Journal of Chromatography, 132 (1977) 27–36

CHROM. 9505

THE SEPARATION OF NONAPEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

K. KRUMMEN and R. W. FREI

Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., 4002 Basle (Switzerland)

(Received July 1st, 1976)

SUMMARY

The separation properties of five nonapeptides on commercial reversed-phase materials have been investigated and the effects of pH, salt concentration and solvent composition have been studied. With appropriate variation of the pH and salt concentration in the mobile phase, it is possible to resolve all of the peptides investigated and their by-products. Mixtures of water and organic solvents (acetonitrile, dioxan, methanol and *n*-propanol) have been used. The choice of the organic solvent does not strongly influence the separation pattern. The simplicity, speed and quality of the separations and the favourable detection limits (*ca.* 30 ng) at 220 nm render this technique suitable to routine quantitative analysis.

INTRODUCTION

The nonapeptides investigated in this study are important pharmaceutical substances. Oxytocine (Ox) and demoxytocine (Demox) are used as birth-inducing and lactation agents, lypressine (Lyp) is an anti-diuretic and ornipressine (Orp) and felypressine (Fely) are used for the treatment of capillary bleeding.

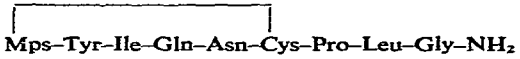
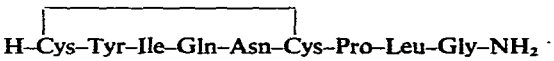
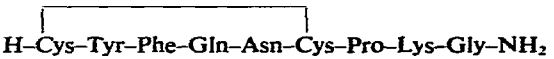
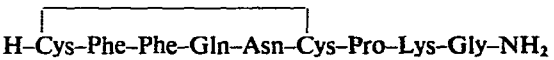
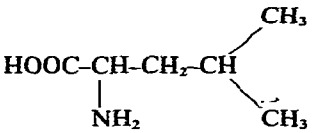
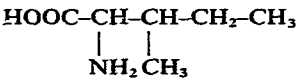
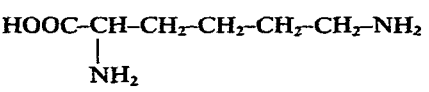
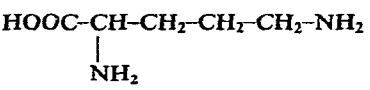
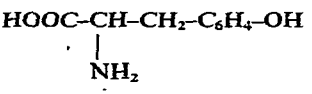
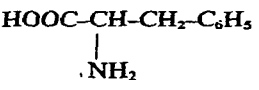
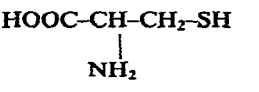
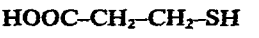
Bioassay techniques are used to test the potency of these peptides in concentrates or in dosage forms. The oxytocic activity is determined by the measurement of the depression of the blood pressure in a chicken or by the uterus contraction in a rat. For the determination of the vasopressoric activity, the blood-pressure assay in rats is often used[1,2]. To date, very few physicochemical techniques are available for assaying peptide compounds. The amino-acid analysis method of Stein and Moore[3] has been applied to concentrated solutions of drug substances following complete hydrolysis of the samples. Electrophoretic and thin-layer chromatographic techniques have occasionally been used for testing the purity of such concentrates[4] or for studying the formation of by-products[5]. Conventional column techniques based on the use of ion-exchange[6] or gel-permeation chromatography[7] have also been reported. In addition, gas chromatographic analysis, after hydrolysis to smaller peptides and subsequent derivatization, has been described[8].

Physicochemical techniques of sufficient accuracy and sensitivity are not avail-

able for the determination of the peptides listed above and their possible by-products and degradation products in dosage forms because of the low dosage of these peptides in the formulations. Therefore, the purpose of this study was to develop a high-performance liquid chromatographic (HPLC) method for the identification and quantification of the five peptides and their possible degradation products and by-products

TABLE I

STRUCTURES OF THE PEPTIDES INVESTIGATED

Demoxytocine	
Oxytocine	
Lypressine	
Ornipressine	
Felypressine	
Leu	
Ile	
Lys	
Orn	
Tyr	
Phe	
Cys	
Mps	

in concentrates and formulations. Reversed-phase systems were chosen for the investigation because of their mild non-destructive surface interactions and also because of the possibility of working at a detection wavelength in the range 210–220 nm where the mobile phase does not exhibit strong absorption. The ability to inject aqueous samples, such as injection solutions, directly on to the column[9] offered the advantage of a simple sample preparation.

