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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION AND CHARACTERIZATION OF THE ADRENOCORTICO-TROPIN/LIPOTROPIN PRECURSOR AND ITS FRAGMENTS

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SUMMARY

Methods are presented for the effective purification by reversed-phase high-performance liquid chromatography (HPLC) of rat adrenocorticotropin/lipotropin (ACTH/LPH) precursor and its two glycosylated forms. Purification of its NH_2-terminal segment from human and porcine pituitaries is presented together with microsequencing data confirming the identity of the purified peptides. The effective separation of various native fragments related to ACTH and β-LPH from sheep pituitaries is presented. A new putative γ-MSH hormone has been synthesized and purified by reversed-phase HPLC and tryptic peptide mapping performed to establish the identity of the purified peptide.

INTRODUCTION

It is now well established that both adrenocorticotropin (ACTH) and β-lipotropin (β-LPH) and their fragments, β-endorphin, γ-LPH and α-MSH, arise from the specific cleavage of a common precursor molecule, named ACTH/LPH precursor[1–6]. From the complete cDNA sequence of this precursor a new peptide bearing homologies to the MSH sequences[7] has been located within the N-terminal segment of the ACTH/LPH precursor. It was named γ-MSH[6]. Through a series of naturation steps such a precursor undergoes cleavages to release the above mentioned peptides together with an N-terminal fragment which contains γ-MSH within its sequence[4–6].

In order to study these peptides, purification methods are necessary and several approaches, including ion-exchange and gel-permeation methods, have been attempt-ed[4,9,10]. The problems associated with the ion-exchange methods are poor resolution of ACTH and β-endorphin and the adsorption of the ACTH/LPH precursor on the columns[4]. Therefore, the object of this investigation was to develop and evaluate methods of effective rapid purification of this wide spectrum of peptides using reversed-phase high-performance liquid chromatography (RP-HPLC) after their extraction from the pituitary of various species. Several recent papers have shown that peptides

related to ACTH and β-LPH can be separated by RP-HPLC[6-16]. In this report a single ion-pair RP-HPLC system using the triethylamine phosphate (TEAP) buffer introduced by Rivier[12] has been evaluated in its ability to achieve the purification of all the peptides related to the ACTH/LPH precursor. Coupled to the RP-HPLC purification scheme either methods of assessing purity have been used. These included microsequencing, peptide mapping followed by amino acid analysis and electrophoretic analysis.

MATERIALS AND METHODS

The HPLC apparatus used consisted of a Waters Assoc. Model 204 liquid chromatograph, including Model U6K injector, two Model 6000A pumps, Model 660 programmer and Model 450 variable-wavelength detector and Model 730 data module. Waters Assoc. μBondapak CN and C_{18} (30 cm × 4 mm) analytical columns were used. Full scale UV absorbance is expressed in AUFS. All runs were performed at room temperature. The triethylamine phosphate buffer was obtained by bringing the pH of a 0.2 N phosphoric acid to 3.0 with triethylamine (Pierce, Rockford, IL, U.S.A.). The aqueous buffer consisted of 0.02 M TEAP pH 3.0 obtained by diluting 10 × the stock solution. The organic phase consisted of 90% acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and 10% 0.2 N TEAP pH 3. All gradients were linear (curve No. 6 on Model 660 programmer) and are represented by dashed lines on the figures. More specific details can be found in the respective figure legends. All buffers were degassed by sonication and filtered through 0.45-μm millipore filters before use.

The ACTH/LPH precursor from rat pars intermedia was obtained after 20 min pulse incubation of the isolated tissue from 20 rats with 1 mCi of [^{35}S]methionine or 0.5 mCi of [^{3}H]Arg (New England Nuclear, Boston, MA, U.S.A.; specific activity 810 and 21.7 Ci/mmol, respectively) as previously reported[4,5]. In the case of the [^{35}S]Met incubation a desalting on Sephadex G-75 (Pharmacia, Uppsala, Sweden) was performed.

The NH_2-terminal fragment of the ACTH/LPH precursor from anterior lobes of porcine and human pituitaries was obtained following an acetone–HCl extraction procedure[17] and a CM-cellulose purification[4].

All other peptides including ACTH, γ-LPH, β-LPH, β-endorphins were extracted from sheep pituitaries, routinely done in this laboratory. The peptides α-MSH (Ciba-Geigy, Basel, Switzerland), ACTH[1-8] and γ-MSH[1-13] were kindly synthesized for us by Dr. S. St-Pierre (Sherbrooke, Canada).

