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SEPARATION OF SOME POLYPEPTIDE HORMONES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Twenty-one analogues of ACTH, three analogues of LH-RH and four insulins have been successfully separated on a commercial reversed-phase material with tartrate buffer–acetonitrile systems containing sodium 1-butanesulphonate and sodium sulphate as the mobile phase. The effect of the constituent amino acid residues on the order of elution has been studied in detail by using a variety of closely related peptides; the order of elution of a series of peptides, which differ by only one amino acid residue, can in most instances be explained in terms of the difference in the hydrophobicities of the amino acid residues concerned, but in some instances, such as in diastereoisomers or positional isomers, the order of elution must be interpreted in terms of the hydrophobicity of the whole peptide molecule. This chromatographic method has been proved to be very useful for the rapid examination of the purity of these peptide hormones and for the separation of closely related peptides with molecular weights up to *ca.* 6000.

INTRODUCTION

A number of papers describing the separation of native and synthetic peptides by high-performance liquid chromatography (HPLC) have been published[1–8], but there are only a few papers in which the successful separation of peptides containing more than nine amino acid residues has been reported[2,3,6,8]. We have recently found that reversed-phase liquid chromatography can be applied successfully to the separation of closely related analogues of various peptide antibiotics consisting of 8–13 amino acid residues and a C_8–C_{11} fatty acid attached to the N-terminal group[9,10]. This paper describes the separation of larger peptides, including corticotropins (ACTH), insulins and their closely related analogues, by use of an ion-pair reversed-phase liquid chromatographic technique.

A large number of peptides related to the amino acid sequence of ACTH have been synthesized in order to examine the relationships between structures and

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biological activities (*e.g.*, ref. 11). In the present study ACTH-(1–18)-octadecapeptide amide and its analogues were mainly chosen as test samples in order to explore the possibility of the separation of closely related peptides. Experiments on the separation of a series of analogues, in which a particular amino acid residue was substituted by different amino acids, have provided useful information concerning the effect of constituent amino acid residues on the order of elution.

EXPERIMENTAL

Reagents and materials

All of the peptides investigated were synthesized in this laboratory, unless otherwise stated. The ACTH peptides and their analogues employed were as follows: porcine corticotropin (α_p-ACTH)[12], human corticotropin (α_h-ACTH)[12], ACTH-(1–26)-NH_2[13], ACTH-(1–27)-NH_2[13], ACTH-(1–18)-NH_2[14,15] and three of its 1-substituted analogues, [Gly^1]-[16], [βAla^1]-[17] and [Aib^1]-ACTH-(1–18)-NH_2[18], four 10-substituted analogues of ACTH-(1–18)-NH_2[19], [βAla^1, Orn^{15}]-ACTH-(1–18)-NH_2[20], [βAla^1, D-Phe^7, Orn^{15}]-ACTH-(1–18)-NH_2[20], [Aib^1, Orn^{15}]-ACTH-(1–18)-NH_2[20], [Gly^1, Pro^3]-ACTH-(1–18)-NH_2[21], [Aib^1, Nle^4]-ACTH-(1–18)-NH_2[21], [Lys^3, Ser^{11}]-ACTH-(1–18)-NH_2[21], [Gly^1]-ACTH-(1–18)-NH_2 S-oxide[22], [Ala^{10}]-ACTH-(1–10)-$NHNH_2$[19] and [D-Ala^{10}]-ACTH-(1–10)-$NHNH_2$[19]. Most of the structures of these above cited peptides are shown in Figs. 1–5.

Luteinizing hormone-releasing hormone (LH-RH)[23] was prepared by the solid-phase method with benzhydrylamine resin[24] as support, and its two analogues[25]

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                 1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18  19  20
αp-ACTH:  H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-

αh-ACTH:  H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-


                21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38  39
αp-ACTH:  Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH

αh-ACTH:  Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH
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Fig. 1. Primary structures of porcine corticotropin (α_p-ACTH) and human corticotropin (α_h-ACTH).

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  1  2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18
H-X-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-NH2
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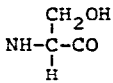
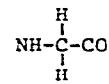
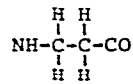
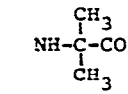
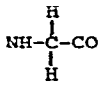
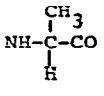
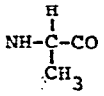
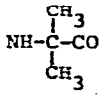
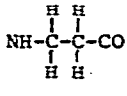
Peptide*	1	2	3	4
X				
	Ser	Gly	βAla	Aib

*1: ACTH-(1-18)-NH_2, 2: [Gly^1]-ACTH-(1-18)-NH_2,
3: [βAla^1]-ACTH-(1-18)-NH_2, 4: [Aib^1]-ACTH-(1-18)-NH_2.

Fig. 2. Structures of 1-substituted analogues of ACTH-(1–18)-NH_2.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-X-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-NH_2

Peptide*	1	5	6	7	8
X					
	Gly	Ala	D-Ala	Aib	βAla

*1: ACTH-(1-18)-NH_2, 5: [Ala^{10}]-ACTH-(1-18)-NH_2, 6: [D-Ala^{10}]-ACTH-(1-18)-NH_2, 7: [Aib^{10}]-ACTH-(1-18)-NH_2, 8: [βAla^{10}]-ACTH-(1-18)-NH_2.

Fig. 3. Structures of 10-substituted analogues of ACTH-(1–18)-NH_2.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H-X-Tyr-Ser-Met-Glu-His-Y-Arg-Trp-Gly-Lys-Pro-Val-Gly-Z—Lys-Arg-Arg-NH_2

Peptide*	X	Y	Z
3	βAla	Phe	Lys
9	βAla	Phe	Orn
10	βAla	D-Phe	Orn
4	Aib	Phe	Lys
11	Aib	Phe	Orn

*3: [βAla^1]-ACTH-(1-18)-NH_2, 9: [βAla^1, Orn^{15}]-ACTH-(1-18)-NH_2, 10: [βAla^1, D-Phe^7, Orn^{15}]-ACTH-(1-18)NH_2, 4: [Aib^1]-ACTH-(1-18)-NH_2, 11: [Aib^1, Orn^{15}]-ACTH-(1-18)-NH_2.

