+}{N}H_2-CH_2-CH_2-\overset{+}{N}H_3$, which is covalently bonded to fully porous silica (LiChrosorb Si-60). This stationary phase with an exchange capacity of 2 mequiv./g (5 mequiv. per column of the size 30 × 0.4 cm) had been introduced previously for simultaneous analysis of nucleobases, nucleosides and

nucleotides[6], and is used for the separation and sequencing of sequence isomeric deoxypentanucleotides[7].

Peptides are usually not retained well on the weak anion-exchanger using aqueous buffers as mobile phases. However, addition of an organic modifier such as acetonitrile to the buffer increases peptide retention. Mixtures of triethylammonium acetate (TEAA) buffer and acetonitrile were used as the eluent for separation of dipeptides[4]. This mobile phase has minimal absorbance at wavelengths in the range of 210 to 225 nm allowing gradient elution and sensitive detection of peptides. More-over, the TEAA buffer is volatile and thus facilitates the easy recovery of eluted peptides for further use and study. Gradient analysis is carried out by increasing the amount of the buffer in the eluent.

The purpose of this paper is to review the applications of this method to peptide separations.

2 SEPARATION OF DIPEPTIDES

Efficient separation of dipeptide mixtures is required in the sequence analysis of peptides using dipeptidyl peptidases, in which enzymatic digestions coupled with the Edman degradation yield overlapping sets of dipeptides[8]. The identification of the released dipeptides is used to reconstruct the sequence of the peptides. Dipeptides have been separated by a variety of techniques, including cation-exchange HPLC[9], and RP-HPLC[10,11]

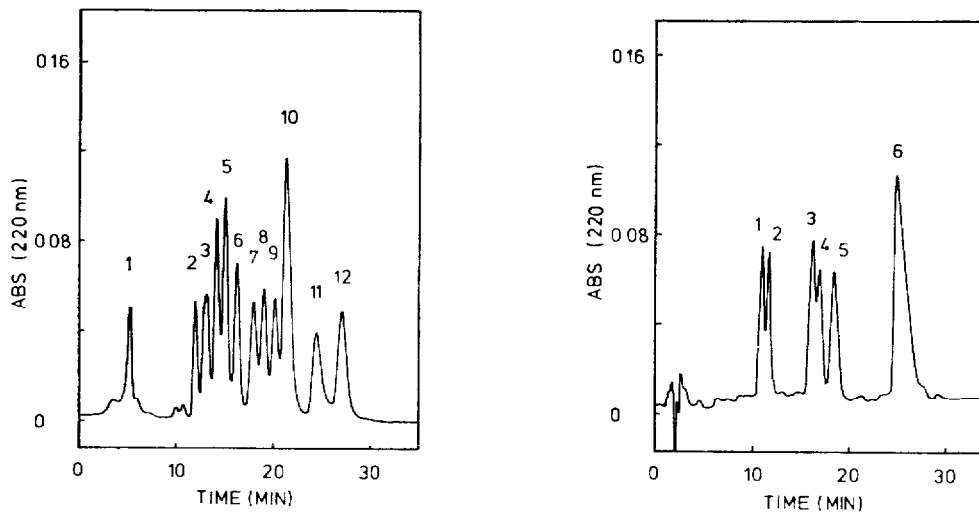Fig. 1 shows the separation by WAE-HPLC of a mixture of sixteen selected

Fig 1. Separation of some selected dipeptides Column, MicroPak AX-10 (10 μm), 30 × 0 4 cm Temperature, 40°C Eluent, 0 01 M TEAA (pH 4 3)–acetonitrile (32.68) Flow-rate, 1 ml/min Peaks 1 = L-Arg-L-Phe, 2 = L-Leu-L-Leu; 3 = Gly L-Ile and L-Leu L-Trp, 4 = L-Ala–L-Ile; 5 = L-Trp Gly, 6 = L-Trp-L-Phe, 7 = L-Val L-Val and L-Ala–L-His, 8 = L-Trp-L-Ala, 9 = L-Ala–L-Thr and L-Met–L-Met; 10 = Gly–Gly and L-Phe L-Phe, 11 = L-Ser-L-Phe, 12 = L-Tyr–L-Tyr

Fig. 2 Separation of some dipeptides containing acidic amino acids Column as in Fig 1 Temperature, 40°C. Eluent, 0 01 M TEAA (pH 3.1)–acetonitrile (60 40). Flow-rate, 1.5 ml/min Peaks 1 = L-Ala-L-Glu, 2 = Gly–L-Glu, 3 = α-L-Glu–L-Ala; 4 = L-Ala–L-Asp, 5 = Gly–L-Asp, 6 = γ-L-Glu–L-Leu

dipeptides[5]. The absorption profile represents a resolution of twelve peaks. The conditions used for the separation shown in Fig 1 do not elute acidic dipeptides containing aspartate and glutamate residues; these species can be eluted by reducing the pH of the aqueous buffer as shown in Fig. 2.

2.1. Sequence isomeric dipeptides

Resolution of sequence isomeric dipeptides is important in peptide sequencing by dipeptidyl peptidases. An isocratic separation of a six-pair mixture of isomeric dipeptides by WAE-HPLC is shown in Fig. 3, another six-pair mixture required use of a gradient to elute all components in reasonable time (Fig 4). Except DL-Leu–DL-Ala, and DL-Ala–DL-Ser, the dipeptides in these mixtures were in L,L-configuration. DL-Leu–DL-Ala and DL-Ala–DL-Ser each gave two peaks (peaks 1 and 4 in Fig. 3 and peaks 6 and 9 in Fig 4, respectively). In each case, the peak with the longer retention time most probably represents the L,L-form as discussed below in separation of diastereomers.

The elution order of a given pair of sequence isomeric dipeptides appears to obey certain rules. The dipeptide with the most hydrophobic residue as the C-terminal amino acid elutes first. For dipeptides containing only aliphatic amino acids, the dipeptide having a shorter side chain in the N-terminal amino acids elutes first. These rules, of course, cannot be generalized without examining all possible pairs of sequence isomeric dipeptides.
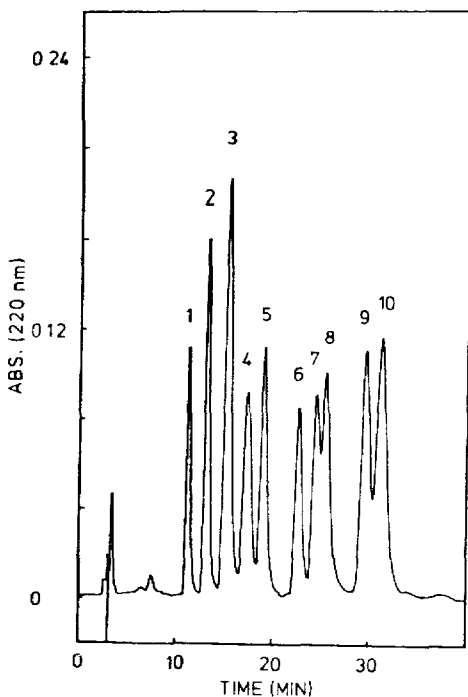