Sodium dodecyl sulfate (SDS)–slab gel polyacrylamide electrophoresis in a discontinous gradient was done according to Laemmli[18] using a 10–30% gradient.

Specific labeling of cysteine residues with [^{14}C]iodoacetamide (NEN) was done following reduction with β-mercaptoethanol in the presence of 8 M urea[19].

Amino acid sequence analysis of radiolabeled peptides was performed on a Beckman 890B sequenator using a 0.3 M quadrol program[4,5,10] in the presence of 3 mg of polybrene (Aldrich, Milwaukee, WI, U.S.A.).

RESULTS

Purification of rat biosynthetic pars intermedia
    ACTH/LPH precursor. Following a 20-min pulse in incubation of rat pituitary pars intermedia with either [^{35}S]Met or [^{3}H]Arg, extraction of the cells with 5 N acetic acid[4,5] and desalting, the pattern of incorporated radioactivity is seen in Fig. 1A, C and D. Fig. 1Da represents the separation on SDS slab gel electrophoresis of the [^{35}S]Met labeled proteins extracted. Fig. 1Db represents the same SDS pattern following purification by RP-HPLC on a μBondapak CN column collecting fractions and counting an aliquot (1/50) as shown in Fig. 1A (fraction 90–95).
    It is seen that two labelled molecules of apparent molecular weight of 34,000 (34K) and 36,000 (36K) are purified. These two glycosylated variants of the ACTH/LPH precursor have been previously reported[5]. Tryptic digestion and performic acid oxydation[5] of the purified peptides (fraction 90–95) was performed. Upon peptide mapping of the digest on a μBondapak C^{18} column, as shown in Fig. 1B, two peaks coeluting with synthetic oxydized β-LPH (61–69) and ACTH (1–8) are seen, regions of the molecule expected to contain methionines[5]. Hence this proves that the precursor purified contains the ACTH and β-LPH molecules within its sequence.
    Next the problem of separating the two glycosylated forms of the precursor was tackled. For this it was found that using the same buffer system on a μBondapak CN column but collecting smaller fractions one can separate, though with difficulty, the two forms of the precursor as shown in Fig. 1C, which depicts such a separation on the [^{3}H]Arg neosynthesized ACTH/LPH precursor.
    Figures 1Dc–f show that the separation of the two glycosylated forms is possible by shaving the rising and descending parts of the main peak, though at a loss since the intermediary forms containing major 36K, minor 34K (Fig. 1Dd) and *vice versa* (Fig. 1De) are predominant.

