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ISOLATION OF PITUITARY PEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EXPANSION OF THE RESOLVING POWER OF REVERSED-PHASE COLUMNS BY MANIPULATING pH AND THE NATURE OF THE ION-PAIRING REAGENT

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SUMMARY

The reversed-phase liquid chromatographic behaviour of pituitary peptides at various pH values was compared. The retention times of peptides at pH 1, 3 and 4.5 were essentially as expected for acidic solvent systems. However, at pH 5.5 or 7, radically different elution characteristics were observed. This is probably brought about by the increase in polarity that accompanies ionization of the peptide carboxyl groups at higher pH values. Basic hydrophobic ion-pairing reagents, such as tetrabutylammonium ion, can be used to maximize differences in polarity. We have previously shown that the use of acidic hydrophobic ion-pairing reagents at low pH (*e.g.* trifluoroacetic and heptafluorobutyric acids) enhances the basic polarity of peptides. The combination of both approaches facilitated the reversed-phase purification of posterior pituitary glycopeptide and α-N-acetyl-β-endorphin$_{1-27}$ from an extract of 200 rat neurointermediate pituitaries. The separation of these two peptides at low pH, which is particularly difficult, was achieved by the sequential use of solvent systems containing trifluoroacetic acid (0.1%), heptafluorobutyric acid (0.13%) and finally triethylamine acetate (0.01 *M*, pH 5.5).

INTRODUCTION

Our previous studies have been aimed at developing a general approach to the isolation and purification of peptides from endocrine tissues[1] using the combination of reversed-phase tissue extraction techniques[2] with the resolving power of reversed-phase high performance liquid chromatography (RP-HPLC). The sequential use of trifluoroacetic and (TFA) and heptafluorobutyric acid (HFBA) as hydrophobic ion-pairing reagents introduces the elements of cation-exchange chromatography into the purification scheme[3]. This approach has had notable success in the purification of an insulin-like peptide from porcine pituitaries[4], the preparation of highly purified human β-lipotropin[5] and the isolation of epidermal growth factor from mouse sub-

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maxillary gland[6]. We have found that the purification of certain pituitary peptides tests this method to the limit. For instance the separation of posterior pituitary glycopeptide from α-N-acetyl-β-endorphin$_{1-27}$ is particularly difficult[7]. This paper illustrates that RP-HPLC at elevated pH (5.5. or 7) in combination with the use of basic hydrophobic ion-pairing reagents (*i.e.* tri- or tetraalkylammonium ions) can be used to exploit the acidic character of peptides. This approach greatly expands the resolving power of reversed-phase columns.

MATERIALS AND METHODS

Formic acid (88% solution), glacial acetic acid and phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Tetramethylammonium hydroxide and tetrabutylammonium hydroxide (40% solution) were obtained from Sigma (St. Louis, MO, U.S.A.). Triethylamine and HFBA (both sequenal grade) were from Pierce (Rockford, IL, U.S.A.). Laboratory grade TFA was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Chromatography was performed on a Beckman HPLC system, consisting of two 110A pumps with microprocessor control and an injector. Column eluates were monitored for absorbance at 235 nm or 210 nm with a Waters Assoc. Model 450 variable-wavelength detector and at 280 nm with a Beckman Model 330 fixed-wavelength detector. An LKB Redirac was used to collect column fractions. C_{18} Sep-Pak cartridges and the C_{18} μBondapak and I-125 HPLC columns were from Waters Assoc., Milford, MA, U.S.A.

Preparation of HPLC solvents

Stock solutions of 1% (v/v) TFA (*i.e.* 0.13 *M*), 1.3% (v/v) HFBA (*i.e.* 0.1 *M*) and 0.84% (v/v) phosphoric acid (*i.e.* 0.1 *M*) were prepared. Buffered stock solutions were prepared by titrating acid (formic, acetic or phosphoric) against base (triethylamine, tetrabutylammonium hydroxide or tetramethylammonium hydroxide) to the appropriate pH. UV-absorbing contaminants were removed from these solutions by passing them through a bed of octadecylsilyl-silica (ODS-silica) as described previously[3]. Stock solutions were stored at 5°C. HPLC-grade acetonitrile was from Fisher Scientific; HPLC-grade water was prepared as described previously[3].