Fig 3 Separation of sequence isomeric dipeptides Column details in Fig 1 Peaks 1 = DL-Leu–DL-Ala, 2 = Gly–L-Phe, 3 = L-Ala–L-Leu, Gly–L-Met and L-Ala L-Phe, 4 = Gly–L-Try and DL-Leu–DL-Ala, 5 = L-Ala–L-Tyr, 6 = L-Phe–Gly; 7 = L-Tyr–Gly, 8 = L-Met–Gly, 9 = L-Phe–L-Ala, 10 = L-Tyr–L-Ala
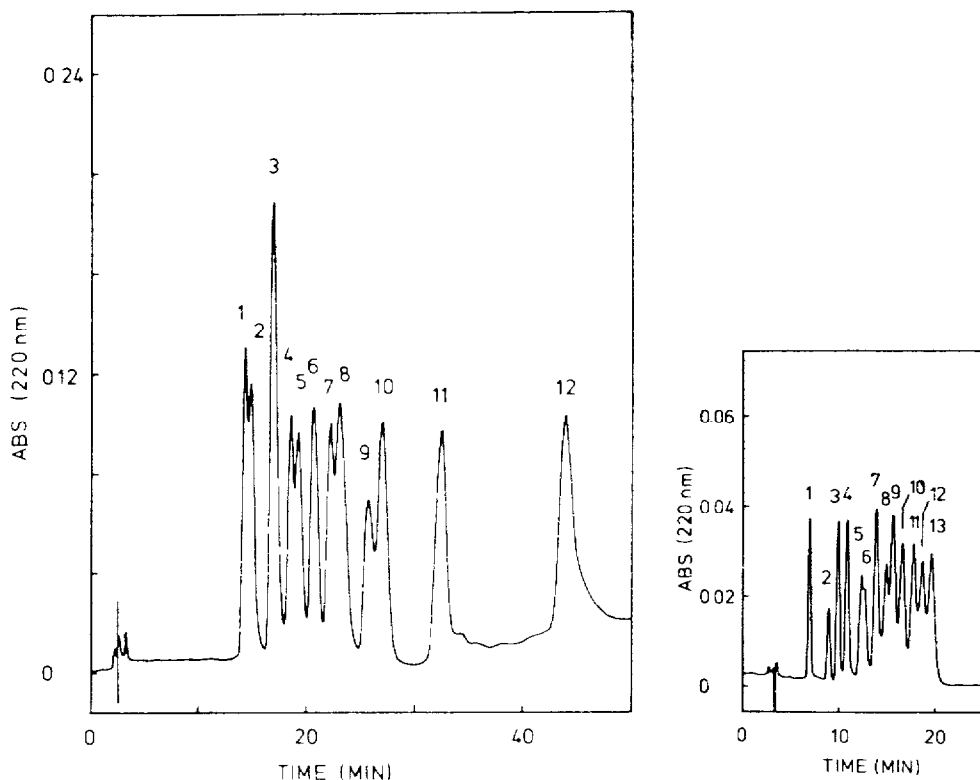
Fig 4. Separation of sequence isomeric dipeptides Column as in Fig. 1. Temperature, 50°C Eluent A, acetonitrile, B, 0.01 M TEAA (pH 4 3), gradient program step 1, isocratic with 70% A and 30% B for 25 min, step 2, linear gradient of 0 4% B per min. Flow-rate, 80 ml/h. Peaks 1 = Gly–L-Leu, 2 = L-Leu–Gly, 3 = L-Ala–L-Met and Gly-L–Val, 4 = L-Val–L-Phe, 5 = L-Val–Gly; 6 = DL-Ala–DL-Ser; 7 = L-Ala–Gly, 8 = Gly–L-Ala, 9 = DL-Ala–DL-Ser, 10 = L-Phe–L-Val, 11 = L-Met–L-Ala, 12 = L-Ser–L-Ala

Fig 5 Separation of diastereomeric dipeptides. Column as in Fig 1. Temperature 45°C. Eluent, 0 01 M TEAA (pH 4.3)–acetonitrile (35.65) Flow-rate, 1 ml/min. Peaks: 1 = DL-Leu–DL-Phe, 2 = DL-Ala–DL-Phe; 3 = DL-Leu–DL-Ala; 4 = DL-Ala–DL-Val, 5 = DL-Leu–DL-Phe; 6 = L-Ala–L-Phe, 7 = DL-Ala–DL-Ala and DL-Ala–DL-Val, 8 = DL-Leu–DL-Ala; 9 = DL-Ala–DL-Ser, 10 = DL-Ala–DL-Asn; 11 = DL-Ala–DL-Ala, 12 = DL-Ala–DL-Ser; 13 = DL-Ala–DL-Asn

2.2. Diastereomeric dipeptides

A number of DL,DL-peptides were shown to be separable by WAE-HPLC into their diastereomers[5] as Fig. 5 illustrates. All DL,DL-dipeptides examined except DL-Ala–DL-Phe were resolved into two peaks. The DL-Ala–DL-Phe mixture apparently does not contain the L,L-configuration because L-Ala–L-Phe shows a longer retention time (peak 6 in Fig. 5) than DL-Ala–DL-Phe (peak 2 in Fig. 5). Since individual standards of all four diastereomers were not available for these dipeptides, peak assignments were based on the elution order of the available four Ala–Ala diastereomers. In this case, the D,L- and L,D-isomers elute early as a single peak (peak 7, Fig. 5), while the D,D- and L,L-isomers coelute later (peak 11, Fig. 5). This elution order of diastereomeric dipeptides is the same found for these compounds on a cation-exchanger[11]; but the opposite of that observed in RP-HPLC[12].
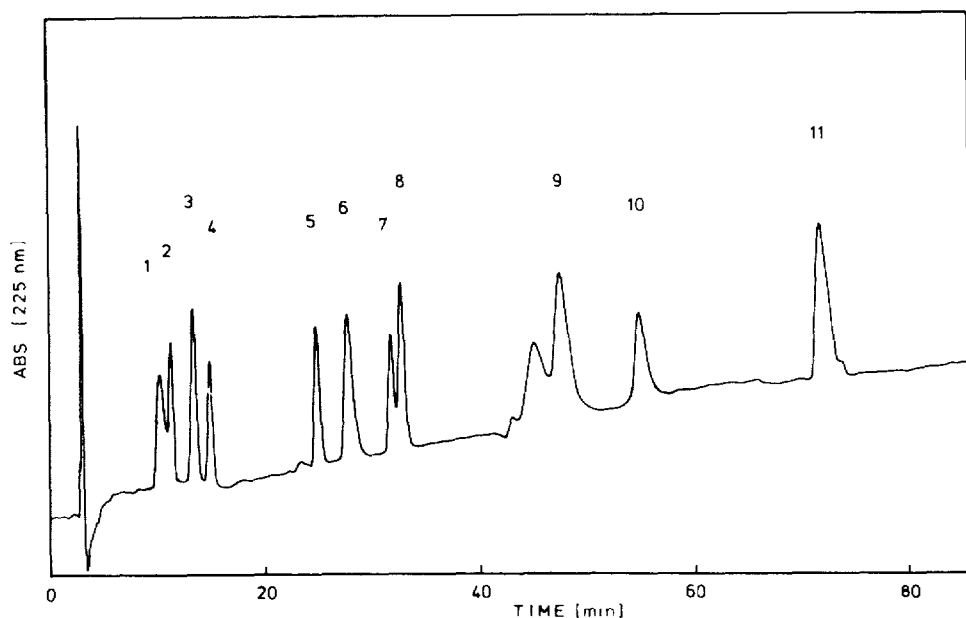
Fig. 6 Separation of various peptides Column as in Fig. 1 Temperature, 30°C. Eluent A, acetonitrile, B, 0.01 M TEAA (pH 6 0), gradient program. linear starting from 25% B at a rate of 1% B per min Flow-rate, 1 ml/min Peaks 1, somatostatin, 2, proctolin; 3, neurotensin, 4, Met-enkaphalin, 5, bradykinin potentiator c, 6, Lys–Glu–Thr–Tyr–Ser–Lys, 7, α-endorphin, 8, EAE-peptide, 9, glucagon, 10, ribonuclease s-peptide; 11, IgE-peptide

3 SEPARATION OF PEPTIDES

The WAE-HPLC method has recently been applied to the separation of longer peptides with a slight change in the pH of the TEAA buffer[13]. The separation by this method of a multicomponent peptide mixture is shown in Fig. 6. The peak identification is given in the legend of Fig. 6. A good peak symmetry was obtained for all peptides in this mixture The amino acid analysis of eluted peptides confirmed the authenticity of peaks in Fig. 6 (for sequences, see ref. 13).

