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Note

Reversed-phase high-performance liquid chromatography of neuropeptides related to adrenocorticotropin, including a potent adrenocorticotropin 4—9 analogue (ORG 2766)

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Neuropeotides are fragments of pituitary and brain polypeptides that influence brain functions [1]. One class of these peptides is comprised of fragments derived from adrenocorticotropic hormone (ACTH; Fig. 1). In both the pituitary gland and the brain, ACTH can be enzymatically generated from a precursor molecule of molecular weight 31,000 and further processed into α-melanocyte-stimulating

	1	5	10	15	20
Human	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-				
Porcine					
Ovine, Bovine					

	21	25	30	35	39
Human	Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe				
Porcine			-Leu-		
Ovine, Bovine				-Gln-	

Fig. 1. Structure of human ACTH and comparison with ACTH from other species.

hormone (α-MSH, $N^{\alpha 1}$-acetyl, $C^{\alpha 13}$-ACTH 1—13-amide) and corticotropin-like intermediate lobe peptide (CLIP; ACTH 18- 39) [2, 3]. Accumulating evidence exists that ACTH and α-MSH as well as N-terminal fragments that are virtually devoid of classical endocrine activities, affect adaptive behaviour in laboratory animals and in man through direct actions on the central nervous system [1, 4, 5]. In addition, analogues of N-terminal fragments of ACTH, e.g. [Met$(O_2)^4$,D-Lys^8, Phe^9]ACTH 4—9 (ORG 2766), which exhibit remarkably increased

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behavioral potencies as compared to their parent peptides, have been synthesized [6, 7]. From these observations it has been postulated that endogenous ACTH can act as a precursor which is enzymatically converted into smaller, behaviourally active fragments [1]. In the investigation of this hypothesis, the application of reversed-phase high-performance liquid chromatography (HPLC) is virtually indispensable. This technique has been established as a powerful tool for the separation of complex peptide mixtures [8—11]. Resolution of various ACTH-related peptides has been described using a large variety of mobile phase combinations, including ammonium acetate and/or acetic acid [10, 12], phosphoric acid [9, 13], trifluoroacetic acid (TFA) [14–17], heptafluorobutyric acid (HFBA) [15], or triethylammonium phosphate (TEAP) [18] in the aqueous solvent. The addition of lyophilizable compounds such as ammonium acetate, TFA or HFBA in the mobile phase allows good peptide recoveries for further analysis procedures, in contrast to the use of phosphate or TEAP.

In this paper we report the resolution of ACTH 1—39 and various fragments, including peptides that have not yet been investigated, by reversed-phase HPLC with the ion-pairing reagent TFA in the mobile phase. In addition, using ammonium acetate buffers, we present the separation between numerous N-terminal fragments of ACTH as well as between an ACTH 4–9 analogue (ORG 2766) and several of its peptide metabolites that could arise upon enzymatic digestion [19].

EXPERIMENTAL

Chemicals and peptides

All chemicals were of analytical grade. Ammonium acetate and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, G.F.R.). Glacial acetic acid was supplied by Fluka (Buchs, Switzerland). Methanol of HPLC grade was purchased from Baker (Deventer, The Netherlands).

pACTH 1—39 was isolated from porcine pituitary glands by Organon International (Oss, The Netherlands). Human ACTH 18—39 (CLIP; corticotropin-like intermediate lobe peptide) was a generous gift of Dr. P.J. Lowry (St. Bartholomew's Hospital, London, Great Britain). All other peptides were synthesized, purified and kindly donated by Dr. H.M. Greven and Dr. J.M. van Nispen (Organon, Oss, The Netherlands).