EXPERIMENTAL

Reagents

The structures of the peptides investigated are given in Table I. Uvasol® quality acetonitrile and methanol, analytical grade dioxan and *n*-propanol (E. Merck, Darmstadt, G.F.R.) and doubly-distilled water were used. Buffer solutions were made by dilution of Merck buffer ampoules according to instructions. The following reversed-phase materials were used: Nucleosil® C_8 and C_{18}, 5-μm particle size (Macherey, Nagel & Co., Düren, G.F.R.); Spherisorb® S5 ODS, 5-μm particle size (Phase Separations Ltd., Queensferry, Great Britain); RP 8®, 10-μm particle size (E. Merck). The columns were packed by slurry techniques described elsewhere[10,11]. Columns of 7.5, 15 and 25 cm length (3 and 4 mm I.D.) were used. Experimental details are given with the respective chromatograms.

Apparatus

A Hewlett-Packard UFC-1000 liquid chromatograph, equipped with an Orlita® pump, was used in conjunction with a Perkin-Elmer LC 55 UV detector. The samples were introduced to the column by means of a loop injector (Altex, Berkeley, Calif., U.S.A.; 3000 p.s.i.). A Laboratory Data System 3352 B (Hewlett-Packard) was used for the evaluation of the data. A strong absorption in the UV region of the spectrum was observed at *ca.* 200 nm. A detection wavelength in the range 210–220 nm was chosen for practical reasons.

RESULTS AND DISCUSSION

Reversed-phase materials

The order of separation of the peptides investigated was the same on all of the solid supports tested. The changes in retention values are shown in Table II. A typical separation effected on an RP 8 column is given in Fig. 1. The order of separation Lyp + Orp (not resolved), Ox, Fely, Demox is the same as that observed on a Nucleosil® column (see Fig. 2). When operating with these conditions, it can be shown that nipagine and trichlorobutanol, often present as preservatives in liquid formulations, can be readily separated from the peptides. The separation of Lyp and Orp, which differ only by one methyl group in the amino acid at position 8 (see Table I), was difficult and could only be achieved at pH $\geqslant$ 9 (see *Influence of pH*). A chromatogram of Lyp and Orp carried out at pH 10 is shown in Fig. 3. A disadvantage of working at such a high pH is that the reversed-phase material decomposes within a few days owing to attack of the base on the silica matrix.

The particle size of the reversed-phase material had a considerable influence on the resolution power. The separation efficiency obtained with a column of 15 cm

TABLE II

k' VALUES FOR DIFFERENT COLUMN MATERIALS AND MOBILE PHASES 1–3 AT pH 7

Mobile phases: 1 = phosphate buffer pH 7–acetonitrile (4:1); 2 = phosphate buffer pH 7–acetonitrile (33:7); 3 = phosphate buffer pH 7–acetonitrile (3:1).

Compound	*RP 8*		*Nucleosil C_8*		*Nucleosil C_{18}*	*Ratio of k' values*	
	1	*2*	*1*	*3*	*1*		
	k'_1	k'_2	k'_3	k'_4	k'	k'_2/k'_1	k'_3/k'_4
Ornipressine	1.4	2.8	2.7	1.1	2.3	2.0	2.5
Lypressine	1.4	2.8	2.7	1.1	2.3	2.0	2.5
Oxytocine	3.9	9.0	7.0	2.2	7.3	2.3	3.2
Felypressine	5.6	11.7	9.5	2.8	10.0	2.1	3.4
Demoxytocine	10.8	29.0	17.5	4.3	21.1	2.7	4.1
Nipagine M (*p*-hydroxybenzoic acid methyl ester, preservative)	5.9	8.4	10.5	6.6	—	1.4	1.6
Trichlorobutanol (preservative)	11.7	15.0	19.8	12.2	17.9	1.3	1.6