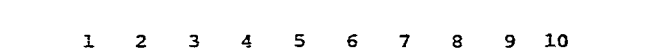
Fig. 4. Analogues of ACTH-(1–18)-NH_2.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H-X-Tyr-Y-Z-Glu-His-Phe-Arg-Trp-Gly-W-Pro-Val-Gly-Lys-Lys-Arg-Arg-NH_2

Peptide*	X	Y	Z	W
2	Gly	Ser	Met	Lys
12	Gly	Pro	Met	Lys
4	Aib	Ser	Met	Lys
13	Aib	Ser	Nle	Lys
1	Ser	Ser	Met	Lys
14	Lys	Ser	Met	Ser

*2: [Gly^1]-ACTH-(1-18)-NH_2, 12: [Gly^1, Pro^3]-ACTH-(1-18)-NH_2, 4: [Aib^1]-ACTH-(1-18)-NH_2, 13: [Aib^1, Nle^4]-ACTH-(1-18)-NH_2, 1: ACTH-(1-18)-NH_2, 14: [Lys^1, Ser^{11}]-ACTH-(1-18)-NH_2.

Fig. 5. Analogues of ACTH-(1–18)-NH_2.

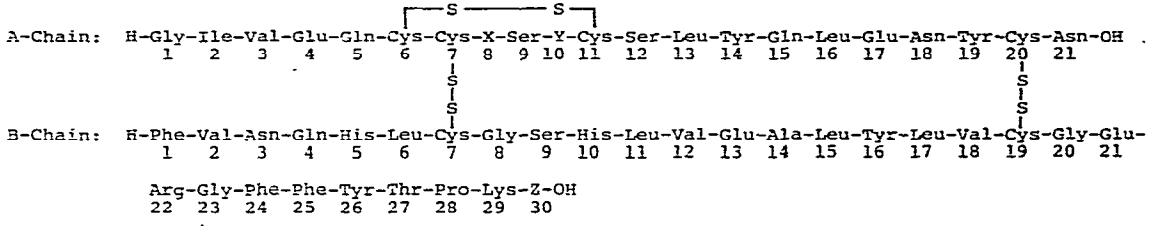


Peptide	X	Y	Z
LH-RH	Trp	Ser	Tyr
[Gly^4, Phe^{3,5}]-LH-RH	Phe	Gly	Phe
[Gly^4, Phe^5]-LH-RH	Trp	Gly	Phe

Fig. 6. Analogues of LH-RH.

were synthesized by the conventional solution method. These are shown in Fig. 6. A preparation of LH-RH synthesized by the solution method was also obtained from Protein Research Foundation (Osaka, Japan).

The structures of the four insulins investigated are shown in Fig. 7. Bovine insulin was obtained from Calbiochem (San Diego, Calif., U.S.A.). Porcine insulin was kindly donated by Dr. M. A. Root of Lilly Research Laboratories. These two insulins were dezinced and purified on a QAE-Sephadex A-25 column according to Schlichtkrull *et al.*[26]. Human insulin[27,28] and [Thr^{B30}]-bovine insulin[28] were prepared by a semi-synthetic method from porcine and bovine insulin, respectively.



Insulin	A-Chain X	A-Chain Y	B-Chain Z
Human	Thr	Ile	Thr
Porcine	Thr	Ile	Ala
Bovine	Ala	Val	Ala
[Thr^{B30}]-Bovine	Ala	Val	Thr

Fig. 7. Structures of insulins.

HPLC-quality acetonitrile (Wako, Osaka, Japan) and reagent-grade sodium 1-butanesulphonate (Eastman-Kodak, Rochester, N.Y., U.S.A.) were used. Reagent-grade L (+)-tartaric acid (Kanto, Tokyo, Japan) was used as a constituent of the mobile phase. Water was purified by means of an ion-exchange column, reverse osmosis and finally single distillation.

Apparatus

The apparatus consisted of a Waters Assoc. Model 6000A pump, a Rheodyne Model 7120 injector and a Japan Spectrooptics UVIDEC-100 variable-wavelength UV detector. The column (20 cm × 4 mm I.D.) was packed with Nucleosil 5C$_{18}$,

particle size 5 μm (Macherey, Nagel & Co., Düren, G.F.R.) by the technique recommended by the manufacturer with Slurry Solvent B (Macherey, Nagel & Co.).

Procedure

ACTH, LH-RH and their analogues were dissolved in distilled water to give concentrations of 0.1–1 mg/ml. Insulins were dissolved in 0.005 *M* tartrate buffer (pH 3.0) to the same concentration. The solutions were kept at −20° when not in use. Amounts of sample injected were usually of the order of micrograms.

All experiments were carried out at room temperature. The flow-rate was 1.0 ml/min throughout and the inlet pressures were 1800–2500 p.s.i., depending on the content of acetonitrile. The detector was operated at 220 nm and at an attenuation range of 0.008–0.002 a.u.f.s.

The mobile phase was prepared by mixing the following two solutions in an appropriate ratio to obtain a desired content of acetonitrile: solution A, 0.005 *M* tartrate buffer (pH 3.0) containing 0.005 *M* sodium 1-butanesulphonate and 0.05 *M* sodium sulphate; solution B, a mixture of equal volumes of acetonitrile and 0.005 *M* tartrate buffer (pH 3.0) containing 0.01 *M* sodium 1-butanesulphonate and 0.1 *M* sodium sulphate. The mobile phase was filtered through a 1- or 0.5-μm membrane filter and degassed prior to use. The content of acetonitrile in the mobile phase was adjusted in order to control the retention times of the samples. The contents of acetonitrile in the eluting agent are given in the figure captions.

RESULTS AND DISCUSSION

As shown in Fig. 1, α_p-ACTH and α_h-ACTH are both nonatriacontapeptides with molecular weights of *ca.* 4500. The only structural difference between the two lies in the amino acid residue in position 31, which is Leu in α_p-ACTH and Ser in α_h-ACTH. The important structural characteristic of these hormones for chromatography is that they have a cluster of basic amino acid residues in positions 8, 11, 15–18 and 21, whereas no basic amino acid residues are contained in the C-terminal half (positions 22–39) of their molecules.