Equipment

The HPLC assembly (Waters Assoc., Milford, MA, U.S.A.) consisted of two Model 6000A solvent delivery pumps, a Model 600 solvent programmer for gradient elution, a universal U6K injector and a reversed-phase μBondapak C_{18} column (300 × 3.9 mm I.D., particle size 10 μm). The column effluent was monitored continuously for UV absorbance at 210 nm (1.0 a.u.f.s.) with a Schoeffel variable-wavelength UV monitor (Model 770) equipped with an 8-μl flow-through cell. UV absorbance was recorded on a two-channel range recorder (BD9, Kipp en Zonen, Delft, The Netherlands) with a chart speed of 0.5 cm/min. Solvents were filtered and degassed using Pyrex filter holders with 0.5-μm pore diameter filters from Millipore (Bedford, MA, U.S.A.).

Chromatography

Mixtures of pACTH 1—39 and its fragments were chromatographed at ambient temperature by gradient elution with 0.09 *M* TFA, pH 1.9 (solvent A) and methanol (solvent B). Upon injection of a peptide sample, the column was eluted at 1 ml/min with a linear gradient from 60% A (40% B) to 30% A (70% B) over 25 min.

Separation of N-terminal fragments of ACTH 1—39 was performed at ambient temperature with a 30-min convex gradient (program 5) of 0.01 *M* ammonium acetate adjusted to pH 4.2 with glacial acetic acid (solvent X) and methanol containing 1.5 ml of acetic acid per liter (solvent Y); initial conditions X/Y = 95:5, final conditions X/Y = 50:50. The flow-rate was 2 ml/min.

Chromatography of an ACTH 4—9 analogue (ORG 2766) and several of its peptide metabolites was carried out at 2 ml/min with a 30-min linear gradient of 0.01 *M* ammonium acetate, pH 4.2 (solvent X) and methanol (solvent Y) as mobile phases; initial conditions X/Y = 95:5, final conditions X/Y = 65:35.

RESULTS AND DISCUSSION

The resolution of ACTH 1—39 and related peptides following RP-HPLC on a μBondapak C_{18} column with a linear gradient of 0.09 *M* TFA and methanol, is shown in Fig. 2. The chromatographic conditions are adapted to those intro-

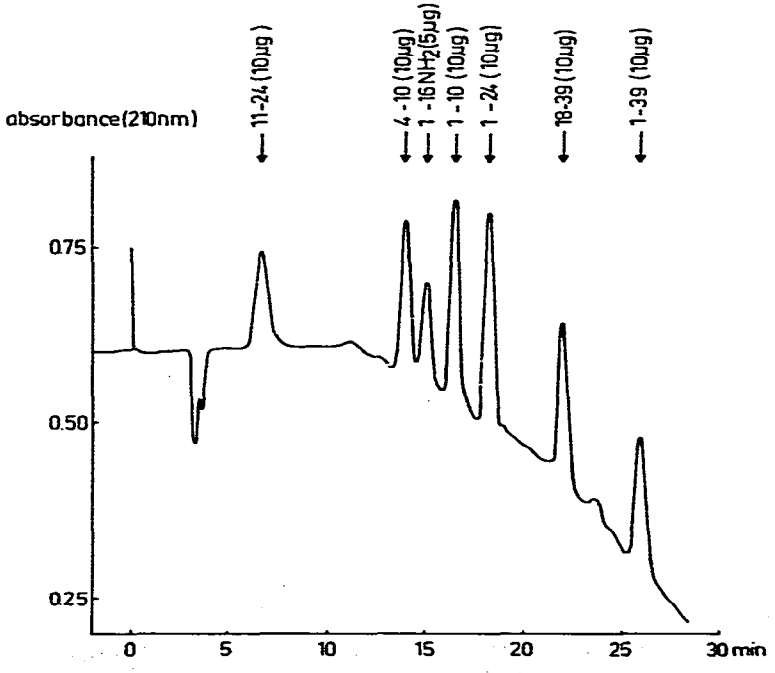

Fig. 2. Chromatography of a mixture of ACTH 1—39 and various fragments (5—10 μg of each peptide) on μBondapak C_{18}. Elution was performed with a 25-min linear gradient of 0.09 *M* TFA, pH 1.9 (A) and methanol (B). Initial conditions, A/B = 6:4; final conditions, A/B = 3:7. The flow-rate was 1 ml/min. UV absorbance was measured at 210 nm (1.0 a.u.f.s.). The abbreviations refer to ACTH 1—39 as the basic sequence (see Fig. 1).