3.1. Acidic peptides

Peptides containing a number of acidic amino acid residues with no compensating basic residues either did not elute from the column or had unacceptably long retention times when the elution conditions described in Fig. 6 were used. Such acidic peptides were chromatographed using an isocratic flow of dilute formic acid instead as the eluent[13], as is shown in Fig. 7. Both ribonuclease s-peptide (RSP) and IgE-peptide (peaks 1 and 2, respectively) could also be eluted with the solvent system described in Fig. 6. Although RSP contains three acidic amino acid residues (for sequences of peptides in Fig. 7, see ref. 13), it elutes much earlier than the other peptides in this mixture. This might be due to the compensation of acidic residues by the basic amino acids. Dilute formic acid solutions used as eluent for these separations are compatible with the column and pumps used and also volatile, allowing
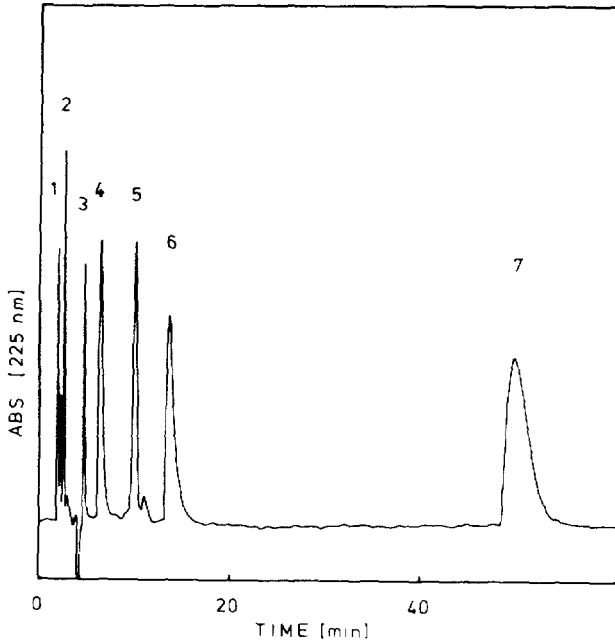
Fig 7 Separation of some acidic peptides Column as in Fig 1 Temperature, 60°C Eluent, 0 04 *M* formic acid (pH 2 6) Flow-rate, 1 ml/min Peaks 1, ribonuclease s-peptide, 2, IgE-peptide, 3, glutathionie (oxidized form), 4, Phe–Leu–Glu–Glu–Ile, 5, delta sleep-inducing peptide, 6, γ-Glu–Leu, 7, γ-Glu–Glu.
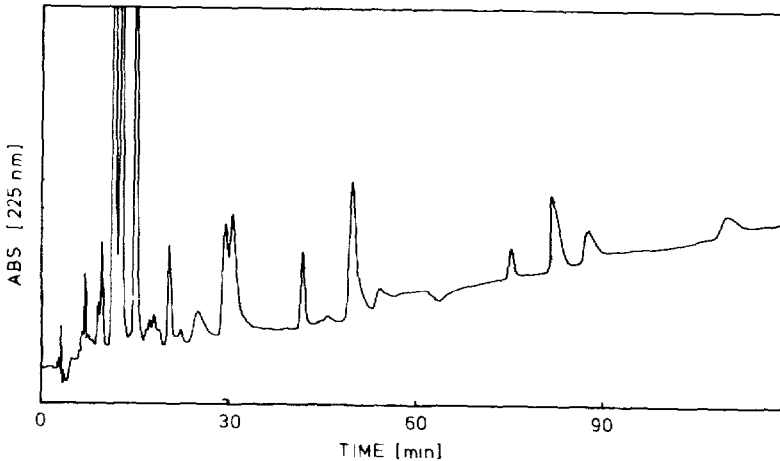

Fig 8 Separation of a tryptic digest of horse heart cytochrome *c* Column details as in Fig 6 except gradient program linear starting from 25% B at a rate of 0 6% B per min to 50% B, then 4 5% B per min to 100% B Amount of injection, *ca.* 10 nmol of cytochrome *c*.

easy recovery of eluted peptides. As with the TEAA buffer, peptide recoveries were found to be 80% or greater[13].

3.2. Enzymatic digests of peptides

Enzymatic digestion is frequently employed in sequence analysis of peptides and proteins. For this reason, the WAE-HPLC method was also applied to separation of peptides resulting from tryptic digestion of some proteins[13]. Fig. 8 shows the separation of a tryptic digest of horse heart cytochrome c. The elution became isocratic at 100% buffer after 60 min, and another four peaks were observed. The number of the peaks detected in Fig. 8 corresponds closely to the number of fragments expected from the known sequence of horse heart cytochrome c upon trypsin digestion[14]. Assignment of the peptide fragments in Fig. 8 was, however, not carried out. Since some digestion products contain a number of acidic amino acid residues, the separation of the digestion mixture was also undertaken using the conditions given in Fig. 7. As Fig 9 shows, the majority of the fragments had no or little retention using formic acid as eluent, however, four other peptides with significant retention were observed.

Fig. 10 shows the separation of a tryptic digest of reduced and alkylated lysozyme[13]. Again, the number of major peaks detected corresponded closely to the number of fragments expected from tryptic digestion of lysozyme[15]. In this case, no additional peptide fragments were observed when formic acid was used for elution.
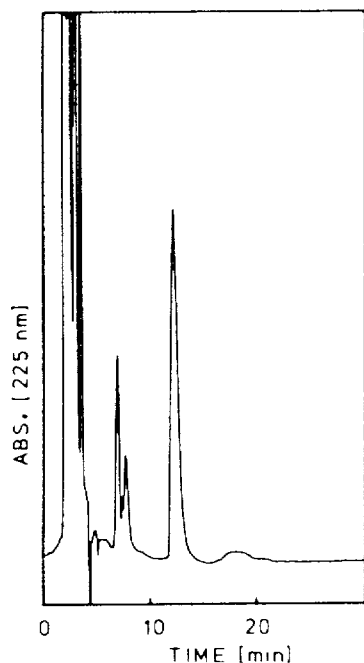

Fig. 9 Separation of a tryptic digest of horse heart cytochrome c Column details as in Fig 7. Amount of injection ca 10 nmol of cytochrome c
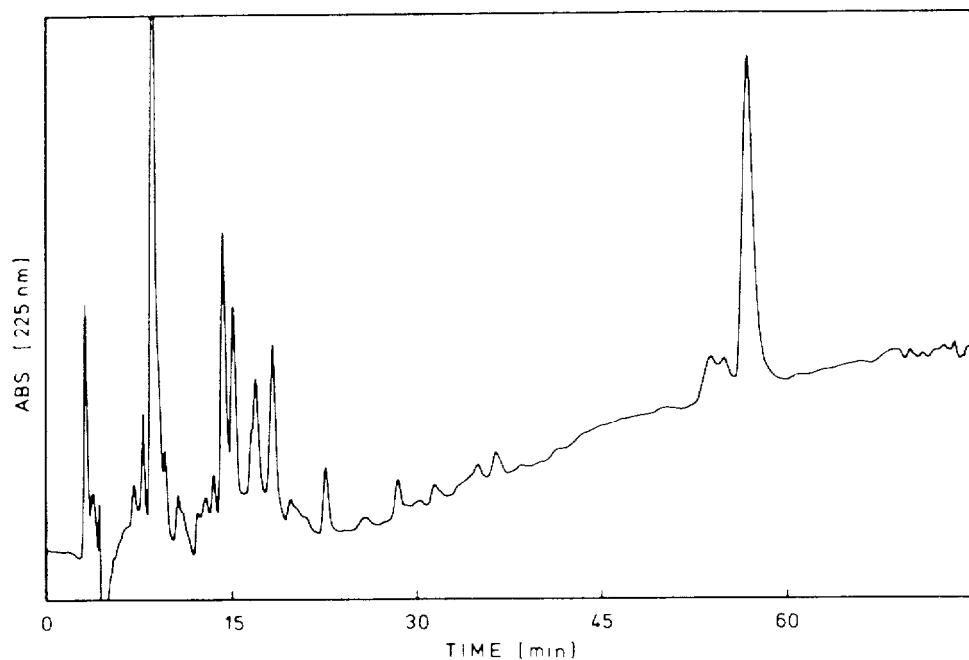
Fig 10. Separation of a tryptic digest of reduced and alkylated lysozyme Column details as in Fig 6
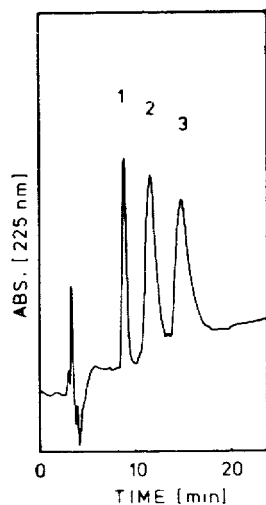except temperature 40°C Amount of injection *ca.* 10 nmol of lysozyme