Chromatograms of synthetic α_p-ACTH, α_h-ACTH and a mixture of them are given in Fig. 8. The reproducibilities of the peak heights and retention times for replicate injections of identical amounts were good provided that the content of acetonitrile in the mobile phase was strictly controlled. The chromatograms of α_p-ACTH and α_h-ACTH reveal that the purity of these synthetic hormones is fairly good, if not very satisfactory. A preparation of natural α_p-ACTH purchased from Sigma (St. Louis, Mo., U.S.A.) was of much lower purity, even after purification by carboxymethylcellulose column chromatography, although the HPLC pattern is not shown here. α_h-ACTH was eluted much faster than α_p-ACTH (Fig. 8C). This result indicates that the separation of these two hormones is attributable to the difference by a single amino acid residue in position 31 between their 39 amino acid sequences. The order of elution of α_h-ACTH and α_p-ACTH agrees with that predicted from the difference between Ser and Leu in reversed-phase chromatography.

The hexacosa- and heptacosapeptides corresponding to the first 26 and 27 amino acid residues of ACTH had retention times of 18.0 and 19.8 min, respectively, under the conditions employed. In this instance an additional Ala residue resulted in

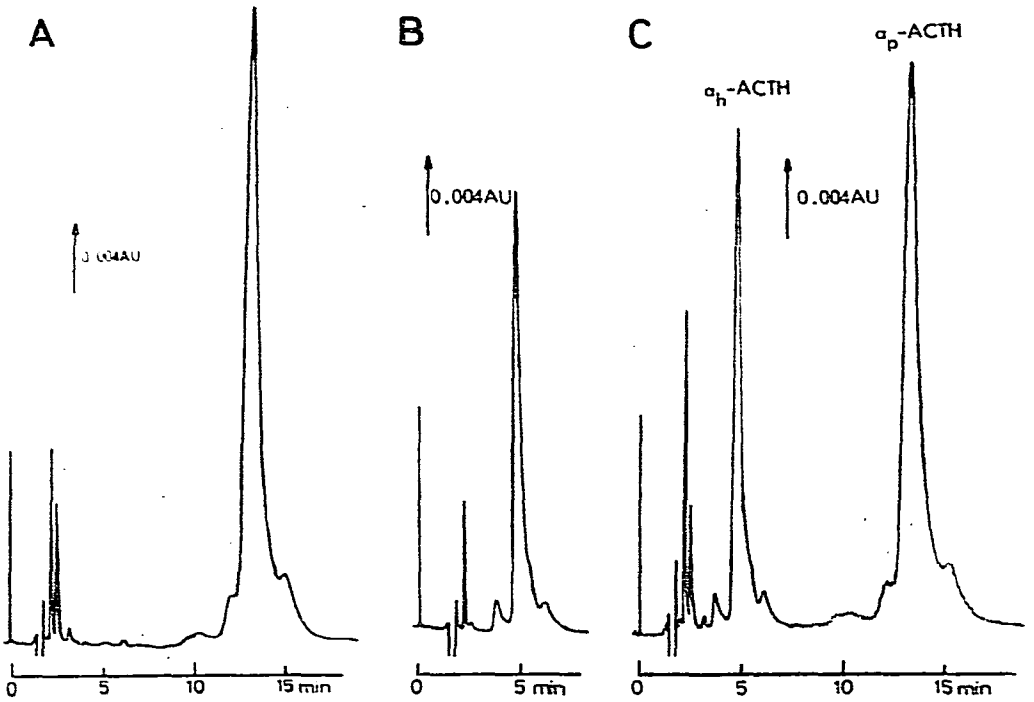


Fig. 8. HPLC of synthetic ACTHs. A, α_p-ACTH (5.6 μg); B, α_h-ACTH (2.0 μg); C, a mixture of α_p-ACTH and α_h-ACTH. Content of acetonitrile in the mobile phase: 30%.

the separation of ACTH-(1–27)-NH_2 from ACTH-(1–26)-NH_2, although the separation was not complete. The content of acetonitrile in the mobile phase used (18%) was much lower than that used for the separation of α_h-ACTH and α_p-ACTH. This means that the amino acid residues in positions 22–39 make the whole molecule of ACTH more hydrophobic than the shorter peptides.

Chromatograms of a mixture of four 1-substituted analogues of ACTH-(1–18)-NH_2 are given in Fig. 9. When the acetonitrile content of the mobile phase was 17%, [Gly^1]-ACTH-(1–18)-NH_2 and [βAla^1]-ACTH-(1–18)-NH_2 could not be separated and only [Aib^1]-ACTH-(1–18)-NH_2 was completely separated from the other three. On reducing the content of acetonitrile to 16%, a slightly better resolution between [Gly^1]-ACTH-(1–18)-NH_2 and [βAla^1]-ACTH-(1–18)-NH_2 was obtained, as shown in Fig. 9B. The structural differences among these four peptides are shown in Fig. 2. The main chain in [βAla^1]-ACTH-(1–18)-NH_2 is one methylene unit longer than that in [Gly^1]-ACTH-(1–18)-NH_2. The elution times of these four peptides increased in the order Ser < Gly $\lesssim$ βAla $\ll$ Aib with respect to the N-terminal residue. This order agrees with that expected from the hydrophobicity of the amino acid residue concerned.