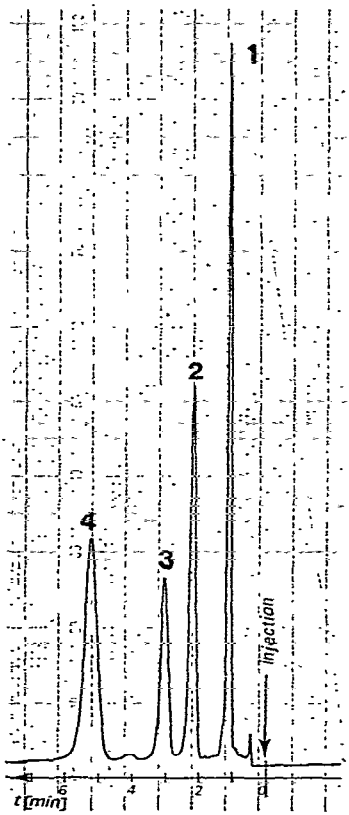


Fig. 1. Separation of nonapeptides at pH 7. Peaks: 1 = Ornipressine and lypressine (not separated); 2 = oxytocine; 3 = felypressine; 4 = demoxytocine. Conditions: column (25 cm × 0.3 cm I.D.) of RP 8 (10-μm particle size); mobile phase, 20% acetonitrile in phosphate buffer (pH 7); flow-rate, 3.0 ml/min; pressure at column inlet, 170 atm; temperature, ambient; UV monitor at 215 nm.

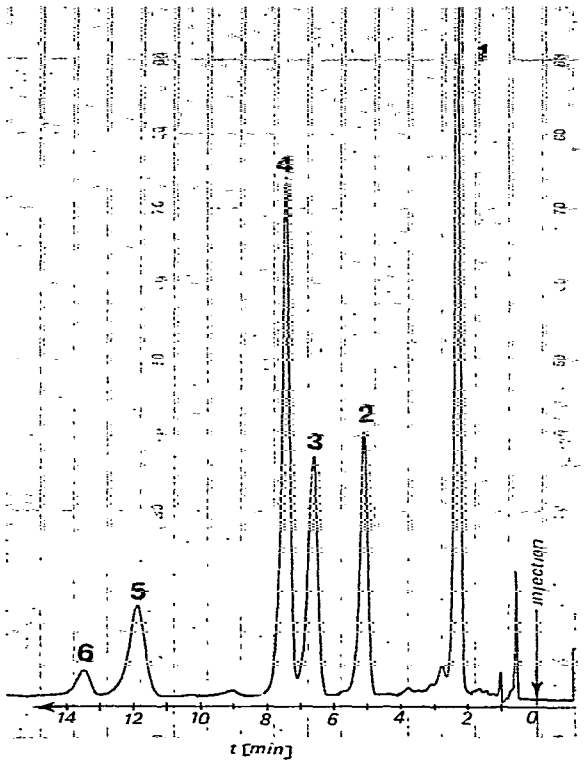


Fig. 2. Separation of nonapeptides and preserving agents at pH 7. Peaks: 1–3 as in Fig. 1; 4 = nipagine M; 5 = demoxytocine; 6 = trichlorobutanol. Conditions: column (15 cm × 0.4 cm I.D.) of Nucleosil C_8 (5-μm particle size); mobile phase, as in Fig. 1; flow-rate, 2.0 ml/min; pressure at column inlet, 230 atm; temperature, ambient; UV monitor at 210 nm.

length, packed with Nucleosil of 5-μm particle size, was nearly the same as that obtained with a column of 25 cm length, packed with RP 8 material of 10-μm average particle size (see Figs. 4 and 5). On the Nucleosil column (Fig. 4), by-products eluting earlier than Lyp were better separated from the main peak, while by-products eluting after Lyp were better resolved on the RP 8 column.

Good separations from their by-products were also observed for Ox, Demox and Orp. The conditions used in Figs. 4 and 5 were found to be optimal for such separations. Since they also gave a good separation for the preserving agent trichlorobutanol, the conditions are suitable for routine practical applications.