Fig 11 Separation of several bradykinins. Column details as in Fig 6 except gradient program: linear
starting from 25% B at a rate of 1.7% B/min Amount of injection, *ca.* 2 µg per peptide Peaks: 1,
bradykinin, 2, Met,Lys-bradykinin, 3, Lys-bradykinin

4 SEPARATION OF CLOSELY RELATED PEPTIDES

The WAE-HPLC method was also shown to be useful in separating closely related peptides. Some applications are discussed below.

4.1. Bradykinins

Separation of several bradykinins[13] is shown in Fig. 11. Bradykinin gave a symmetrical peak (peak 1), whereas Met,Lys-bradykinin and Lys-bradykinin (peaks 2 and 3, respectively) yielded somewhat broad peaks (for sequences, see ref. 13).

4.2. Angiotensins

Angiotensins (A) are hormones with important biological activities[16,17]. These compounds are closely related peptides and differ from one another in most cases by only one amino acid residue. The WAE-HPLC method was successfully applied to separation of angiotensin[18]. Fig. 12 shows the separation of a mixture of twelve angiotensins by gradient elution Peak identification is given in the legend of Fig. 12. Recoveries of 90 to 98% for all A's tested have been obtained[18]. Two A's (peak 6) were not resolved at that temperature. They could, however, be separated by elevation of column temperature as discussed below
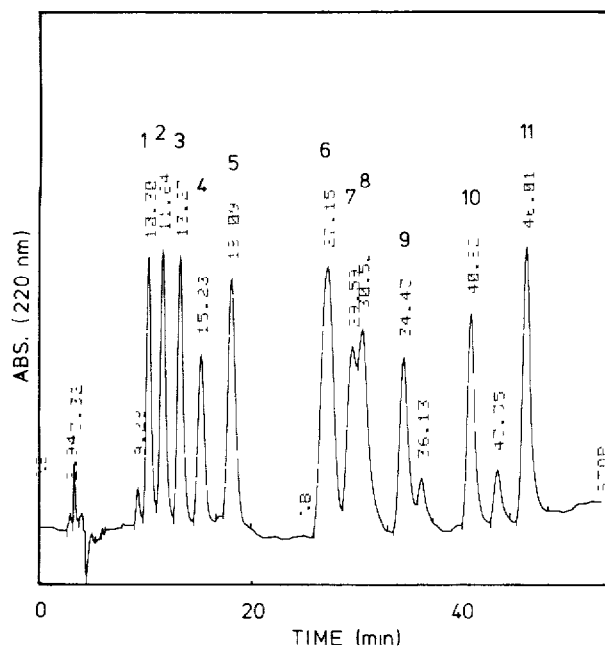

Fig. 12 Separation of angiotensins Column details as in Fig. 6 except gradient elution starting from 24% B at a rate of 0.1% B/min for 25 min, then 0 5% B/min and column temperature 26°C, amount of injection per peptide, approximately 1 μg (1 nmol based on A II). Peaks: 1, A III; 2, (Val⁴)–A III; 3, A III inhibitor; 4, (Asn¹–Val⁵)–A II, 5, (Sar¹–Ile⁸)–A II; 6, (Sar¹–Ala⁸)–A II, (Sar¹–Gly⁸)–A II, 7, (Sar¹–Thr⁸)–A II; 8, (Sar¹–Val⁵–Ala⁸)–A II, 9, A II, 10, A I, 11, (Val⁵)–A II
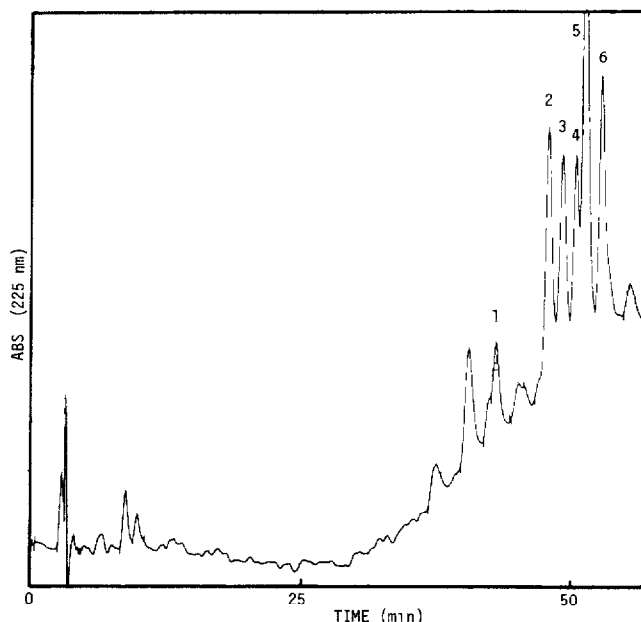
Fig 13 Separation of some diastereomers of angiotensin I. Column details as in Fig 6 except temperature. 50°C, and gradient program linear starting from 24% B at a rate of 0.08% B/min for 25 min, then 0 25% B/min. Peaks 1, (D-*allo*-Ile[5])-A I; 2, (D-Leu[10])-A I; 3, (D-Phe[8])-A I, 4, (D-Pro[7])-A I, 5, A I, 6, (D-Asp[1]-Ile[8])-A I

4.3. Diastereomers of angiotensin I

A number of diastereomers of angiotensin I (AI) were separated by WAE-HPLC[19], as Fig. 13 illustrates. Peak identification is given in the legend of Fig. 13. In that work, separation of individual diastereomers of AI from their impurities was also demonstrated. As an example, separation of (D-Phe[8])-AI from its impurities is shown in Fig. 14. Peak 5 represents (D-Phe[8])-AI and other peaks corresponds to impurities characterized by fractional additions of combinations of Asp, Tyr or Arg, fractional deletions of His or reduced amounts of Ile, Val and Tyr (for more details see ref. 19).

4.4. Analogues and diastereomers of neurotensin

Neurotensin (NT) is a recently discovered peptide hormone which exhibits a broad spectrum of biological activity[20]. A large number of fragments, diastereomers and analogs of NT have recently been synthesized and tested for their biological activity[21].