Fig. 10A shows the separation of five 10-substituted analogues of ACTH-(1–18)-NH_2 (Fig. 3) with mobile phase containing 18% of acetonitrile. They were almost completely separated from one another, except for ACTH-(1–18)-NH_2 and [Ala^{10}]-ACTH-(1–18)-NH_2. The last two peptides were well separated, as shown in

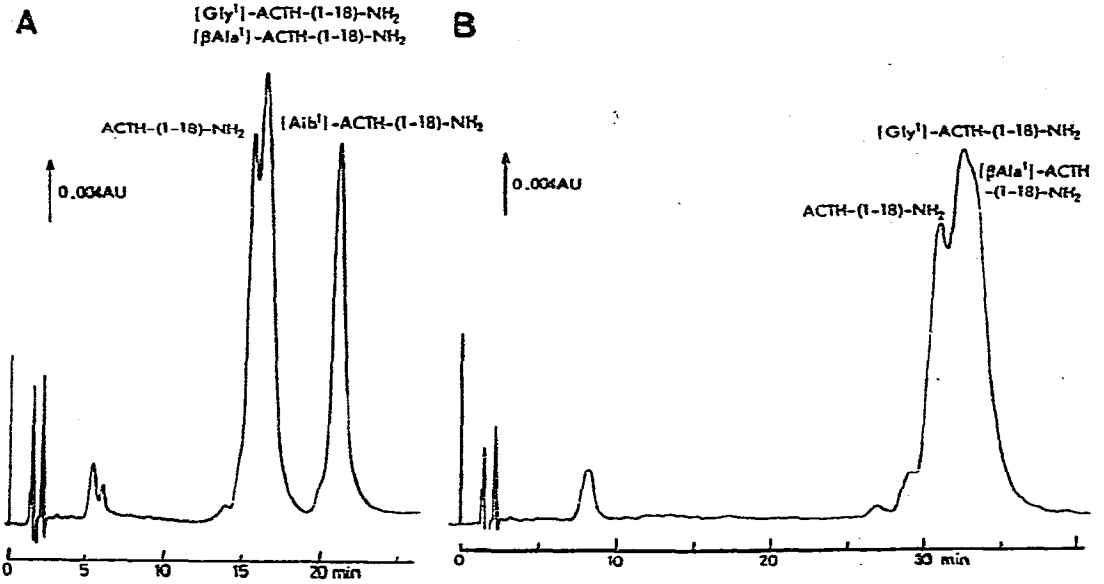


Fig. 9. Chromatograms of mixtures of 1-substituted analogues of ACTH-(1–18)-NH_2. Content of acetonitrile in the mobile phase: A, 17%; B, 16%. [Aib¹]-ACTH-(1–18)-NH_2 was not injected in run B.

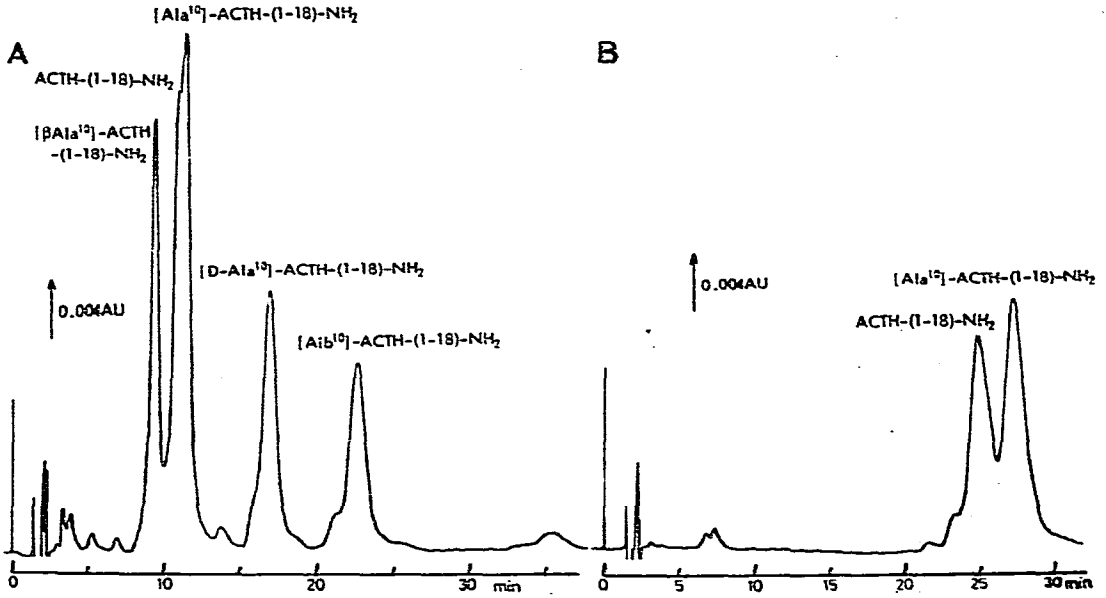


Fig. 10. Chromatograms of mixtures of 10-substituted analogues of ACTH-(1–18)-NH_2. Content of acetonitrile in the mobile phase: A, 18%; B, 16%.

Fig. 10B, when the acetonitrile content of the mobile phase was reduced to 16%. The structural relationship between these two peptides may be similar to that between [Gly¹]-ACTH-(1–18)-NH_2 and [βAla¹]-ACTH-(1–18)-NH_2 (Fig. 9) with respect to the extra methylene group which is positioned outside the peptide chain.

The chromatogram shown in Fig. 10A reveals two interesting points. One is concerned with the separation of diastereoisomers, [Ala^{10}]-ACTH-(1–18)- NH_2 and [D-Ala^{10}]-ACTH-(1–18)-NH_2, and the other with the order of elution of [βAla^{10}]-ACTH-(1–18)-NH_2, ACTH-(1–18)-NH_2 and [Ala^{10}]-ACTH-(1–18)-NH_2. The former will be discussed later in connection with another separation experiment on diastereoisomers. [βAla^{10}]-ACTH-(1–18)-NH_2 was eluted faster than ACTH-(1–18)-NH_2, which has a glycine residue in position 10, despite the fact that the former has one more methylene unit than the latter. This order of elution is reversed in comparison with the pair [Gly^1]-ACTH-(1–18)-NH_2 and [βAla^1]-ACTH-(1–18)-NH_2 (Fig. 9). This suggests that the order of elution is affected not only by the difference of a particular amino acid residue but also by the whole molecule. Insertion of an extra group in an *endo* position in a peptide causes a so-called "frame shift" and alters the steric relations of the side-chains of peptide. This would lead to a significant conformational change in the molecule, and could be a reason why the addition of a methylene group makes [βAla^{10}]-ACTH-(1–18)-NH_2 less hydrophobic than ACTH-(1–18)-NH_2. The difference in the chromatographic behaviours of the two isomers [Ala^{10}]-ACTH-(1–18)-NH_2 and [βAla^{10}]-ACTH-(1–18)-NH_2 also may not be explained solely by hydrophobicity of the residue in position 10. There must be an additional effect combined with some conformational difference in the peptides.