Mobile phase

Influence of solvent composition. The influence of the following organic solvents was tested at pH 10: methanol, acetonitrile, dioxan and *n*-propanol. The ratios of organic solvent to water were chosen in order to give k' values between 2 and 10. The results are shown in Fig. 6.

It can be seen that the same elution order Orp, Lyp, Demox occurred for all four systems, and that the selectivity factor α depends only to a minor degree on the

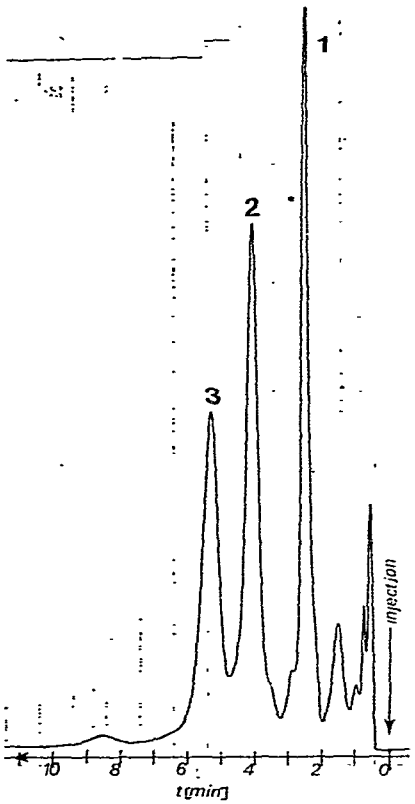
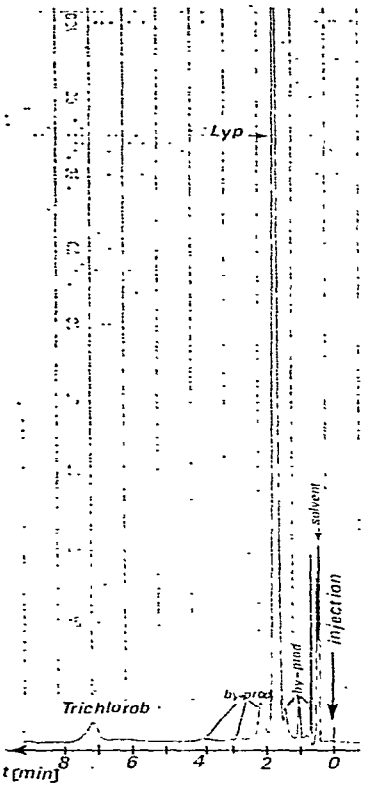


Fig. 3. Separation of oxytocine (1), ornipressine (2) and lypressine (3). Conditions: column (7.5 cm × 0.3 cm I.D.) of Spherisorb S5 ODS (5-μm particle size); mobile phase, 17.5% acetonitrile in borate buffer (pH 10); flow-rate, 1.0 ml/min; pressure at column inlet, 60 atm; temperature, ambient; UV monitor at 220 nm.

choice of the solvent. The only exception is Ox which can overlap with Orp or Lyp. This can be overcome by appropriate choice of the salt concentration (see *Influence of salt concentration*).

Since *n*-propanol gave the best separation of Orp and Lyp, the same solvent system was tried at a lower pH, resulting in a complete loss of resolution of these substances. For convenience, acetonitrile was finally chosen for further work because of its lower viscosity. As would be expected, the ratio of organic solvent to water strongly influenced the k' values of the peptides (k' increasing with decreasing organic solvent concentration, see Table II). For the excipients, nipagine and trichlorobutanol, this influence was less pronounced.