Separation of a number of diastereomers and analogues of NT was achieved by WAE-HPLC[22], as shown in Figs. 15 and 16, respectively. Peak identification is given in the legend of the figures. Excellent recoveries between 90 and 98% of NT's tested were obtained[22]. Except for (D-Arg[9])-NT (peak 5 in Fig. 15), all diastereomers were completely separated from NT (peak 6 in Fig. 15). Also remarkable is the excellent resolution of (Phe[11])-NT (peak 4) from (D-Phe[11])-NT (peak 1). Five analogues of NT were also completely resolved from NT (peak 3 in Fig. 16).
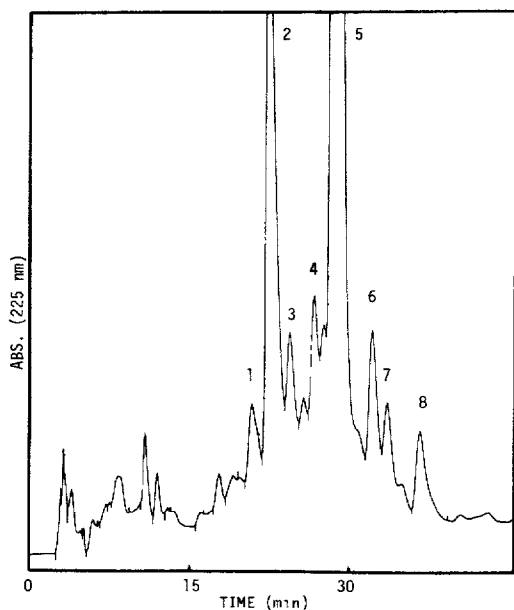
Fig. 14. Separation of (D-Phe[8])–A I from its impurities Column details as in Fig. 6 except temperature: 30°C, and gradient program starting from 25% B at a rate of 0 5% B/min Peak 5 represents (D-Phe[8])–A I. For other details, see ref 19
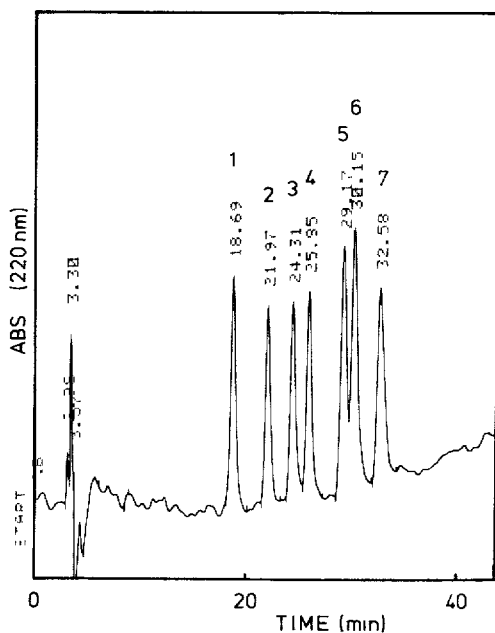
Fig. 15 Separation of some diastereomers of neurotensin Column details as in Fig 6 except temperature: 50°C, and gradient program linear starting from 23% B at a rate of 0.3% B/min. Amount of injection per peptide, ca 1 nmol Peaks 1, (D-Phe[11])–NT, 2, (D-Tyr[11])–NT, 3, (D-Pro[10])–NT, 4, (Phe[11])–NT, 5, (D-Arg[9])–NT, 6, NT, 7, (D-Glu[4])–NT
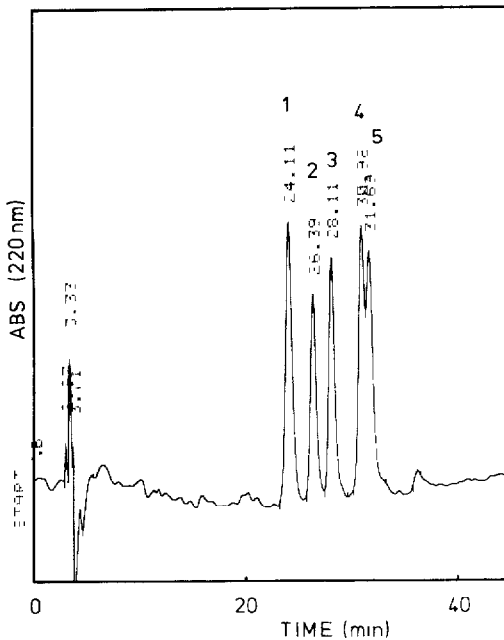
Fig 16. Separation of some analogs of neurotensin Column details as in Fig 15 except temperature 40°C Peaks 1, (Phe[11])–NT, (Trp[11])–NT, 2, (Leu[11])–NT; 3, NT, 4, (Lys[8])–NT; 5, (Lys[9])–NT

5 EFFECT OF pH ON RETENTION

Separation of dipeptides by WAE-HPLC was carried out at pH 4.3 of the TEAA buffer[5]. For larger peptides, however, a pH value of 6.0 was found more suitable in terms of retention, peak symmetry and resolution[13]. The separation selectivity of the stationary phase used thus shows a quite strong dependence on the pH value of the buffer. For this reason, pH values between 4 and 6 should be explored to improve separation by WAE-HPLC of a given mixture of peptides.

The retention of acidic peptides depends even more strongly on the pH value of the eluent, as Fig. 17 clearly shows. A strong dependence of k' values of delta sleep-inducing peptide on pH above *ca.* 2.9 was observed. To keep a constant solvent strength, a 0.04 M formic acid solution was used and pH of the eluent was increased by adding triethylamine.

6. EFFECT OF TEMPERATURE

A strong effect of column temperature on retention and resolution of peptides separated by WAE-HPLC was observed As an example, the dependence of retention times of angiotensins on temperature is given in Table 1 (ref. 18). An increase in column temperature from 26 to 50°C differently affected retention times of individual peptides. For instance, retention times of three A IIIs, (Asn[1]–Val[5])–A II, A II, A I, and (Val[5])–A II were increased by an increase in temperature. On the other hand, the increase of temperature caused a decrease in retention times of all five A's con-
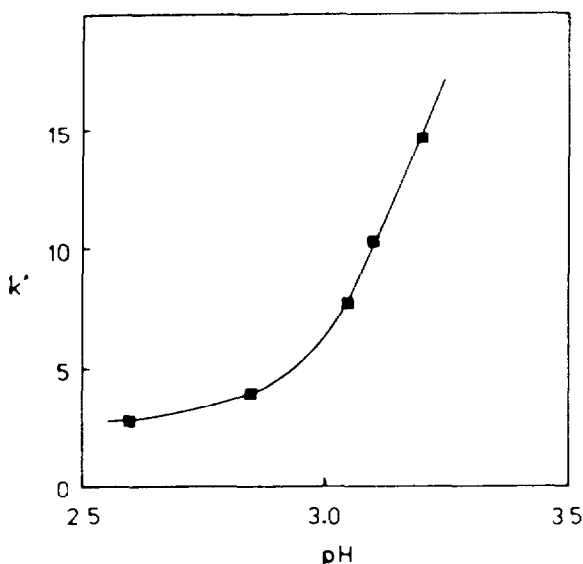
Fig. 17 Dependence of the k' value of DSIP (peptide No 5 in Fig. 7) on the pH value of the eluent. Column as in Fig 1 Eluent, 0 04 M formic acid, the pH value was adjusted by adding triethylamine. Flow-rate. 1 5 ml/min Temperature, 60°C

taining sarcosine. Consequently, resolution between the peptides of this particular mixture was greatly affected by a change in column temperature. For example, (Sar¹–Ala⁸)-A II and (Sar¹–Gly⁸)-A II coeluted at 26°C as Fig. 12 shows. But, they could be completely separated from each other at 50°C.