Fig. 11 shows three chromatograms of the successful separation of peptide epimers. HPLC permitted for the first time the detection and determination of [Ala^{10}]-

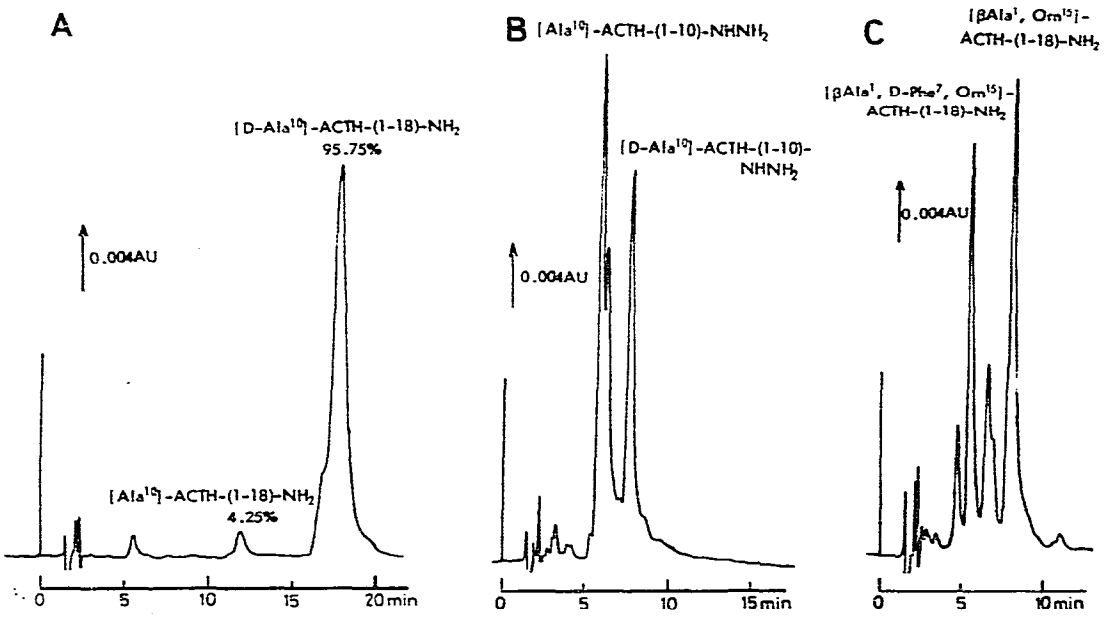


Fig. 11. Separation of some peptide epimers. A, Determination of the content of [Ala^{10}]-ACTH-(1–18)-NH_2 in a [D-Ala^{10}]-ACTH-(1–18)-NH_2 preparation. B, Separation of [Ala^{10}]-ACTH-(1–10)-$NHNH_2$ and [D-Ala^{10}]-ACTH-(1–10)-$NHNH_2$. The peak on the shoulder of the [Ala^{10}]-ACTH-(1–10)-$NHNH_2$ peak is due to an impurity in [Ala^{10}]-ACTH-(1–10)-$NHNH_2$. C, Separation of [βAla^1, D-Phe^7, Orn^{15}]-ACTH-(1–18)-NH_2 and [βAla^1, Orn^{15}]-ACTH-(1–18)-NH_2. Minor peaks other than those of two epimers are due to impurities present in the [βAla^1, D-Phe^7, Orn^{15}]-ACTH-(1–18)-NH_2 preparation. Content of acetonitrile in the mobile phase: A, 18%; B, 22.5%; C, 18%.

ACTH-(1–18)-NH_2 in a preparation of [D-Ala^{10}]-ACTH-(1–18)-NH_2. Thus far, no spectroscopic or other chromatographic method has ever been useful for this purpose. It seemed likely that the 4.5% of [Ala^{10}]-ACTH-(1–18)-NH_2 found in the [D-Ala^{10}]-ACTH-(1–18)-NH_2 preparation originated from the D-alanine used as starting material, because the content of the L-isomer in [D-Ala^{10}]-ACTH-(1–18)-NH_2 was almost the same as that of L-alanine in the D-alanine used. The different retention times observed for [Ala^{10}]-ACTH-(1–18)-NH_2 and [D-Ala^{10}]-ACTH-(1–18)-NH_2 in Figs. 10 and 11 may be explained as a result of a small change in the acetonitrile content of the mobile phase. [Ala^{10}]-ACTH-(1–10)-$NHNH_2$ and [D-Ala^{10}]-ACTH-(1–10)-$NHNH_2$ are intermediates in the syntheses of [Ala^{10}]-ACTH-(1–18)-NH_2 and [D-Ala^{10}]-ACTH-(1–18)-NH_2, respectively, and the epimeric position is at the C-terminal amino acid residue. The acetonitrile content of the mobile phase had to be increased to 22.5%, as shown in Fig. 11B, although there are eight less constituent amino acids in these epimers than in the octadecapeptides mentioned above. The order of elution of these two decapeptide epimers was similar to that of the octadecapeptides derived from these two, *i.e.*, the L-isomer was eluted faster than the D-isomer*.