Influence of salt concentration. The effect of the salt concentration was tested at pH 7 and pH 10. At both these values the k' values decreased with increasing salt concentration. This change in retention was particularly pronounced with Fely and was least with Ox. By varying the salt concentration at pH 10, it was possible to separate Ox from Orp and Lyp (Table IIIB). At very low salt concentrations the elution of Fely, Orp and Lyp was difficult. The separation of Lyp from Orp cannot be affected



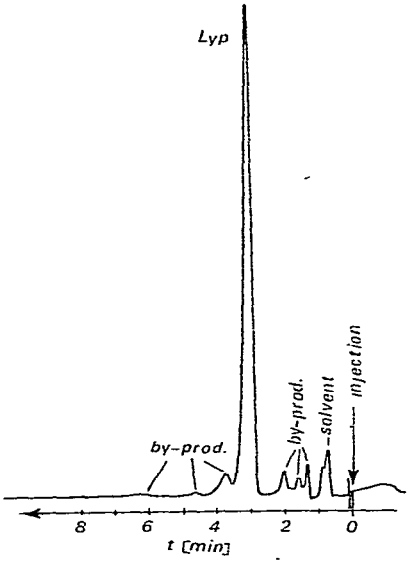


Fig. 4. Chromatogram of 100 μl of lypressine solution (100 I.U./ml). Conditions: column (15 cm × 0.3 cm I.D.) of Nucleosil C_8 (5-μm particle size); mobile phase, as in Fig. 1; flow-rate, 1.20 ml/min; pressure at column inlet, 230 atm; temperature, ambient; and UV monitor at 220 nm; sensitivity, 2.0 a.u.f.s.

Fig. 5. Chromatogram of 100 μl of lypressine solution (100 I.U /ml). Conditions: column, as in Fig. 1; mobile phase, 17.5% acetonitrile in phosphate buffer (pH 7); flow-rate, 3.0 ml/min; pressure at column inlet, 200 atm; temperature, ambient; UV monitor at 215 nm; sensitivity, 1.0 a.u.f.s.

by variations of this parameter. The k' values of the two preserving agents investigated were independent of the salt concentration.

An explanation of these results can be deduced from observations reported for proteins[12]. At low ionic strength a strong increase in the solubility of the protein occurred with increasing salt concentration (salting-in effect). This increase reaches a maximum and then decreases at even higher concentrations (salting-out effect). Similar trends were observed in the chromatographic behaviour of amino acids on Porapak Q reversed-phase material with water–acetone and water–methanol mobile phases[13]. Thus, it seems reasonable to assume that the increase in the solubility of peptides with increasing salt concentration in the mobile phase is responsible for this decrease in k' values.

Influence of pH. The effect of pH was tested in the range of pH 5–12. The influence was particularly noticeable for the separation of the pair Lyp–Orp. A plot of α values ($\alpha = k'_{Lyp}/k'_{Orp}$) as a function of pH is shown in Fig. 7. A satisfactory sepa-