For each solvent system, solution A consisted of 1:10 dilution of the appropriate stock solution in water. Solution B consisted of an 80% (v/v) solution of aqueous acetonitrile containing a 1:10 dilution of the appropriate stock solution. Linear gradients of acetonitrile were generated by mixing solutions A and B by means of the 110A pumps.

Extraction of rat pituitaries

The pituitaries of 650 female Sprague Dawley rats (Canadian Hybrid Farms, Stanstead, Canada) were extracted as described previously[1]. The extraction medium consisted of 1 *M* hydrochloric acid containing 5% (v/v) formic acid, 1% (w/v) sodium chloride and 1% (v/v) TFA. The extract of the neurointermediate lobes (NILs) was passed through ODS-silica cartridges as described previously[1]. The eluate, consisting of 35 ml of 80% acetonitrile–0.1% TFA, was stored at −20°C.

Loading of HPLC columns

As in our previous work samples were pumped directly onto the reversed-phase columns[1]. This method, which was used throughout this study, is particularly valuable in ensuring that maximal recoveries are achieved in the preparative experiments. For column loading, a Milton-Roy Simplex pump attached to the HPLC system was used.

Characterization of solvent systems

For the characterization of the various solvent systems 250 μl of the eluate from the ODS-silica cartridges (*i.e.* equivalent to five NILs) was diluted to 5 ml with the appropriate solution A and pumped onto the column. In each instance the column was eluted with a linear gradient of 1.6 to 41.6% acetonitrile over a 60-min period at a flow-rate of 1.5 ml/min. The presence of corticotropin-like intermediate-lobe peptide (CLIP, $ACTH_{18-39}$) and α-melanotropin (α-MSH) in column fractions was assessed by radioimmunoassays as described previously[8]. The elution positions of γ-lipotropin (γ-LPH) and the acidic joining peptide (*i.e.* the 77–94 sequence of the ACTH/β-LPH precursor[7,9] were determined by their characteristic absorbances at 210 and 280 nm[7].

Preparative isolation of NIL peptides

A 10.75-ml portion of the eluate from the ODS-silica cartridge (*i.e.* equivalent to 200 NILs) was concentrated to 2 ml under reduced pressure at room temperature in a National Vacuum oven (Fisher Scientific) attached to a water aspirator. The concentrate was then pumped directly onto two C_{18} μBondapak columns connected in series. The columns were eluted for 3 h with a linear gradient of 1.6 to 61.6% acetonitrile containing 0.1% TFA throughout. The flow-rate was 1.5 ml/min and 1-ml fractions were collected. The eluate was monitored continuously for absorbance at 235 and 280 nm. The predictable nature of the UV recordings (see refs. 7 and 10) permitted identification of fractions containing posterior pituitary glycopeptide and α-N-acetyl-β-endorphin$_{1-27}$. Purification of these peptides was performed as described in the legends to Figs. 2–4.

Peptide characterization

The molecular weight estimation and tryptic mapping of the isolated peptides was carried out as described previously[10]. Amino acid analysis of the isolated peptides and of their tryptic fragments was performed on a Beckman System 6300 high-performance analyzer.

RESULTS AND DISCUSSION

RP-HPLC of a pituitary extract at different pH values

Extracts of rat NIL constitute a useful standard peptide mixture for testing different RP-HPLC columns and solvent systems. All the major peptide components observed in the fractions of rat NILs are related to the ACTH/β-LPH precursor and to precursors for oxytocin and vasopressin[7,11,12]. We have observed this preparation to be highly reproducible. It contains numerous peptides of molecular weights ranging from 1000 to 15,000 daltons. Both acidic and basic peptides are represented in the

mixture as well as many post-translational modifications[7,13]. Using the same reversed-phase column (Waters C_{18} μBondapak), solvent gradient (1.6 to 41.6% acetonitrile over 60 min) and flow-rate (1.5 ml/min), the behaviour of an extract of five rat NILs was compared in solvent systems with pH values ranging from 1 to 7 (Fig. 1). In each system, peak shapes were inferior to those in solvent systems containing 0.1% TFA or 0.13% HFBA[7]. This defect can be overcome somewhat by doubling the