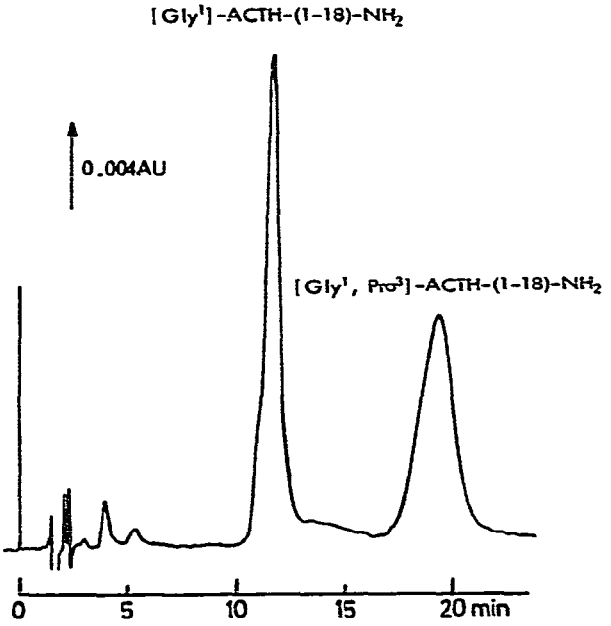


Fig. 12. Separation of [Gly^1]-ACTH-(1–18)-NH_2 and [Gly^1, Pro^3]-ACTH-(1–18)-NH_2. Content of acetonitrile in the mobile phase: 16%.

The separation of [βAla^1, D-Phe^7, Orn^{15}]-ACTH-(1–18)-NH_2 and [βAla^1, Orn^{15}]-ACTH-(1–18)-NH_2 (Fig. 4) was also possible with 18% of acetonitrile, as is clearly shown in Fig. 11C. In this instance the D-isomer was eluted faster than the L-isomer. The D-Phe analogue prepared was now found by HPLC to be impure, but the impurities could not easily be detected by thin-layer chromatography (TLC) with 1-butanol–acetic acid–pyridine–water systems as solvent. Peptide epimers

* The use of a D(−)-tartrate buffer did not alter the order of elution.

consisting of more than ten amino acid residues are generally very difficult to separate by other methods. The same kind of separation of peptide diastereoisomers by the reversed-phase HPLC has recently been published[8]. Although the conformational differences between these epimers are not known, the easy separation of these epimers by reversed-phase chromatography suggests that the replacement of an amino acid residue with its optical isomer appreciably affects the molecular conformation even if the epimeric position is terminal, because in reversed-phase chromatography the hydrophobicity of the molecule is the most important factor governing the order of elution.

[Gly^1]-ACTH-(1–18)-NH_2 and its 3-substituted analogue (Fig. 5) were separated with 18% of acetonitrile, as shown in Fig. 12. The different retention times observed for [Gly^1]-ACTH-(1–18)-NH_2 in Figs. 9 and 12 may be attributable to a small difference in the acetonitrile content of the mobile phase, as mentioned above. Pro substituted for Ser in position 3 is considered to be more hydrophobic than the latter. [Aib^1]-ACTH-(1–18)-NH_2 and [Aib^1, Nle^4]-ACTH-(1–18)-NH_2 (Fig. 5) were also easily separated under the same conditions and the retention times were 13.2 and 27.7 min, respectively. The Nle-containing analogue, which was shown to be considerably more hydrophobic than the parent peptide, is prepared by the replacement of sulphur by a methylene group in the 2-methylthioethyl side-chain of Met in position 4.

Attempts were also made to separate two pairs of ACTH peptides, each differing by only one methylene group in position 15, *viz.*, a mixture of [βAla^1]-ACTH-(1–18)-NH_2 and [βAla^1, Orn^{15}]-ACTH-(1–18)-NH_2 and a mixture of [Aib^1]-ACTH-(1–18)-NH_2 and [Aib^1, Orn^{15}]-ACTH-(1–18)-NH_2 (see Fig. 4). They were subjected to HPLC under the same conditions as above. In both instances, however, the two peptides could not be separated even when the content of acetonitrile was reduced to 16%. The retention time of the former mixture was 10.8 min and that of the latter was 13.2 min with 18% of acetonitrile. The relationship between the 4-aminobutyl and 3-aminopropyl side-chains of Lys and Orn is similar to that between βAla in [βAla^1]-ACTH-(1–18)-NH_2 and Gly in [Gly^1]-ACTH-(1–18)-NH_2. The mixture of ACTH peptides with Lys and Orn in position 15 was the only example that showed no separation under the conditions used.

The chromatogram shown in Fig. 13 indicates that ACTH-(1–18)-NH_2 and [Lys^5, Ser^{11}]-ACTH-(1–18)-NH_2 are separable; the latter has the structure obtained by exchanging the amino acid residues in positions 3 and 11 of the former (Fig. 5). The separation of these two isomers cannot be explained in terms of the difference in the hydrophobic properties of their constituent amino acid residues but in terms of the difference in their molecular hydrophobicities.

As ACTH has one Met in position 4 which is liable to be oxidized to Met S-oxide (Fig. 1), the S-oxide could be one of the impurities observed in the chromatograms of ACTH analogues. The S-oxide of [Gly^1]-ACTH-(1–18)-NH_2 was eluted much faster than its parent compound, as shown in Fig. 14. Judging from this observation the minor peaks observed between 5 and 6 min in Fig. 9A may be assigned to the S-oxides.

There are two methods for the synthesis of polypeptides, the solution and the solid-phase techniques. Preparations of LH-RH synthesized by the two methods were subjected to HPLC in order to establish whether there are any differences between

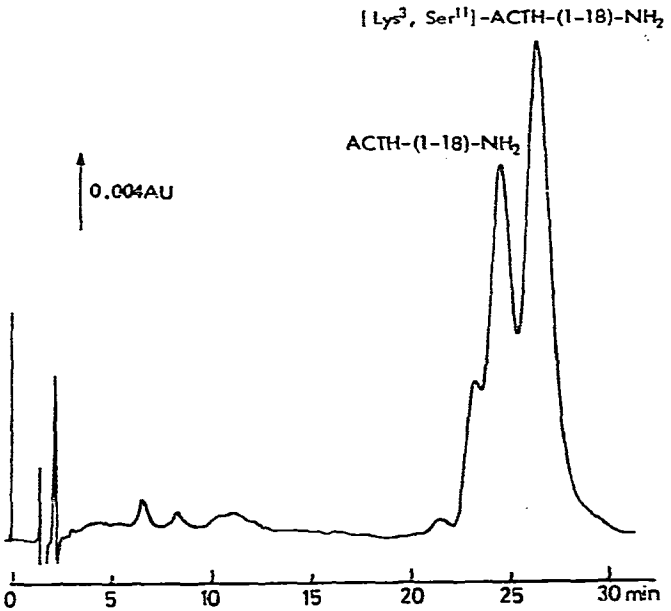


Fig. 13. Separation of two isomeric octadecapeptides with the same amino acid compositions but different sequences. Content of acetonitrile in the mobile phase: 16%.