Retention times of analogs and diastereomers of NT were also significantly affected by column temperature as demonstrated in Table 2 (ref. 22). In this case, an

TABLE 1

DEPENDENCE OF RETENTION TIMES OF ANGIOTENSINS ON TEMPERATURE

Other column details as in Fig 12

Angiotensin	Retention time (min) at			
	26°C	30°C	40°C	50°C
A III	10 3	10.5	11 0	11.6
(Val⁴)–A III	11 6	11.8	12 2	12.8
A III inhibitor	13 2	13 3	13 4	13.7
(Asn¹–Val⁵)–A II	15 2	15 6	16 2	17.1
(Sar¹ Ile⁸)–A II	18.1	17 8	17 1	17 1
(Sar¹–Ala⁸)–A II	27.2	26 3	25 0	24 3
(Sar¹–Gly⁸)–A II	27 2	26 3	26.2	26 8
(Sar¹–Thr⁸)–A II	29 6	28 8	27 8	26 8
(Sar¹ Val⁵–Ala⁸)–A II	30 5	29 5	27 8	26 8
A II	34 4	35.5	38 3	40 8
A I	40 8	42.8	45 8	48 0
(Val⁵)–A II	46 0	47.7	49 8	51.1

TABLE 2

DEPENDENCE OF RETENTION TIMES OF NEUROTENSINS ON TEMPERATURE

Other column details as in Fig 15

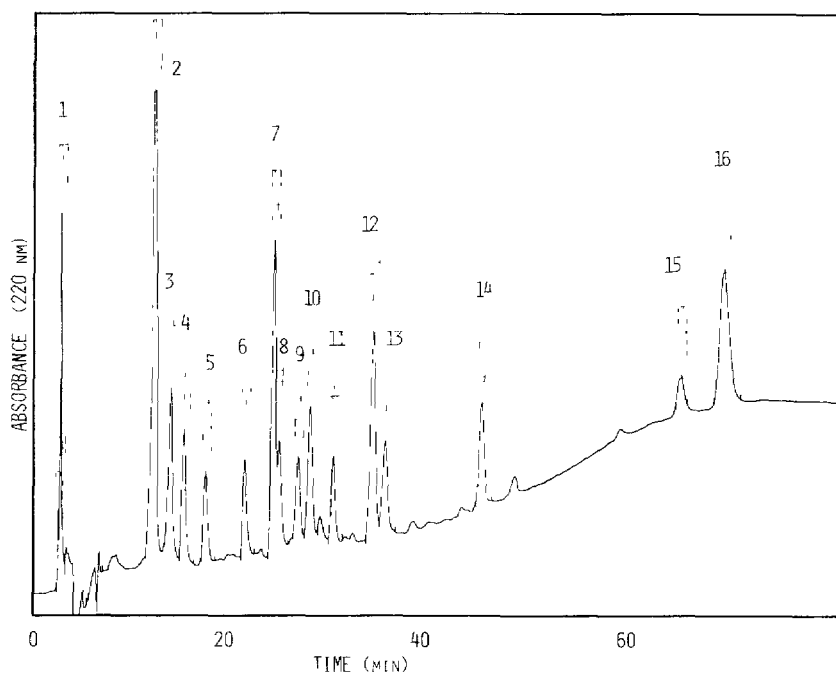| Neurotensin	Retention time (min) at		
	30°C	40°C	50°C
Fig. 15.			
(D-Phe[11])–NT	16.1	17 5	18 7
(D-Tyr[11])–NT	19.2	20 7	22.0
(D-Pro[10])–NT	21 8	23 1	24.3
(Phe[11])–NT	23 3	24 3	25.9
(D-Arg[9])–NT	26 5	27.4	29 2
NT	27 6	28.2	30 2
(D-Glu[4])–NT	29 9	31.2	32 6
Fig. 16			
(Phe[11])–NT	23 4	24 1	25 9
(Trp[11])–NT	23 4	24 1	25 9
(Leu[11])–NT	25.8	26 4	27 6
NT	27.6	28 1	30 0
(Lys[8])–NT	30.3	31 0	32.8
(Lys[9])–NT	30 9	31 7	33.4

Fig. 18 Separation of a tryptic digest of RSMBP by WAE-HPLC Column details as in Fig 6 except temperature 40°C, and gradient program: linear starting from 23% B at a rate of 0 7% B/min for 40 min, then 1% B. Peak identification and sequences are given in Table 3

increase in temperature from 30 to 50°C caused an increase in retention times of all NT's tested.

7. COMPARISON OF RP- AND WAE-HPLC METHODS

RP- and WAE-HPLC methods for peptide separations were recently compared using a tryptic digest of rat small myelin basic protein (RSMBP)[23]. This small protein isolated from rat brain contains 127 amino acid residues[24] and expected to yield twenty-one peptide fragments and two arginine molecules upon digestion with trypsin. The tryptic digest of RSMBP was analyzed by WAE-HPLC using the conditions given in ref. 13 and by RP-HPLC using a solvent system containing 0.1% trifluoroacetic acid (TFA) in water and acetonitrile[25].

Fig. 18 shows the separation by WAE-HPLC, where a resolution of sixteen peaks was observed. Peak identification and sequences are given in Table 3, which indicates that the identified fragments cover the total sequence of RSMBP. Fig. 19 shows the separation by RP-HPLC of another aliquot of the same sample. The amino acid sequences of the peptides and their positions in the total sequence are given in Table 4. By deduction from the known sequence of RSMBP[24], the number of peptides accumulated near the void volume should total nine, because peaks 2–13 in Fig. 19 correspond to the remaining twelve tryptic peptides. The results showed that neither method resolved all of the peptides. All but two peptides were retained on the WAE column and several peaks contained two peptides, whereas some peptides had little or no retention on the RP column. Different selectivities of these two sep-

TABLE 3

PEAK IDENTIFICATION AND SEQUENCES IN FIG. 18

| Peak	Sequence	Position in sequence
1	His–Arg	32, 33
	Gly–Arg	103, 104
	2 Arg	54, 127
2	Phe–Ser–Trp–Gly–Gly–Arg	111–116
3	His–Gly–Phe–Leu–Pro–Arg	26–31
4	Asn–Ile–Val–Thr–Pro–Arg	89–94
5	Gly–Leu–Ser–Leu–Ser–Arg	105–110
6	Ser–Gly–Ser–Pro–Met–Ala–Arg	120–126
7	Arg–Pro–Ser–Gln–Arg	5–9
	Thr–Thr–His–Tyr–Gly–Ser–Leu–Pro–Gln–Lys	64–73
8	Ser–Gln–Arg	74–76
9	Ac–Ala–Ser–Gln–Lys	1–4
	Gly–Ala–Pro–Lys	50–53
10	Thr–Pro–Pro–Pro–Ser–Gln–Gly–Lys	95–102
11	His–Gly–Ser–Lys	10–13
	Gly–Ser–Gly–Lys	55–58
12	Tyr–Leu–Ala–Thr–Ala–Ser–Thr–Met–Asp–His–Ala–Arg	14–25
13	Phe–Phe–Ser–Gly–Asp–Arg	44–49
	Asp–Ser–Arg	117–119
14	Asp–Ser–His–Thr–Arg	59–63
15	Asp–Thr–Gly–Ile–Leu–Asp–Ser–Ile–Gly–Arg	34–43
16	Thr–Gln–Asp–Glu–Asn–Pro–Val–Val–His–Phe–Phe–Lys	77–88
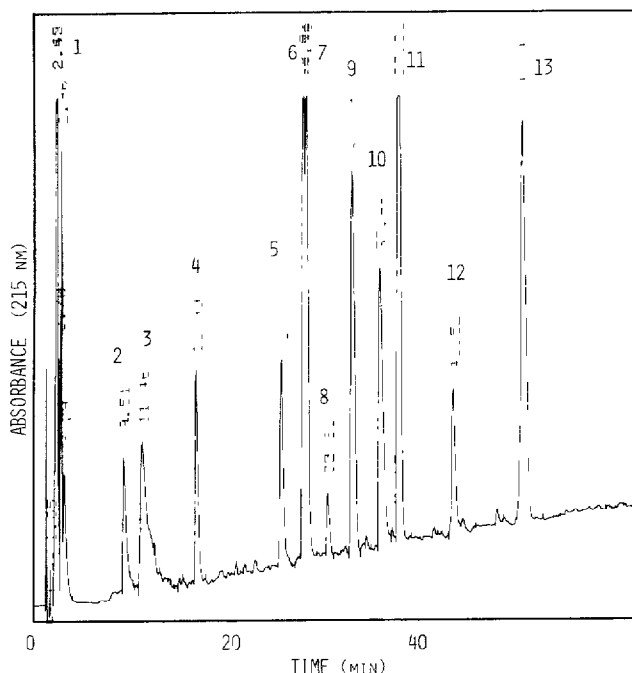
Fig. 19 Separation of a tryptic digest of RSMBP by RP-HPLC Column: Supelcosil LC-8-DB (5 μm), 15 × 0 46 cm Temperature 30°C Eluents A, 0 1% TFA in water, B, 0 1% TFA in acetonitrile Gradient program. linear starting from 0% B at a rate of 0 5% B/min Flow-rate 1 2 ml/min. Peak identification and sequences are given in Table 4