duced by Bennett et al. [14]. The use of the hydrophilic ion-pairing reagent TFA, which is volatile and is transparent at 210 nm, can result in sharp and symmetric peptide peaks. The elution order of the peptides investigated is in agreement with that found by others [14—17]. It seems to be correlated more with the total hydrophobicity of the peptide than with the chain length. For example, $C^{\alpha 16}$-ACTH 1—16-amide has a shorter retention time than its fragment ACTH 1—10, probably because the presence of three hydrophilic lysine residues within the C-terminal sequence 11—16 (Fig. 1) more than compensates for the effect of increased chain length. Similar considerations might explain the elution of ACTH 11—24 before the shorter ACTH sequences 4—10 and 1—10. Under the conditions used in Fig. 2, no resolution was accomplished between the peptide pairs ACTH 11—24 and ACTH 4—7, ACTH 4—9 and ACTH 4—10, ACTH 1—24 and α-MSH, and no baseline separation between pACTH 1—39 and pACTH 25—39. However, by increasing the gradient time from 25 min to 50 min, baseline separation was obtained between α-MSH and ACTH 1—24 as well as between pACTH 25—39 and pACTH 1—39 (data not shown).

Using TFA in the mobile phase for HPLC analysis, the chromatographic profile of ACTH-related peptides appeared to be independent of both the amounts of peptides applied to the column and of injection volumes up to 1 ml. Decreased resolution was observed when the TFA concentration was lower-