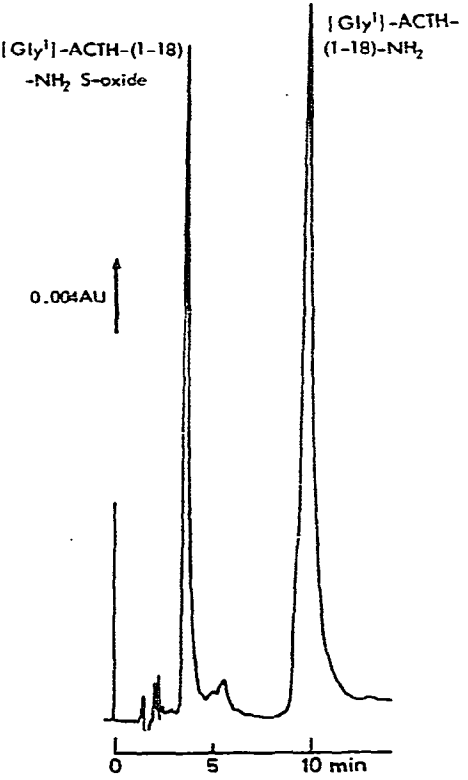


Fig. 14. Separation of [Gly¹]-ACTH-(1–18)-NH_2 and its S-oxide. Content of acetonitrile in the mobile phase: 18%.

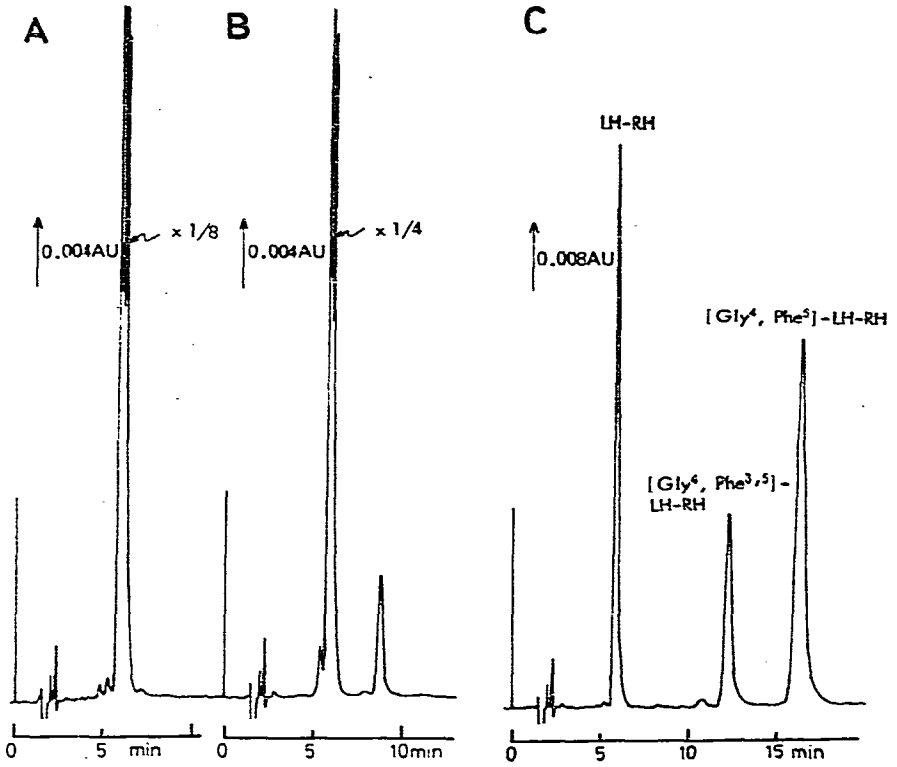


Fig. 15. Chromatograms of LH-RH and analogues. Content of acetonitrile in the mobile phase: 21%. A, LH-RH synthesized by the solid-phase method (1.7 μg); B, LH-RH synthesized by the solution method (0.96 μg); C, a mixture of LH-RH, [Gly⁴,Phe³,⁵]-LH-RH and [Gly⁴,Phe⁵]-LH-RH.

them. Fig. 15A is a chromatogram of LH-RH which was synthesized by the solid-phase method and purified very carefully and extensively. Fig. 15B shows an HPLC pattern of LH-RH synthesized by the solution method.

Fig. 15A shows that the solid phase-synthetic product is of high purity. Numerous but small amounts of impurities were detected only when a large amount of sample was injected on to the column. In contrast, Fig. 15B reveals that the product of the solution method contains a relatively small number of impurities but their amounts are not always small. These observations clearly indicate that in the synthesis of peptides with about ten amino acid residues the carefully controlled solid-phase method can replace the solution method. However, the biological activities of impurities are not always proportional to their amounts and it is not easy to judge which method is generally better.

Fig. 15C shows the separation of three analogues of LH-RH (Fig. 6). These peptides proved to be very pure compared with the ACTH analogues examined above. The order of elution of the three analogues suggests that Trp is more hydrophobic than Phe .