aration principles were demonstrated by collecting the peptides near the void column of the RP column (designated as peak 1 in Fig. 19) and injecting them onto the WAE column A resolution of six peaks corresponding to nine peptides and two arginine's was observed[23]. Except for the two dipeptides, all peptide fragments were retained on the WAE column in contrast to the RP column, and three of them could be obtained in pure form (for details, see ref. 23). In another instance, two peptides, which were slightly resolved on the RP-column (peaks 6 and 7 in Fig. 19), were collected and injected onto the WAE column. Fig. 20 shows the complete separation of these peptides on the WAE column. These examples excellently demonstrate the combined use of RP- and AE-HPLC methods for isolation of peptides, which were not resolved by using only one method. It is important to note that the RP-HPLC has not been exhaustively studied by using solvent systems other than the TFA system and the total capability of this technique is not judged here

In some other instances, as might be expected, RP-HPLC provided better resolution of certain peptides than did the WAE-HPLC methods (for more details, see ref. 23). The results obtained in that work strongly suggested that the combined use of these two methods of separation, which utilize different selectivities, can provide even more resolving power and thus yield a high probability for the complete separation of a given mixture of peptides into its components.

The combined use of RP- and WAE-HPLC was also demonstrated by Lemke

TABLE 4

PEAK IDENTIFICATION AND SEQUENCES IN FIG 19

| Peak	Sequence	Position in sequence
1	Ac–Ala–Ser–Gly–Lys	1–4
	His–Gly–Ser–Lys	10–13
	His–Arg	32–33
	Gly–Ala–Pro–Lys	50–53
	2 Arg	54, 127
	Gly–Ser–Gly–Lys	55–58
	Asp–Ser–His–Thr–Arg	59–63
	Ser–Gln–Arg	74–76
	Gly–Arg	103–104
	Asp–Ser–Arg	117–119
2	Arg–Pro–Ser–Gln–Arg	5–9
3	Thr–Pro–Pro–Pro–Ser–Gln–Gly–Lys	95–102
4	Ser–Gly–Ser–Pro–Met–Ala–Arg	102–126
5	Asn–Ile–Val–Thr–Pro–Arg	89–94
6	Thr–Thr–His–Tyr–Gly–Ser–Leu–Pro–Gln–Lys	64–73
7	Phe–Phe–Ser–Gly–Asp–Arg	44–49
8	Gly–Leu–Ser–Leu–Ser–Arg	105–110
9	Tyr–Leu–Ala–Thr–Ala–Ser–Thr–Met–Asp–His–Ala–Arg	14–25
10	His–Gly–Phe–Leu–Pro–Arg	26–31
11	Phe–Ser–Trp–Gly–Gly–Arg	111–116
12	Asp–Thr–Gly–Ile–Leu–Asp–Ser–Ile–Gly–Arg	34–43
13	Thr–Gln–Asp–Glu–Asn–Pro–Val–Val–His–Phe–Phe–Lys	77–88
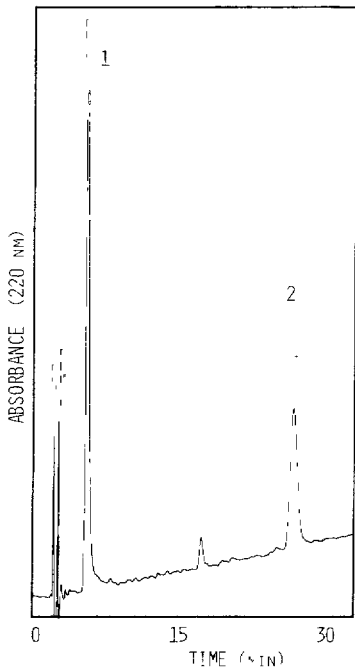
Fig 20 Separation of peptides eluted from RP column (peaks 6 and 7 in Fig. 19) by WAE-HPLC Column details as in Fig 18 except for gradient program. starting from 45% B at a rate of 0 5% B/min Peaks 1 and 2 correspond to peaks 6 and 7 in Fig. 19, respectively
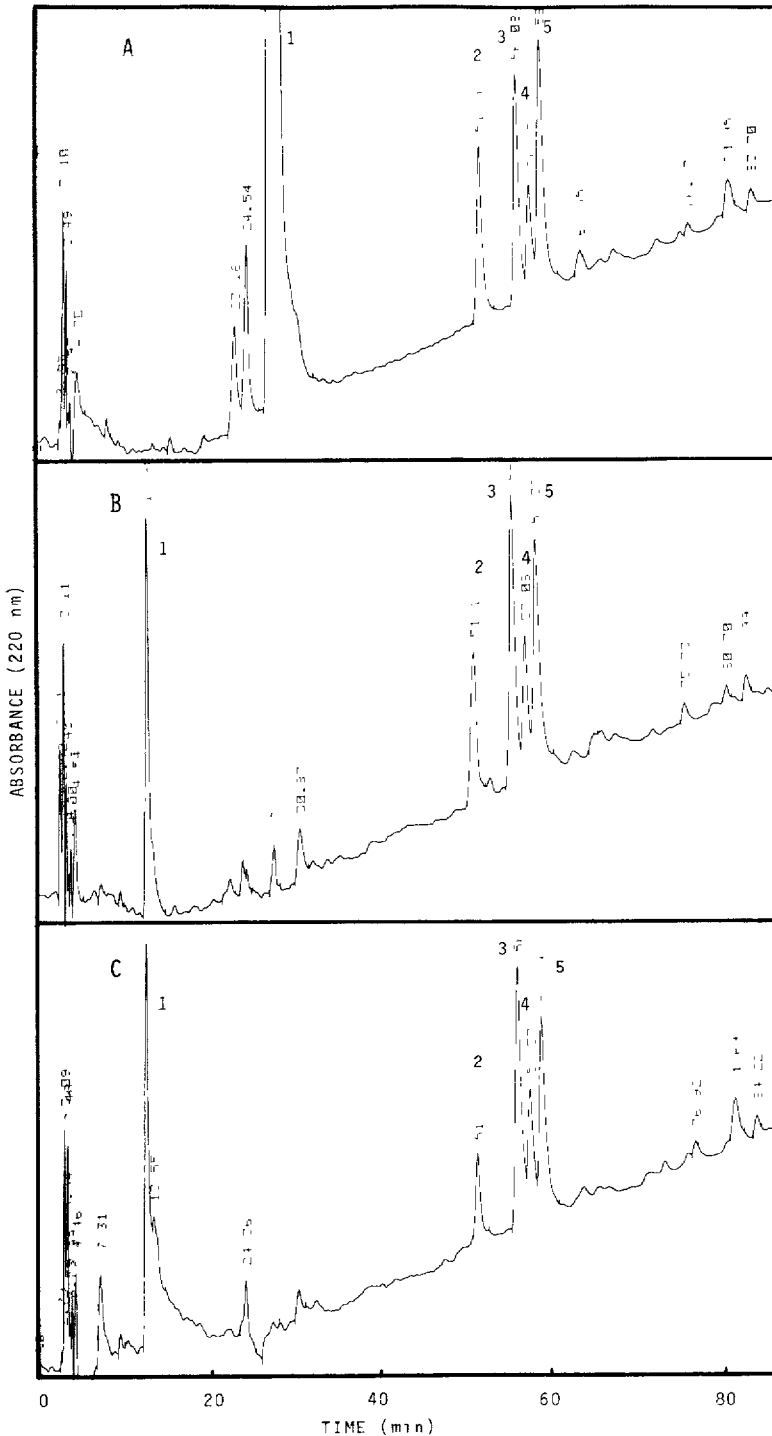
Fig 21 (A) Separation of γ-irradiated L-Ala–L-Ala by WAE-HPLC Column, MicroPak AX-5 (5 μm), 30 × 0 4 cm, temperature, 40°C, eluent and flow as in Fig. 6, gradient elution starting from 25% B with a rate of 0 75% B/min (B) and (C) γ-irradiated L-Ala–L-Ala after hydrolysis with aminopeptidase M and leucine aminopeptidase, respectively.