Purification of NH$_2$-terminal segment of ACTH/LPH from human and porcine anterior pituitaries
    The details of the experimental procedures used to extract and partially purify these peptides will form the object of a forthcoming publication[20]. From the amino acid sequence of the bovine ACTH/LPH precursor reported[6], it can be deduced that the NH$_2$-terminal segment of the rat cellular form[8] of the precursor contains cysteine residues at positions 2, 8, 20, 24 of the peptide chain. It is also known that in both the pars intermedia and pars distalis of the pituitary, the ACTH/LPH precursor undergoes a series of maturation steps leading to the formation of an NH$_2$-terminal segment with an apparent molecular weight on SDS gels of about 19,000 (19K) (ref. 5) together with β-LPH, ACTH for the pars distalis and α-MSH/β-endorphin/ γ-LPH for the pars intermedia.
    In our search for such an NH$_2$-terminal segment we used the method of labeling cysteine residues with [^{14}C]iodoacetamide[19] followed by amino acid sequence in order to follow our purification procedures. The product obtained from both human and porcine pituitary possessing the correct sequence anticipated was then finally purified by RP-HPLC on a μBondapak C$_{18}$ column using the TEAP system (see Materials and methods).
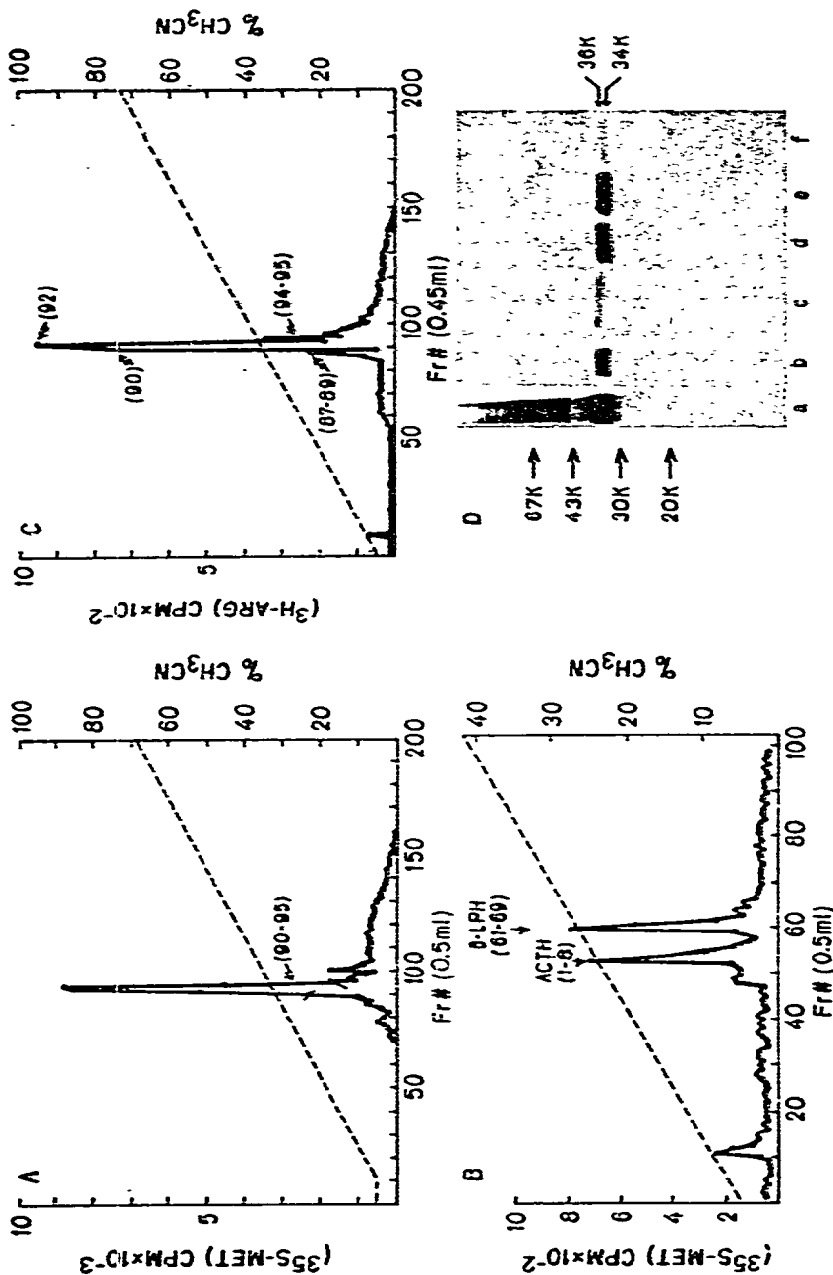
Fig. 1. Separation of rat biosynthetic pars intermedia ACTH/LPH precursor labeled either with [^{35}S]Met or [^{3}H]Arg. Fr #= Fraction number. A, Preparative RP-HPLC on a μBondapak CN column of [^{35}S]Met-labeled proteins extracted from rat pars intermedia and desalted (see Materials and methods). Elution was done with 5% CH$_3$CN isocratically for 5 min followed by an acetonitrile gradient of 0.68%/min at 1 ml/min. Fractions of 0.5 ml were collected and aliquots (1/10) were counted. B, RP-HPLC on a μBondapak C$_{18}$ column of 24-h tryptic digest followed by performic acid oxidation^{s} of purified [^{35}S]Met labeled ACTH/LPH precursor (fraction 90-95 of Fig. 1A). The positions of the oxidized markers ACTH (1–8) and β-LPH (61–69) are shown. The linear gradient has a slope of 0.83% acetonitrile/min at 1 ml/min. Fractions of 0.5 ml were collected and counted. C, Preparative RP-HPLC on a μBondapak CN column of [^{3}H]Arg ACTH/LPH precursor. Elution conditions are identical to those in Fig. 1A except that 0.45-ml fractions were collected and aliquots (1/10) were counted. D, SDS-slab polyacrylamide gel electrophoresis autoradiograms^{s} of various purified fractions from Figs. 1A and 1C. a, [^{35}S]Met ACTH/LPH before purification on μBondapak CN; b, [^{35}S]Met ACTH/LPH after purification on μBondapak CN, fractions 87-89, 90, 92 and 94-95, respectively. c-f, [^{3}H]Arg labeled ACTH/LPH from Fig. 1C, fractions 87-89, 90, 92 and 94-95, respectively.