Both bovine and porcine insulins are usually commercially available as crystals containing zinc. In this study it was found that an insulin containing zinc and the corresponding zinc-free insulin gave identical chromatograms, indicating that the

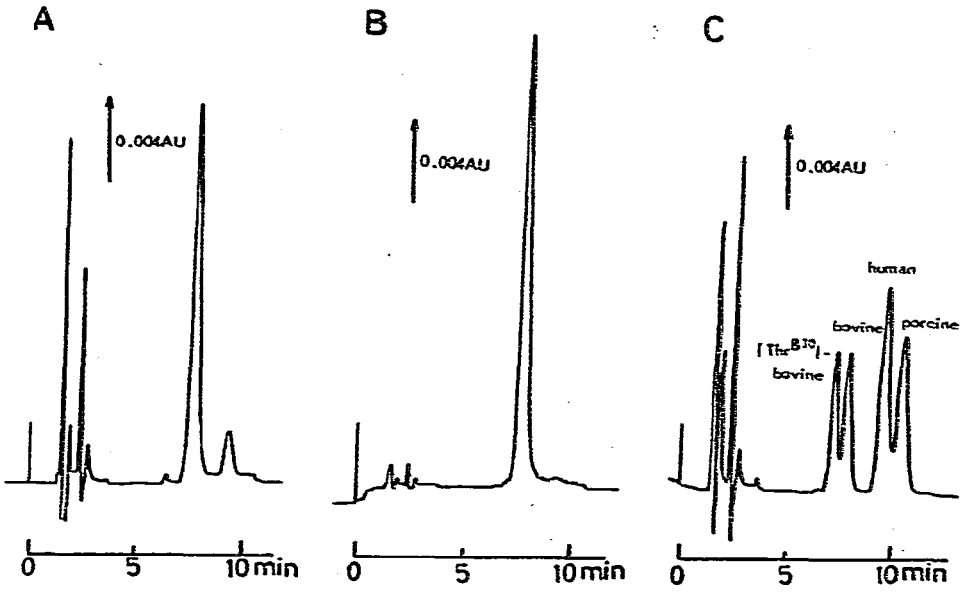


Fig. 16. Chromatograms of insulins. Content of acetonitrile in the mobile phase: 29.2%. A, Bovine insulin (Calbiochem, 3 μg); B, purified bovine insulin (3 μg); C, a mixture of purified [Thr^{B30}]-bovine, bovine, human and porcine insulins.

zinc insulin was dissociated into free insulin under the chromatographic conditions. The chromatogram of bovine insulin purchased from Calbiochem (Fig. 16A) showed the presence of at least two minor components. Bovine insulin purified on a QAE-Sephadex A-25 column (monocomponent insulin[26]) is shown in Fig. 16B, where the insulin is found to be almost free of impurities. Insulins (Fig. 7) consist of two peptide chains, the A-chain containing 21 amino acid residues and the B-chain containing 30 amino acid residues, connected by two disulphide bridges. Their molecular weights are *ca.* 6000. Human insulin differs from porcine insulin by a single amino acid residue in position B30. Ala occupies position B30 in porcine insulin, and Thr in human hormone. Bovine insulin differs from porcine hormone by two amino acid residues on the A-chain. Positions A8 and A10 are the sites of difference; Ala and Val are present in bovine insulin and Thr and Ile are in porcine hormone. Fig. 16C shows a good separation of four kinds of pure insulins, [Thr^{B30}]-bovine, bovine, human and porcine insulins, thus demonstrating that our HPLC system can separate these closely related polypeptides which differ from each other by one out of 51 amino acid residues. Porcine insulin shows a retention time considerably longer than that of bovine insulin. This seems to be partly due to the difference in hydrophobicity between the Ile and Val in position A10 rather than the Thr and Ala in position A8, because Thr has an extra methylene group compared with Ala, but it also has a hydroxyl group.

The relationship between the orders of elution and structures of peptides have been discussed above with reference to each chromatogram. In most chromatograms the order of elution of closely related peptides can be explained in terms of the hydrophobicity of the amino acid residues which lead to differences among these peptides. In some instances, however, this interpretation is invalid. For instance,

[Gly^1]-ACTH-(1–18)-NH_2 is eluted faster than [βAla^1]-ACTH-(1–18)-NH_2 (Fig. 9B), and this order of elution is reversed with ACTH-(1–18)-NH_2 and [βAla^{10}]-ACTH-(1–18)-NH_2 (Fig. 10A). The replacement of one amino acid residue by its enantiomer may alter the conformation of the molecule, thus making a large difference in the partition coefficients of the epimers (Fig. 11). A pair of peptides such as that shown in Fig. 13, with the same amino acid composition but different sequences, should also have different conformations and, therefore, may have different affinities to the stationary phase.

All ACTH peptides and their analogues investigated were synthesized by the solution method and carefully purified by CM-cellulose column chromatography and/or partition chromatography. Most of them were found to be homogeneous on TLC (see references cited above). In the present HPLC analyses of these peptides, however, a few minor peaks other than the main peak were observed in every chromatogram, indicating that HPLC is a very useful means for evaluating in a short time the purity of peptides with molecular weights up to *ca.* 6000. In the chromatograms the peak areas must be approximately proportional to the amounts of peptides if the elution is monitored at 220 nm, because the relative absorbances of a series of analogues should be very similar. However, the amounts of samples indicated in the figure captions are not very accurate and have not been corrected for the water content of the samples. Therefore, one should not estimate the absolute purity of the peptide samples from their peak areas.

Ion-pair reversed-phase chromatographic conditions were chosen because ACTH contains many basic amino acid residues. Simple reversed-phase chromatographic conditions can also be applied to LH-RHs and insulins. In fact, a trialkylammonium phosphate buffer–acetonitrile system has been used successfully as a mobile phase for various peptides including LH-RH and insulin[6]. Some other eluting agents, *e.g.*, phosphate buffer (0.01 *M*, pH 3.0)–acetonitrile and tartrate buffer (0.005 *M*, pH 3.0)–acetonitrile, were tested for the separation of insulins, but the resolution was poorer than that obtained with the mobile phase used throughout this study. It should be noted that the retention times varied considerably unless the relative content of acetonitrile in the mobile phase was carefully kept constant, but slight changes in pH or the concentration of the buffer were not critical. These observations are similar to those reported by Rivier[6].

CONCLUSION

It has been shown that the separation of closely related peptides such as ACTH, LH-RH, insulin and their analogues can be effected successfully by use of a chemically bonded C_{18} stationary phase and a mixture of acetonitrile and tartrate buffer containing sodium 1-butanesulphonate and sodium sulphate as the mobile phase. This technique is also very useful for the rapid examination of the purities of peptides.

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