et al.[26]. Separation of all peptides contained in a subtilisin digest of purple membrane protein bacteriorhodopsin was achieved when peptide fragments derived from a RP-column were injected onto a WAE-column under the conditions given in ref. 5.

Furthermore, Margolis and Dizdaroglu (see above and ref. 19) compared the results of separation of AI diastereomers by WAE-HPLC with those obtained by RP-HPLC[27]. This comparison also strongly suggested[19] that these two HPLC methods of different principles are complementary and their combined use can confidently assess the purity of a given peptide preparation.

8. SEPARATION OF RADIATION-INDUCED PRODUCTS OF PEPTIDES

The WAE-HPLC method was recently applied to separation and isolation of radiation-induced products of aliphatic peptides[28]. Digestibility by exopeptidases of
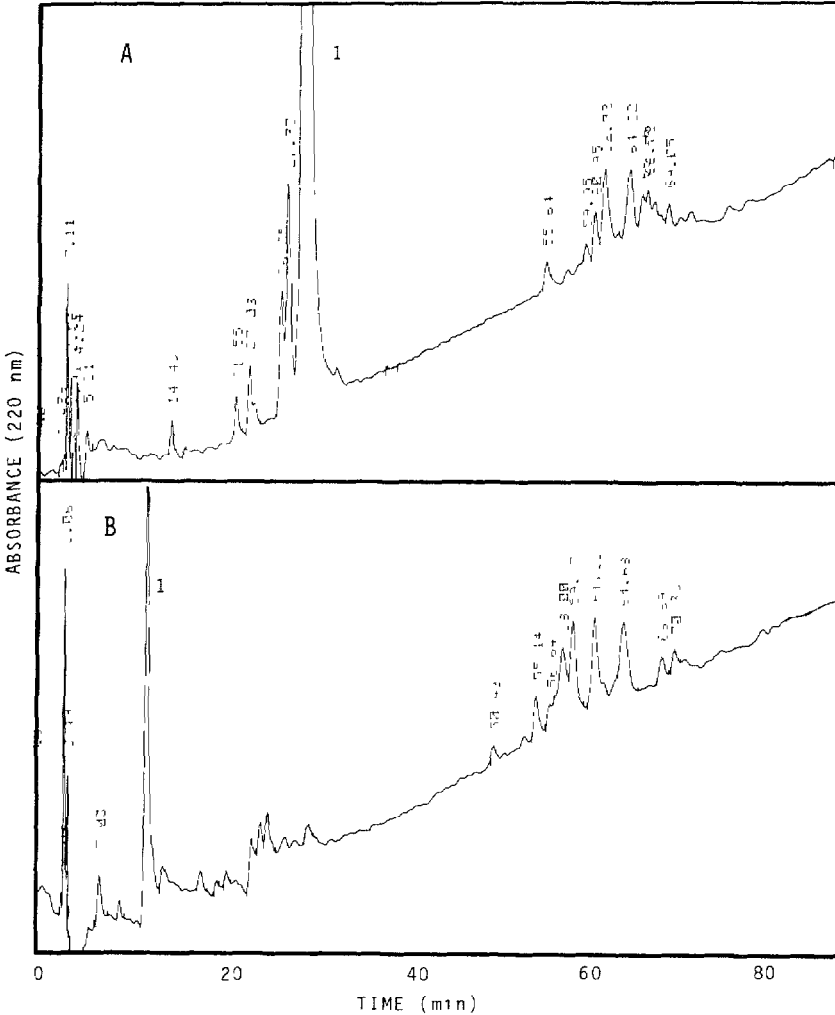

Fig 22. (A) Separation of γ-irradiated tetra-L-Ala by WAE-HPLC Column details as in Fig 21. (B) γ-irradiated tetra-L-Ala after hydrolysis with aminopeptidase M

peptides cross-linked by ionizing radiation was also investigated by using the same method[29]. Fig. 21A shows the separation of γ-irradiated L-Ala–L-Ala. Peak 1 represents this dipeptide and peaks 2–5 correspond to products of radiation-induced crosslinks, which were identified subsequently by gas chromatography–mass spectrometry[28]. Figs. 21B and C show the elution profiles of γ-irradiated L-Ala–L-Ala after digestion with aminopeptidase M and leucine aminopeptidase, respectively. Hydrolysis with two carboxypeptidases yielded similar chromatograms (not shown here) These results clearly indicate the non-digestibility of the crosslinked products under the conditions which hydrolyzed completely L-Ala–L-Ala. WAE-HPLC of γ-irradiated L-Ala–L-Ala–L-Ala–L-Ala (tetra-L-Ala) also showed the presence of radiation-crosslinked products (Fig. 22A)[28]. Partial digestion of these products by amino- and carboxypeptidases was obtained as revealed by their HPLC analysis under the same conditions[29]. As an example, Fig. 22B shows the HPLC profile of γ-irradiated tetra-L-Ala after digestion with aminopeptidase M.

While WAE-HPLC was the method of choice for the analysis of γ-irradiated aliphatic peptides, RP-HPLC yielded better results in analysis of γ-irradiated aromatic peptides[30] and was also successfully used for investigation of their enzymatic digestibility[29].

9 CONCLUSIONS

This survey shows that the WAE-HPLC method recently developed is an excellent approach for peptide separations A broad range of peptides including diastereomeric and other closely related peptides can be chromatographed and successfully separated. The buffer used allows detection of peptides at wavelengths in the range 210–225 nm. Its volatility facilitates a convenient and efficient recovery of salt free peptides for further use and study. The column used provides high recoveries of chromatographed peptides. Because of these features, the WAE-HPLC method could be quite useful for peptide separation and identification in the rapidly developing field of HPLC–mass spectrometry[31,32].

Furthermore, this review indicates that, in some instances, RP- and WAE-HPLC are complementary. This means that their combined use can provide the optimal separation of a given peptide mixture into its components or assess the purity of a peptide preparation.

10 ACKNOWLEDGEMENTS

11. SUMMARY

In this survey, the principles and applications of a method recently developed for peptide separations are given. This method uses a bonded weak anion-exchange column and mixtures of volatile triethylammonium acetate buffer and acetonitrile as

eluent. Its applications to the separation of a large number of peptides including diastereomeric and other closely related peptides are discussed. Separation of the enzymatic digests of some proteins is also presented. The complementary use of this method to the reversed-phase methods is outlined and their combined use for separation of enzymatic digests of proteins and assessment of purity of synthesized peptides is demonstrated. The results reviewed show that the weak anion-exchange method is an excellent approach for peptide separations and could be an important partner of reversed-phase methods for achieving optimal results.

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