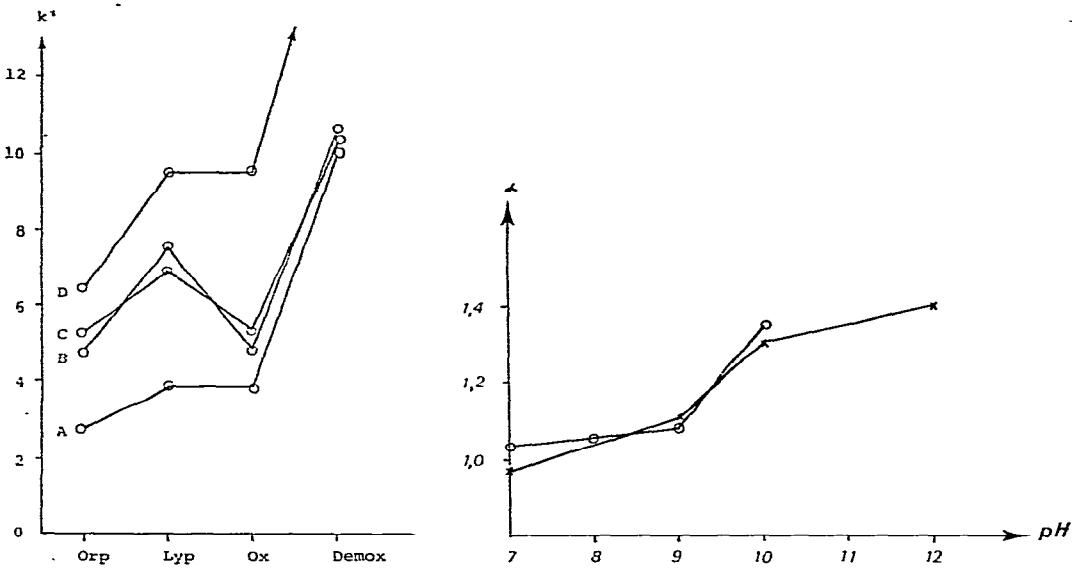


Fig. 6. Influence of solvent composition on the resolution of the nonapeptides. Mobile phases: A = borate buffer (pH 10)–water–acetonitrile (2:2:1); B = borate buffer (pH 10)–water–*n*-propanol (9:9:2); C = borate buffer (pH 10)–water–methanol (3:3:4); D = borate buffer (pH 10)–water–dioxan (17:17:6). Column, Nucleosil C_{18} (5-μm particle size).

Fig. 7. Influence of pH on the resolution of ornipressine and lypressine. Mobile phases: (○) buffer solution–acetonitrile (4:1); (×) buffer solution–methanol (7:3 to 11:8). Column, Nucleosil C_8 (5-μm particle size).

TABLE III

INFLUENCE OF SALT CONCENTRATION ON k' VALUES

(A) pH 7: mobile phase, phosphate buffer pH 7–water–acetonitrile (x:80−x:20 where x = 10, 40, 60 or 80 ml); column, Nucleosil C_{18} (10-μm particle size).

Phosphate concentration in the mobile phase (10^{-2} M)	*k'*						
	Orp	*Lyp*	*Ox*	*Fely*	*Demox*	*Trichlorobutanol*	*α (Ox/Lyp)*
0.67	6.3	6.3	10.1	28.5	29.0	18.6	1.60
2.67	3.0	3.0	8.0	13.0	23.8	18.1	2.67
4.00	2.4	2.4	7.1	10.4	20.5	17.6	2.96
5.33	2.3	2.3	7.3	10.0	21.1	17.9	3.17

(B) pH 10: mobile phase, tris(hydroxymethyl)aminomethane buffer (0.1 *M*, pH 10)–acetonitrile (3:1); column, Nucleosil C_8 (5-μm particle size).

Sodium chloride concentration in the mobile phase (M)	*k'*		
	Orp	*Lyp*	*Ox*
0	∞	∞	2.4
0.05	2.2	2.9	2.3

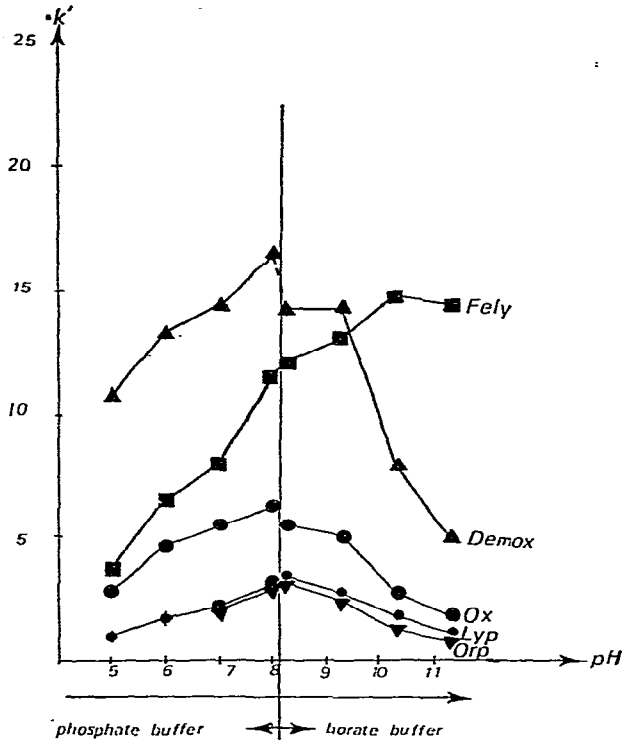


Fig. 8. Influence of pH on the k' values of the nonapeptides. Mobile phases: 20% acetonitrile in phosphate buffer, 0.02 *M* (Soerensen), from pH 5 to pH 8; 20% acetonitrile in borate buffer, 0.02 *M* (Soerensen), from pH 8 to pH 11. Sodium chloride was added to both mobile phases to give a concentration of 0.05 *M*. Column, Nucleosil C_{18} (10-μm particle size).