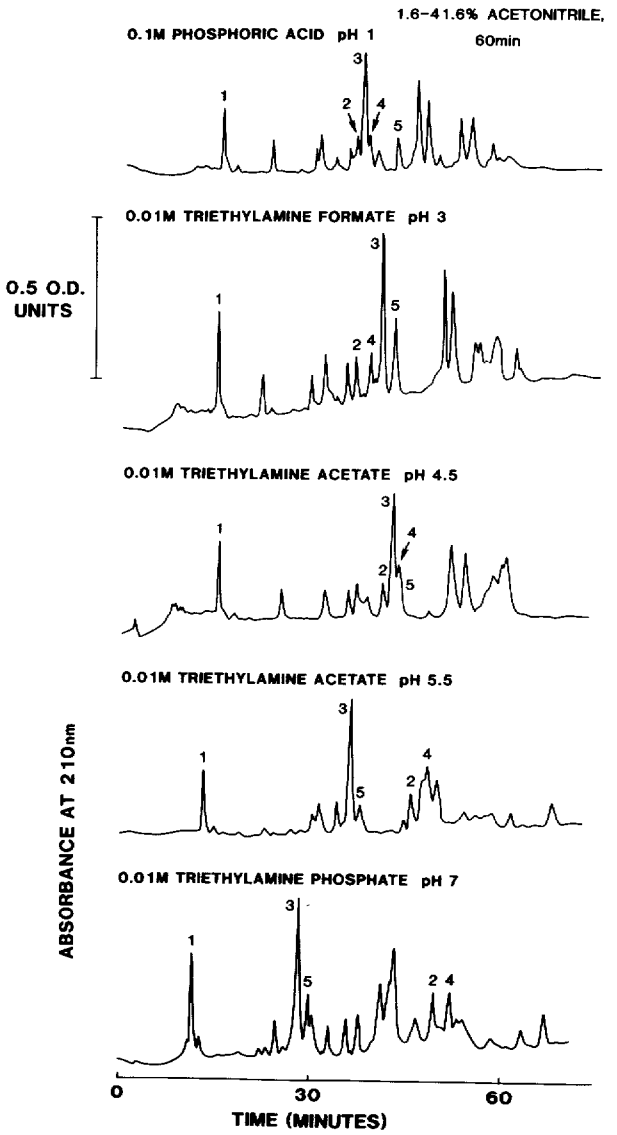

Fig. 1. A comparison of the properties of solvent systems of different pH. In each instance an extract of five NILs was subjected to RP-HPLC with a linear gradient of 1.6 to 41.6% acetonitrile over a 60-min period. Peptides were identified as follows: 1 = acidic joining peptide; 2 = monoacetyl-α-MSH; 3 = γ-LPH; 4 = diacetyl-α-MSH; 5 = CLIP.

steepness of the acetonitrile gradient (*i.e.* 40% acetonitrile per hour instead of 20% acetonitrile per hour), which results in narrower peaks. Column selectivity at pH 1, 3 or 4.5 is similar to that with 0.1% TFA throughout[7]. However, at pH 5.5 or 7 column selectivity is radically changed. This effect is appreciated by referring to peaks 1 (acidic joining peptide), 2 (monoacetyl-α-MSH), 3 (γ-LPH), 4 (diacetyl-α-MSH) and 5 (CLIP). Above pH 5 the retention times of peaks 1, 3 and 5 decrease, whereas those for peaks 2 and 4 increase. This effect can be explained in terms of the number of acidic groups found in each peptide (including free carboxyl termini). Whereas both forms of α-MSH contain only one glutamate residue the other peptides contain numerous acidic residues (*i.e.* 6 out of 18 residues for acidic joining peptide, 12 out of 38 residues for γ-LPH and 5 out of 22 residues for CLIP). As the pH of the solvent system passes through the p*K* for the aspartate and glutamate residues (*i.e.* between 3 and 5) the polarity of peaks 1, 3 and 5 increases markedly and their retention times are reduced. The degree of ion-pairing by each buffer cannot be kept constant in a study such as this. Matters are further complicated by the suppression of ionization of both buffer and peptide that inevitably accompanies the use of organic solvents. However, it is clear that the manipulation of pH alone has potential as part of a peptide purification scheme.

