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USE OF ION-EXCHANGE Sep-Pak CARTRIDGES IN THE BATCH FRACTIONATION OF PITUITARY PEPTIDES

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SUMMARY

Silica-based ion-exchange Sep-Pak cartridges, packed with either carboxymethyl (CM) cation-exchanger or a quaternary methyl ammonium (QMA) anion exchanger, are now available. The feasibility of using ion-exchange Sep-Pak cartridges for the fractionation of pituitary peptides was investigated. Extracts of bovine posterior pituitaries were fractionated at either pH 5 or pH 7 by pairs of cation and anion exchangers, connected in series. The capacity to bind peptides was well correlated with the theoretical charge calculated for a variety of peptides. At pH 5 the entire tissue extract could be fractionated into either basic or acidic pools. In contrast, at pH 7 only the more basic or acidic peptides were retained by the respective ion exchangers. The rest of the peptides passed through both ion exchangers and were recovered in the neutral pool. The ion-exchange fractionation principle was used to facilitate the purification of ^{35}S-labelled intermediate pituitary glycopeptides, prepared by incubating mouse intermediate lobes in explant culture with ^{35}S-labelled sulphate. ^{35}S-labelled glycosylated forms of Lys$^{1}\gamma_{3}$MSH, corticotropin-like intermediate lobe peptide, and the amino terminal or 16K fragment of pro-opiomelanocortin (*i.e.* $16K_{1-74}$) were fractionated into separate pools such that they could be purified to homogeneity in a single step by reversed-phase high-performance liquid chromatography (RP-HPLC). Purification by conventional means would require at least two RP-HPLC steps. Thus, radiolabelled peptides can be purified with the minimum of chromatographic manipulation, thereby ensuring maximal recoveries.

INTRODUCTION

Previous studies from this laboratory have been concerned with rapid methods for extracting peptides from endocrine tissues and maximizing yields through various reversed-phase high-performance liquid chromatography (RP-HPLC) purification schemes[1,2]. A standard methodology has evolved, including reversed-phase extraction of tissue homogenates with octadecylsilyl-silica (ODS-silica) cartridges (C_{18} Sep-Pak), followed by sequential use of a variety of RP-HPLC solvent systems, designed to exploit in a systematic manner the hydrophobic, basic and acidic character of the peptides to be purified[2]. For tissues that are rich sources of biologically active pep-

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tides (*e.g.*, pituitary gland, parathyroid gland) complete purification can be achieved using only reversed-phase liquid chromatography. For tissues in which the concentrations of bioactive peptides are very low (*e.g.*, brain) this purification method is not adequate. For this type of tissue, crude fractionation procedures must be precede high-resolution RP-HPLC. In a previous study we investigated the usefulness of batch fractionation of tissue extracts by ion-exchange HPLC[3]. A simple procedure was developed which facilitated the fractionation of extracts of bovine posterior pituitaries into basic, acidic and neutral pools. In this way, column loads were reduced for the subsequent RP-HPLC purification scheme. For this procedure, the high-resolution properties of the HPLC ion-exchange columns were not used. A Sep-Pak procedure seemed at the time to be more appropriate. Recently, ion-exchange cartidges have become available. They are packed with either Accell quaternary methyl ammonium (QMA) anion-exchange medium or Accell carboxymethyl (CM) anion-exchange medium. In both instances, the functional group is linked to a polymer-coated silica base (37–55 μm particle size, 500-nm pore size). The present study is concerned with determining the feasibility of using these cartridges for batch fractionation of pituitary tissue.

MATERIALS AND METHODS

High-performance liquid chromatography

Chromatography was performed with a Waters Assoc. (Milford, MA, U.S.A.) HPLC system. Column eluates were monitored for UV absorbance at 210 and 280 nm by a Waters Model 450 variable-wavelength detector, and an LDC (Riviera Beach, FL, U.S.A.) fixed-wavelength detector, connected in series. HPLC solvents and reagents were prepared as described previously[1].

Preparation of bovine and mouse posterior pituitary extracts

A stock extract of bovine posterior pituitaries (Pel-Freez Biologicals, Rogers, AR, U.S.A.) was prepared exactly as described previously[2]. Briefly, this involved extraction of twenty pituitaries with an extraction medium consisting of 1 *M* hydrochloric acid, containing 5% (v/v) formic acid, 1% (w/v) sodium chloride and 1% (v/v) trifluoroacetic acid (TFA). This extract was subjected to reversed-phase extraction with ten C_{18} Sep-pak cartridges (Waters). The cartridge eluate in 50 ml acetonitrile–water (80:20, v/v), containing 0.1% (v/v) trifluoroacetic acid (TFA), was divided into ten portions of 5 ml (*i.e.* the equivalent of two pituitaries per portion) and stored at −20°C until required for batch ion-exchange fractionation.