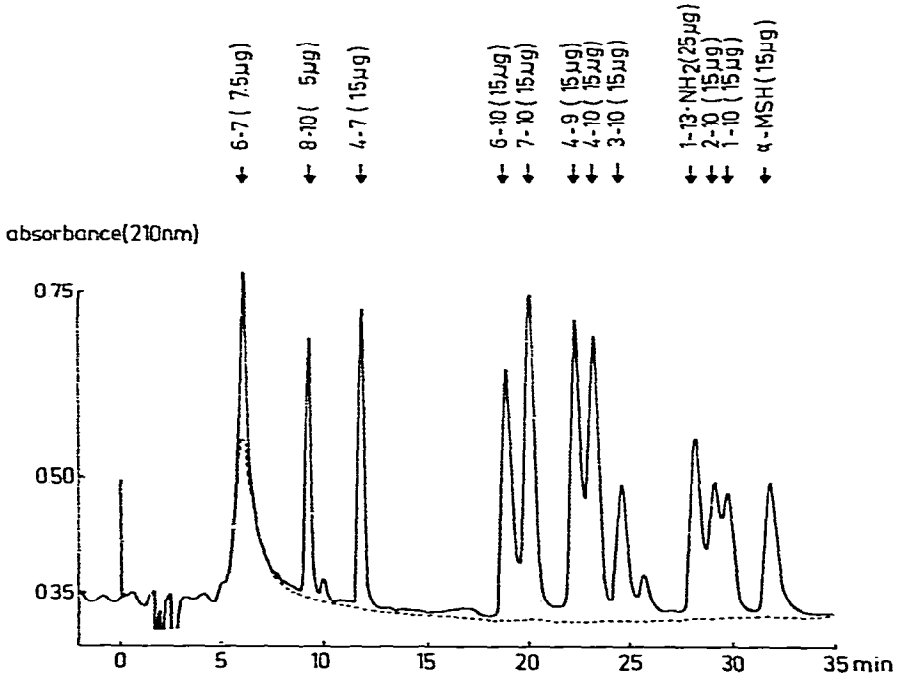

Fig. 3. Separation of a mixture of N-terminal fragments of ACTH (5—25 µg of each peptide, as indicated in the figure) on µBondapak C_{18}. Elution was carried out with a 30-min convex gradient (program 5) of 0.01 *M* ammonium acetate, pH 4.2 (X) and methanol, containing 1.5 ml/l acetic acid (Y). Initial conditions, X/Y = 95:5; final conditions, X/Y = 50:50. The flow-rate was 2 ml/min. UV absorbance was measured at 210 nm (1.0 a.u.f.s.). The baseline in the UV profile of the gradient used is shown by the interrupted line. The abbreviations refer to ACTH 1—39 as the basic sequence (see Fig. 1).

ed from 0.09 *M* to 0.009 *M*, in contrast to previously reported findings [15, 16]. During the lifetime of the columns, peak broadening and increased retention times were noticed for the peptides investigated. Nevertheless, the peak areas remained quite reproducible both on one column with time and from column to column. Extensive washing of the columns restored the original sharp peptide peaks and shorter retention times.

The separation of various N-terminal fragments of ACTH ($\leqslant$ 13 amino acid residues) by reversed-phase HPLC on μBondapak C_{18} using a convex gradient of 0.01 *M* ammonium acetate (pH 4.2) and methanol, is presented in Fig. 3. The high resolving power of the system allows satisfactory resolution of oligopeptides that differ in only one amino acid residue, e.g. ACTH 6- 10 from ACTH 7—10, ACTH 4—9 from ACTH 4—10, and ACTH 2—10 from the sequences 3—10 or 1—10. Of all the peptides studied, only ACTH 5—10 and $C^{\alpha 16}$-ACTH 1—16-amide appear to coelute with ACTH 6—10 and ACTH 4—9, respectively. In general, the retention times are proportional to the peptide chain lengths, as has also been reported for β-endorphin fragments using similar HPLC systems [11]. In addition, the retention times of the peptides depend on their overall hydrophobicity as was found with the resolution of ACTH-like peptides in the TFA system. This is illustrated by the observation that the basic peptide $C^{\alpha 16}$-ACTH 1—16-amide has a lower retention than its shorter, more hydrophobic fragments ACTH 4—10, $C^{\alpha 13}$-ACTH 1—13-amide (des-$N^{\alpha 1}$-acetyl-α-MSH) or ACTH 1—10 (Fig. 3). It is noteworthy that large, basic peptides such as ACTH 1—24 and ACTH 1—39 tend to stick to reversed-phase μBondapak C_{18} columns during elution with mixtures of 0.01 *M* ammonium acetate and methanol, resulting in peak broadening and peak tailing (data not shown). In contrast, using the ion-pairing reagent TFA in the mobile phase, sharp peaks without deterioration of peak shape can be obtained for ACTH 1—24 and ACTH 1--39 (Fig. 2).

The resolution of an ACTH 4—9 analogue (ORG 2766) and its fragments on μBondapak C_{18} with a linear gradient of 0.01 *M* ammonium acetate (pH 4.2) and methanol, is depicted in Fig. 4. The fragments investigated represent peptide intermediates that could arise as radioactive metabolites following enzymatic degradation of [^{3}H-*Phe*^{7}]ORG 2766 [19]. The chromatogram shows excellent separation between these oligopeptides ranging in size from two to seven amino acid residues, sharp and symmetric peptide peaks, and retention times correlating with their chain lengths. Resolution was not achieved between the dipeptides His^6-Phe^7 and Phe^7-D-Lys^8 and between the N-terminal sequences 4—7 and 4—8. Using ammonium acetate in the mobile phase, the separation profiles of the ACTH peptides investigated were affected neither by differences in the amounts of peptide injected nor by differences in injection volumes, similar to the findings observed with the TFA system. An attendant advantage of the ammonium acetate system appeared to be that the peptide peak shapes and retention times hardly changed with increasing column lifetime, in contrast to the results obtained with TFA in the mobile phase. In the ammonium acetate system used, the addition of acetic acid to the organic solvent leads to more stable baseline recordings (compare Fig. 3 with Fig. 4). For gradient elution with the TFA system baseline flattening can be achieved by adding TFA to methanol (3.5 ml/l), although gradual increases of the gradient baseline during