Use of hydrophobic ion-pairing reagents at pH 7

It is clear from the above observations that it should be possible to use basic hydrophobic ion-pairing reagents at pH 7. In this way the elements of anion-exchange chromatography could be introduced into a peptide purification scheme. This approach has been used for a number of years in the RP-HPLC of organic carboxylic acids[14] and polar drugs[15]. More recently, Hancock *et al.*[16] studied the properties of cationic reagents in the RP-HPLC of peptides. Except for that preliminary study little use has been made of these reagents for peptide separation. A comparison was made of the effectiveness of tetramethyl- and tetrabutylammonium phosphate as hydrophobic ion-pairing reagents at pH 7 in fractionating isolated rat mono- and diacetyl-α-MSH and γ-LPH as chromatographic standards (results not shown). The selectivity of the tetramethylammonium phosphate system was similar to that of the triethylamine phosphate system (see Fig. 1) indicating that the former buffer is a weak hydrophobic ion-pairing reagent. In the tetrabutylammonium phosphate the behaviour of the peptide standards indicated that this buffer is, in contrast, a powerful hydrophobic ion-pairing reagent (*e.g.* γ-LPH, which is eluted first in the tetramethylammonium phosphate system, is eluted last in the tetrabutylammonium phosphate system). The manipulation of pH and hydrophobic ion-pairing reagent in the RP-HPLC purification of two rat pituitary peptides is illustrated in the following study.

Isolation and purification of rat NIL peptides

In previous studies we have encountered difficulties in separating several peptides when we used only the TFA and HFBA systems[7]. For instance, the separation of posterior pituitary glycopeptide from α-N-acetyl-β-endorphin$_{1-27}$ (peaks 23 and 24 in Fig. 1 of ref. 7) could only be achieved by a "peak shaving" procedure. The amino acid compositions of these two peptides suggested that posterior pituitary glycopeptide contains considerably more acidic amino acids than endorphin. The following illustrates how RP-HPLC at elevated pH makes this separation very simple.

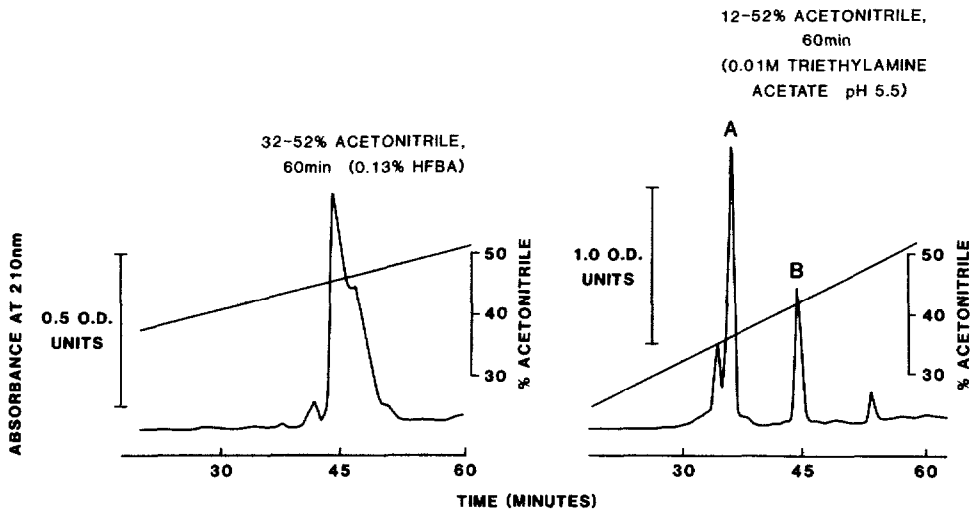

Fig. 2. Left panel: reversed-phase HPLC of fractions containing posterior pituitary glycopeptide and α-N-acetyl-β-endorphin$_{1-27}$ obtained from the initial chromatography of an extract of 200 rat NIL; the column was eluted with a solvent system containing 0.13 % HFBA as indicated. Right panel: reversed-phase HPLC of the major components remaining unresolved by the HFBA solvent system; the column was eluted with a solvent system containing 0.01 *M* triethylamine acetate (pH 5.5) as indicated. Peaks A and B were further purified as described in the text and in the legends to Figs. 3 and 4.