In Fig. 2a, b such a purification of either the cold native porcine material or the [14C]iodoacetamide labeled human peptide is depicted. Incorporation of [14C]-iodoacetamide into material of peaks A and C from Fig. 2a, proved negative, hence showing the absence of cysteine residues in these fractions.
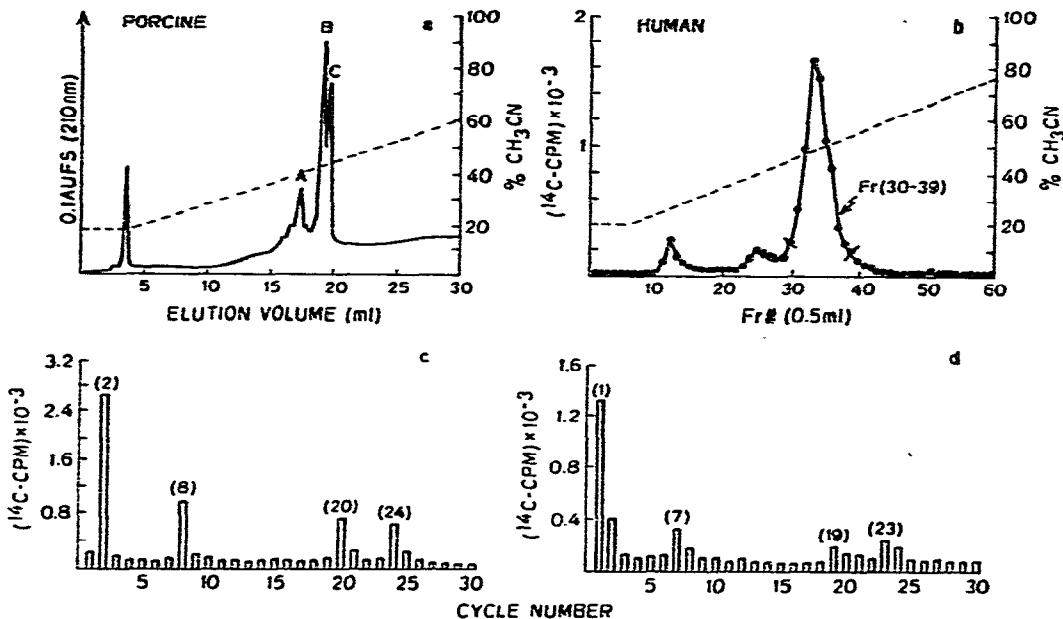

Fig. 2. Purification and microsequencing of porcine and human 19K NH₂-terminal ACTH/LPH segments. a, RP-HPLC on μBondapak C₁₈ of porcine material partially purified[23]. Elution conditions consisted of 20% acetonitrile isocratic for 5 min followed by a gradient of 0.75% acetonitrile/min at 0.5 ml/min; b, RP-HPLC on μBondapak C₁₈ of human material[23] after labeling of Cys residues with [14C]iodoacetamide[22]. Elution conditions are 20% acetonitrile isocratic for 5 min followed by a gradient of 1% acetonitrile/min at 0.5 ml/min. Fractions of 0.5 ml were collected and aliquots (1/10) were counted; c-d, microsequencing of [14C]iodoacetamide labeled porcine material repurified from peak B of Fig. 2a and human material from fraction 30–39 of Fig. 2b. Thiazolinones from each sequenator cycle were counted directly[4,8,10].

Microsequencing characterization

Upon repurification of peak B and microsequencing (Fig. 2c) it is seen that cysteine residues occupy positions 2, 8, 20, 24, exactly as expected from Nakanishi's sequence for the bovine homologue[6]. In the human situation (Fig. 2b) microsequencing of material from fraction 30–39 showed cysteines at positions 1, 7, 19 and 23 (Fig. 2d), hence exhibiting the same sequence as (NH₂-1) terminal region of the reported bovine sequence[6]. This was also observed for the rat homologue[8]. Such micro heterogeneities in sequence has also been observed in other pituitary hormones such as follicle stimulating hormone, growth hormone and luteinizing hormone[21] and is usually due to exopeptidases present during the extraction procedure. The apparent molecular weights of these purified peptides were found to be 19K on SDS–poly-acrylamide gel electrophoresis as expected from the pulse chase experiments in rat pars intermedia[5,8].