ration was only possible at pH $\geq$ 9, independent of the organic solvents used. It seems that a separation can only be effected when the free amino group, in the amino acid in the position 8 (see Table I; lysine for Lyp and ornithine for Orp; pK values, 10.5)[14], is not protonated. The k' values of all of the tested peptides as a function of pH are shown in Fig. 8. The retention times increased with increasing pH up to a maximum at *ca.* pH 8, followed by a gradual decrease at higher pH. Fely is the only peptide that behaves differently. An explanation for this result may be the fact that Fely does not contain the amino acid tyrosine. At pH > 8, the phenolic OH group on tyrosine, which has a pK_a value of 10 (see Table I), is present as the phenolate ion which is less strongly retained on the reversed-phase material. Except for Lyp and Orp, there seems to be little advantage in using pH-variation for the separation of the peptides from each other and from their by-products.

CONCLUSIONS

By varying the pH and the salt concentration in reversed-phase systems, it is possible to separate the nonapeptides investigated. The separation of the peptides from each other and from possible by-products can be easily achieved in the isocratic

mode and in a matter of minutes. The optimum systems suggested for routine application are: (a) Nucleosil C_8 with a mobile phase of water buffered to pH 7–acetonitrile (4:1); (b) RP 8 with a mobile phase of water buffered to pH 7–acetonitrile (33:7).

The separation of Lyp and Orp is not possible in this system but can be carried out at pH $\geqslant$ 9. Additives, such as nipagine and trichlorobutanol, can also be separated. The excellent quality of the separations and the reasonably good detection properties of the peptides at 210–220 nm (detection limits, *ca.* 30 ng per injection) permit the quantitative determination of these peptides in dosage forms. Further work is in progress.

ACKNOWLEDGEMENT

Thanks are due to F. Erni for helpful discussions.

REFERENCES

1 *U.S. Pharmacopoeia*, XIX, pp. 356 and 534.
2 *British Pharmacopoeia*, 1973, pp. A110, A111.
3 S. Moore, in J. Meienhofer (Editor), *Chemistry and Biology of Peptides, Proc. 3rd Amer. Peptide Symp.*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1972, p. 629.
4 E. Jaeger, M. Deffner, K. H. Deimer, R. Scharf and E. Wünsch, in H. Nesvadba (Editor), *Peptides 1971*, North Holland, Amsterdam, 1973, p. 237.
5 H. W. Bossert and P. A. Jaquenoud, in Y. Wolmann (Editor), *Peptides 1974*, Wiley, New York, 1975, p. 233.
6 J. V. Benson, Jr. and J. A. Patterson, in A. Niederwieser and G. Pataki (Editors), *New Techniques in Amino Acid, Peptide and Protein Analysis*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1971, p. 1.
7 A. B. Russel and M. Szelke, in H. Nesvadba (Editor), *Peptides 1971*, North Holland, Amsterdam, 1973, p. 223.
8 B. Kolb, in A. Niederwieser and G. Pataki (Editors), *New Techniques in Amino Acid, Peptide and Protein Analysis*, Ann Arbor Sci. Publ., Ann Arbor, Mich., 1971, p. 129.
9 F. Erni and R. W. Frei, *J. Chromatogr.*, 130 (1977) 169.
10 R. M. Cassidy, D. LeGay and R. W. Frei, *Anal. Chem.*, 46 (1974) 340.
11 H. R. Linder, H. P. Keller and R. W. Frei, *J. Chromatogr. Sci.*, 14 (1976) 234.
12 E. J. Cohn, *Naturwissenschaften*, 20 (1932) 633.
13 A. Niederwieser, *J. Chromatogr.*, 61 (1971) 81.
14 D. M. Greenberg (Editor), *Amino Acids and Proteins*, Thomas, Springfield, Ill., 1951, p. 430.