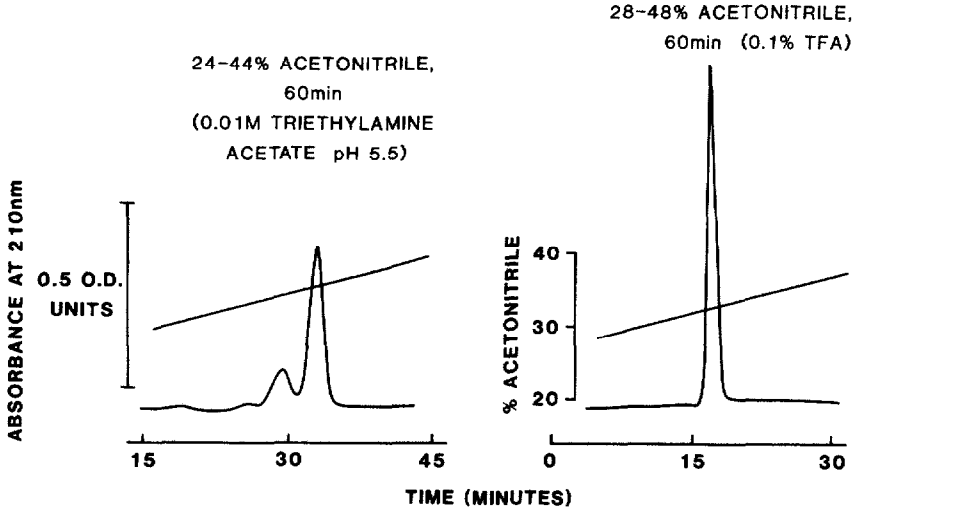

Fig. 3. Repurification of peak A by reversed-phase HPLC. Left panel: Initial repurification with a shallow acetonitrile gradient and 0.01 *M* triethylamine acetate (pH 5.5). Right panel: final purification by means of a 0.1 % TFA solvent system.

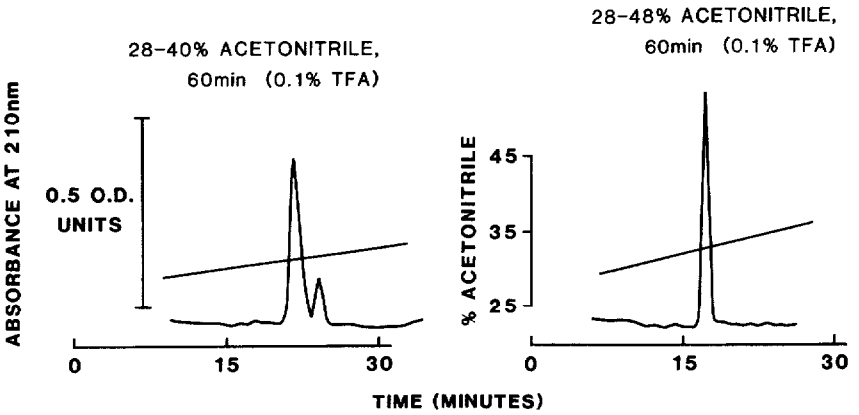

Fig. 4. Final steps in the repurification of peak B by reversed-phase HPLC. Left panel: column eluted with a shallow acetonitrile gradient and 0.1% TFA. Right panel: final purification by means of a 0.1% TFA solvent system.

TABLE I

AMINO ACID COMPOSITIONS AND APPARENT MOLECULAR WEIGHTS OF THE PEPTIDES IN PEAKS A AND B (SEE FIGS. 2–4)

Amino acid	*Peak A*	*Peak B*
Asx	2.8(4)*	1.9(2)**
Thr	2.0(1)	3.0(3)
Ser	2.9(2)	1.9(2)
Glx	7.0(4)	2.2(2)
Pro	2.0(5)	1.1(1)
Gly	2.0(4)	2.2(2)
Ala	5.1(7)	2.1(2)
Val	2.9(2)	1.0(1)
Met	0(0)	1.1(1)
Ile	0(0)	0.8(2)***
Leu	7.1(7)	2.1(2)
Tyr	0.9(1)	1.1(1)
Phe	0(0)	2.1(2)
His	0(0)	0.9(1)
Lys	1.2(0)	3.0(3)
Arg	3.9(2)	0(0)
CHO§	+ +	−
Molecular weight	4170 (4775)§§	2570 (3040)§§§

* Composition expected for bovine posterior pituitary glycopeptide$_{1-39}$ (refs. 19 and 20).

** Composition expected for β-endorphin$_{1-27}$ (refs. 10 and 11).

*** The low value for isoleucine is probably due to the poor hydrolysis of the hydrophobic Ile$_{22}$-Ile$_{23}$ sequence.

§ CHO refers to amino sugar peak: + + indicates the presence of peaks in the amino sugar region of the chromatogram.

§§ Molecular weight expected for bovine posterior pituitary glycopeptide.

§§§ Molecular weight expected for rat β-endorphin$_{1-27}$.