Purification of ovine Met-enkephalin, ACTH (1–8) β-LPH (61–69), γ-MSH (1–13), α-MSH, ACTH

The separation of these various peptides on a single RP-HPLC run on a μ-Bondapak CN column is depicted in Fig. 3A. It is seen that effective separation of ACTH (1–8), Met-enkephalin, γ-MSH, ACTH and β-LPH can be achieved. However in this TEAP system used, three pairs of coelution occurred. Namely, ACTH (1–8)/β-LPH (61–69); ACTH/β-endorphin; γ-LPH/β-LPH.
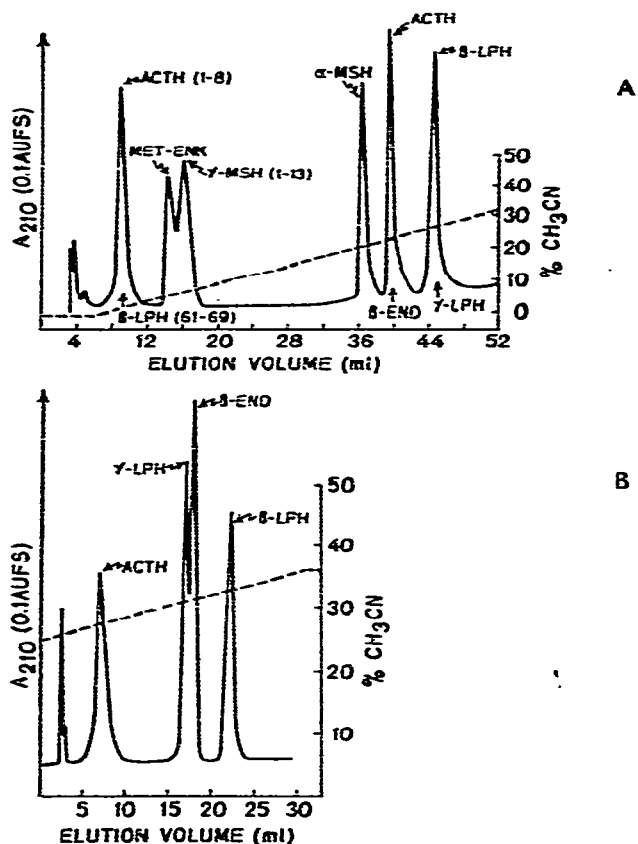

Fig. 3. RP-HPLC of various sheep pituitary peptides related to ACTH and β-LPH segments of the ACTH/LPH precursor. A, μBondapak CN column eluted with 0 % acetonitrile for 6 min followed by a gradient of 0.67 % acetonitrile/min at 1 ml/min. B, μBondapak C₁₈ column eluted with a gradient of 0.37 % acetonitrile/min at 1 ml/min starting at 25 % acetonitrile.

The coelution of γ-LPH (β-LPH 1–58) and β-LPH (1–91) point out the negligible interaction of the carboxy-terminal segment of β-LPH, namely β-endorphin (β-LPH 61–91) with the CN functionality when it is present within the whole β-LPH sequence.

The separation of these pairs could not be achieved on a μBondapak CN

column either by changing the gradient or the pH of the TEAP from 2 to 7. The resolution of these peptides can, however, be obtained on a μBondapak C_{18} column using the same TEAP buffer at pH 3.0 as shown in Fig. 1B and 3B.

Purification of γ-MSH (1-13)

Fig. 4A represents the effective purification of the synthetic peptide γ-MSH (1-13) of sequence H₂N–Tyr–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe–Gly–
1   2   3   4   5   6   7   8   9   10   11   12

Arg–COOH on a μBondapak C_{18} column[6]. The proof of the correct sequence syn-
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thesized is shown in Fig. 4B, where a peptide map of a tryptic digest of this purified peptide on the same column is shown. Following collection of the peptide peaks and amino acid analysis, the nature and position of the tryptic peptides is shown on top of the peaks. It is seen that γ-MSH 1–7, 8–13 and 8–10 are obtained as expected from the sequence. Partial tryptic cleavage at an Arg residue preceded by Asp is common in many tryptic cleavages.
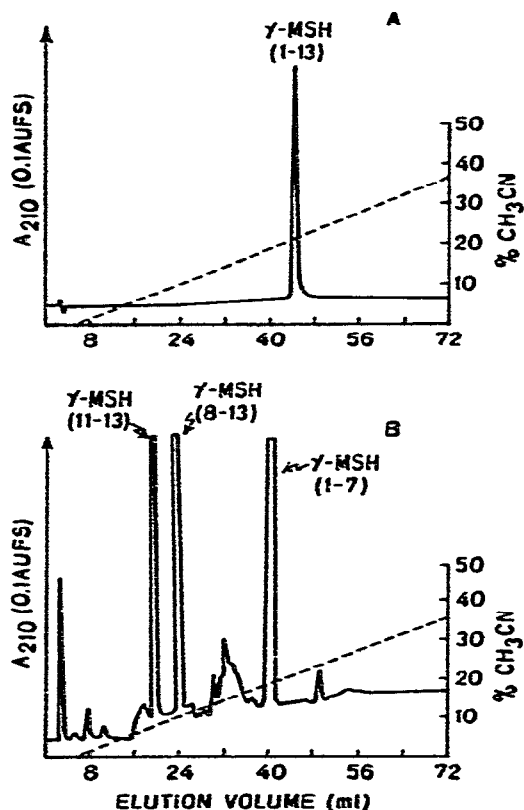