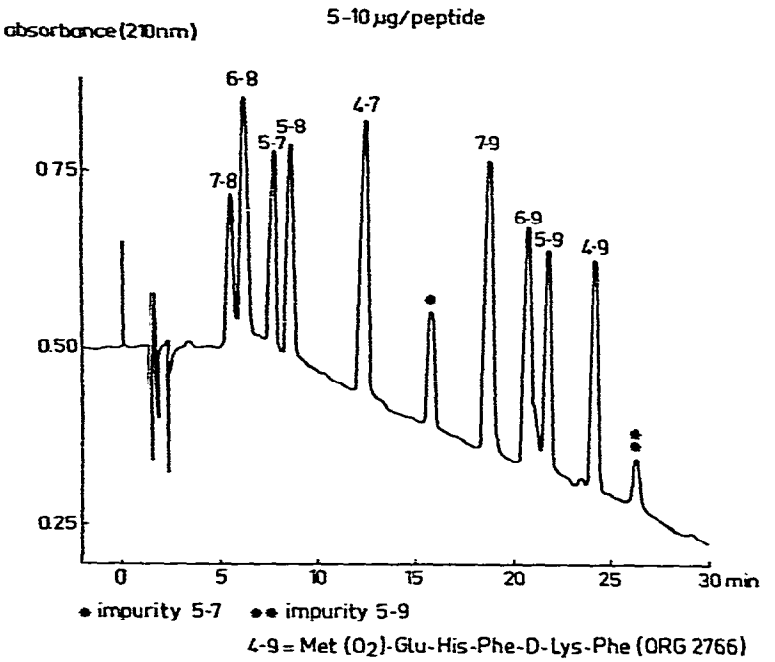

Fig. 4. Chromatography of a mixture of an ACTH 4—9 analogue (ORG 2766) and various fragments (5—10 µg of each peptide) on µBondapak C_{18}. Elution was performed with a 30-min linear gradient of 0.01 *M* ammonium acetate, pH 4.2 (X) and methanol (Y). Initial conditions, X/Y = 95:5; final conditions, X/Y = 65:35. The flow-rate was 2 ml/min. UV absorbance was measured at 210 nm (1.0 a.u.f.s.). The abbreviations refer to Met(O_2)-Glu-His-Phe-D-Lys-Phe = 4—9 (ORG 2766) as the basic structure.

the day have been noticed. Neither the resolution nor the retention times of the peptides studied were influenced by the presence of acetic acid or TFA in the organic solvent.

CONCLUSIONS

The data reported here demonstrate that reversed-phase HPLC employing either TFA or ammonium acetate in the mobile phase provides suitable resolution of ACTH-related neuropeptides. Both TFA and ammonium acetate are easily removed by sample evaporation or lyophilization, allowing good peptide recoveries. Elution systems containing the ion-pairing reagent TFA are particularly successful for the separation of larger and basic peptides such as ACTH 1—24 and ACTH 1—39, which appear to tail in ammonium acetate systems. On the other hand, using ammonium acetate buffers, high resolution can be obtained for ACTH-related oligopeptides, as has been shown for various N-terminal ACTH fragments as well as for an ACTH 4—9 analogue and its shorter fragments.

Reversed-phase HPLC with both TFA and ammonium acetate buffer systems is presently being applied to studies of ACTH 1—39 degradation by membrane preparations from rat brain. In addition, the metabolic fate of [^{3}H-*Phe*^{7}]ORG

2766 in rat plasma is under investigation using ammonium acetate in the mobile phase.

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