The RP-HPLC of the extract of 200 rat NILs was carried out as described under Materials and methods, using the 0.1 % TFA solvent system. Fractions corresponding in elution position to posterior pituitary glycopeptide and α-N-acetyl-β-endorphin$_{1-27}$ were rechromatographed with the 0.13 % HFBA solvent system (Fig. 2, left panel). The peptides were only partially separated and were reloaded onto the same HPLC column which was eluted with an acetonitrile gradient containing 0.01 *M* triethylamine acetate (pH 5.5) throughout[18] (Fig. 2, right panel). In this system the mixture was resolved into two major (peaks A and B) and two minor components. Peaks A and B required further purification on the same HPLC column as illustrated in Figs. 3 and 4. In addition to the systems illustrated in Figs. 3 and 4, peaks A and B were also rechromatographed in the tetrabutylammonium phosphate (pH 7) and HFBA solvent systems, but no apparent further purification was achieved (results not shown).

Peak A and the adjacent contaminant were resolved in the triethylamine acetate system with a shallower acetonitrile gradient. (Fig. 3, left panel). Peak B contained a minor contaminant which only became apparent when the 0.1 % TFA system and a shallow acetonitrile gradient were used (Fig. 4, left panel). Interestingly, this contaminant could not be resolved in the tetrabutylammonium phosphate or HFBA solvent systems. Peaks A and B were finally chromatographed in the TFA system (Figs. 3 and 4, left panels) and subjected to amino acid analysis and molecular weight estimation (Table I). The amino acid composition of the material found in peak A indicates that this peptide is homologous to the posterior pituitary glycopeptides

TABLE II

SEQUENTIAL APPROACH TO PEPTIDE PURIFICATION OF TISSUE PEPTIDES

Chromatographic procedure	*Comments*
(1) Reversed phase tissue extraction using acidic extraction medium and ODS-silica cartridges (ref. 2)	Results in a peptide enriched fraction wich is largely free of proteins and salts.
(2) RP-HPLC with 0.1 % TFA as counter-ion (volatile) (ref. 3)	Initial chromatographic separation. Since TFA is a weak hydrophobic ion-pairing reagent, separation is mainly based upon overall hydrophobicity.
(3) RP-HPLC with 0.13 % HFBA as counter-ion (volatile) (ref. 3)	HFBA acts as a hydrophobic ion-pairing reagent. Separation based mainly upon basic character.
(4) RP-HPLC using 0.01 *M* triethylamine acetate, pH 5.5 (volatile) (ref. 18)	Chromatography at elevated pH reveals acidic character which forms the basis of the separations.
(5) RP-HPLC using 0.01 *M* tetrabutylammonium phosphate, pH 7 (non-volatile)	Tetrabutylammonium ion acts as a hydrophobic ion-pairing reagent. Separation based mainly upon acidic character.
(6) RP-HPLC steps using 0.01 *M* triethylamine acetate, pH 5.5 and/or 0.13 % HFBA	May be necessary for particularly difficult separations.
(7) RP-HPLC using 0.1 % TFA	"Cleanest" solvent for concentrating. May also reveal presence of further contaminants.

found in other species[19,20]. The composition of the material in peak B corresponds to that expected for rat β-endorphin$_{1-27}$[10,11]. Tryptic mapping and analysis of the amino terminal tyrosine residue[10] indicated that this peptide was acetylated at the amino-terminus. This post-translational modification has been shown to be a feature of the endorphins found in the intermediate lobe of the pituitary[21,22].

CONCLUSIONS

The purification of peptides from endocrine tissue is complicated by the losses associated with conventional tissue extraction and chromatographic procedures. The improved recoveries provided by RP-HPLC makes it the method of choice for the isolation and purification of biologically active peptides. This paper illustrates how RP-HPLC can be used to exploit, in a systematic manner, the hydrophobic, basic and acidic character of peptides in a general procedure for peptide purification. This general approach is summarized in Table II. Reversed-phase tissue extraction gives rise to a peptide-enriched fraction which is largely protein- and salt-free. Pumping fractions directly onto the HPLC column between chromatographic steps improves recoveries and makes the multi-step procedure feasible. Finally, the TFA, HFBA and triethylamine acetate solvent systems are volatile, a feature which facilitates biological assay of fractions. It is anticipated that, as in other developments in RP-HPLC, the limits of this approach will quickly be realized. For instance the resolution of closely-related peptides, heterogeneous in terms of carbohydrate content, must await further developments.

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