Fig. 4. Analytical separation of γ-MSH (1–13) and its tryptic fragments on a μBondapak C_{18} column. Elution conditions are identical for both runs namely an isocratic elution at 0% acetonitrile for 2.5 min followed by a gradient of 1% acetonitrile/min at 2 ml/min. In B the peptides from the peaks were collected and subjected to amino acid analysis.

Such a novel peptide has been studied for its biological activities and was found to have interesting activities in both the steroidogenic assay and melanotropic assay[22] and hence showed for the first time that based on sequence homology to α-MSH[6], biological homology of function can be expected.

DISCUSSION

The studies reported in this paper indicate that RP-HPLC using the TEAP buffer system originally developed by Rivier[12] is a very useful method for the separation of a wide spectrum of peptides related to the ACTH/LPH precursor. Recently, on a LiChrosorb AR-8 column, the purification of such a precursor from camel pituitaries was reported[16]. The buffer used was a combination of propanol–pyridine–formic acid, requiring the use of a fluorometric detection system. In the TEAP–CH_3CN system investigated, no post-column derivatization is necessary since this buffer sytem is transparent to UV light above 200 nm (ref. 12).

The separation of the two glycosylated variants of the ACTH/LPH precursor is novel and the availability of these two forms separately could be very useful in understanding their physiological role and in the study of the maturation enzymes responsible for their cleavage into various hormones and pro-hormones[5].

The purification of the NH_2-terminal fragments of the ACTH/LPH precursor from both human and porcine pituitary extracts is also novel and will surely find applications in the development of antibodies to these new fragments useful in clinical studies. Their biological function is as yet not understood, except that they contain the sequence of γ-MSH within their polypeptide chain, a peptide which was shown to exhibit biological function homology to ACTH[22]. The successful combination of RP-HPLC and microsequencing has proved to be a powerful method to follow both the purity and nature of the sought peptide. Hence in absence of a specific radioimmunoassay system for these peptides, RP-HPLC–microsequencing combination following specific labeling of certain key amino acid residues provides a very attractive and definite tool to achieve equivalent if not more definite end results[23].

A method is presented for the separation of Met-enkephalin, ACTH (1–8), β-LPH (61–69), α-MSH, γ-MSH (1–13), ACTH, β-endorphin, γ-L   ᵗ and β-LPH. This problem is a very practical one for the peptide chemist in   ᵗ?d in the separation of these various peptides in biological tissues, and for tho⸱   ⸱sted in biosynthetic studies of these molecules in pituitary and brain[5]. T⸱   elution problem observed is an example of the dangers involved in assigning a gi   ⸱ ⸱eak to a given peptide, and clearly emphasizes the need to confirm the purity and nature of the peptide sought by other methods than RP-HPLC, or by using a different elution system using RP-HPLC. Although on CMC or sephadex G-50 β-LPH and γ-LPH are very well separated[4], ACTH and β-endorphin are not, hence the development of elution conditions to achieve such a separation on μBondapak C_{18} provides a viable alternative.

Peptide mapping using the TEAP–acetonitrile system on μBondapak C_{18} column has been a very useful method for the estimation of the degree of purity and characterization of the peptides purified[9].

In conclusion, the methods proposed should be useful to the research scientist interested in this fascinating ACTH/LPH precursor and its fragments which vary in their biological functions from analgesic to steroidogenic to melanotropic to lipolytic activities. They should also provide methods to assay the presence of these molecules in minute amounts in various tissues, if coupled to a specific radioimmunoassay of the collected fractions.

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