Mouse neurointermediate pituitary explant cultures were also subjected to reversed-phase extraction with C_{18} Sep-Pak cartridges. Tissue and medium were extracted together in 5 ml of extraction medium. The homogenate supernatant was passed through a single C_{18} Sep-Pak, which was washed with 20 ml of 0.1% TFA and eluted with 5 ml of acetonitrile–water (80:20) containing 0.1% TFA.

Explant culture of mouse neurointermediate pituitaries

Male mice (CD) were purchased from Charles River (St. Constant, Quebec, Canada). They were sacrificed by decapitation and brain tissue was removed to reveal the pituitary at the base of the skull. Using fine forceps the neuro-intermediate lobe

(NIL) of the pituitary was dissected free of the anterior lobe *in situ*. This operation was achieved within 30 s of death. For the incorporation of ^{35}S-labelled sulphate, five mouse NILs were first rinsed in Dulbecco's modified Eagle's medium to remove red blood cells. They were then immediately placed in explant culture in 100 μl of sulphate-free medium, supplemented with 1 mCi of carrier-free ^{35}S-labelled sulphuric acid (43 Ci/mg, ICN Biochemicals, Montreal, Canada) at 37°C for 18 h in an atmosphere of oxygen–carbon dioxide (95:5). The sulphate-free medium was made from Earle's balanced salt solution in which magnesium sulphate was replaced by a molar equivalent of magnesium chloride. The medium was also supplemented with essential amino acid and vitamin concentrates to give a final medium identical to Eagle's basal medium but lacking sulphate. All culture media and amino acid and vitamin concentrates were purchased from Gibco Canada (Burlington, Canada).

Ion-exchange fractionation of pituitary peptides with Accell-ion exchange Sep-Pak cartridges

For these studies, both cation-exchange cartridges (quaternary methylammonium) and anion-exchange cartridges (carboxymethyl) were kindly provided by Ed Conrad and Al Pearson of Waters Assoc. (Millford, MA, U.S.A.). Pituitary extracts, obtained by reversed-phase fractionation with C_{18} Sep-Pak cartridges [4 ml of acetonitrile–water (80:20), containing 0.1% TFA], were taken to dryness in a Savant Speed Vac concentrator (Hicksville, NY, U.S.A.). The dried residues were taken up in the appropriate buffer for ion-exchange fractionation with either ammonium acetate (pH 5) or Tris–HCl buffers (pH 7), containing 20% (v/v) acetonitrile. All batch precedures were performed on a pair of Sep-Pak ion exchangers, connected in series. For all studies the cation exchanger was connected ahead of the anion exchanger. Pituitary extracts were passed through both cartridges in 3 ml of either 10 m*M* or 50 m*M* buffer. This was followed by a 6-ml wash of the same buffer. The unretained pool, now in 9 ml of buffer, was designated the neutral pool. The ion-exchange cartridges were then disconnected and each was eluted with 5 ml of 10 m*M* or 50 m*M* buffer containing 1 *M* sodium chloride. The cation-exchange eluate was designated the basic pool, while the anion-exchange eluate was designated the acidic pool. In each instance, the basic, acidic and neutral pools were brought to approximately pH 2 by dropwise addition of 1% TFA.

RP-HPLC analysis of basic, acidic and neutral pools, obtained from ion-exchange fractionation of pituitary extracts

Basic, acidic and neutral pituitary pools were pumped directly into a C_{18} μBondapak RP-HPLC column, as described previously[2]. For the bovine pituitary extracts the column was eluted over 3 h with a linear gradient of acetonitrile–water from 1.6:98.4 (v/v) to 61.6:39.4 (v/v), containing 0.1% TFA throughout, at a flow-rate of 1.5 ml/min. For the mouse pituitary extracts, the same gradient was employed, but the elution time was 1 h. For comparison purposes, a portion of unfractionated pituitary extract was subjected to RP-HPLC under identical elution conditions. For the identification of eluted peptides, material was collected by hand into hydrolysis tubes as they emerged from the column. Following hydrolysis *in vacuo* at 110°C for 18 h, peptides were analysed for their amino acid compositions, using a Series 6300 automated amino acid analyzer (Beckman Instruments, Palo Alto, CA, U.S.A.).

RESULTS AND DISCUSSION

The ease with which ion-exchange Sep-Pak fractionation can be utilized is illustrated in Fig. 1. In pilot experiments, it was found necessary to add 20% (v/v) acetonitrile to all buffers in order to maximize recoveries of peptides. The presence of organic solvent presumably eliminates non-specific hydrophobic binding to the silica-based ion exchangers. Fig. 1 illustrates how bovine posterior pituitaries were fractionated at pH 5 and loaded on the ion exchangers at low ionic strength (10 m*M* buffer). under these conditions, almost the entire tissue extract can be recovered in either the basic or acidic pools (*ca.* 50% of the oxytocin was found in the neutral pool). This behaviour can be correlated well with the theoretical charges of posterior pitutiary peptides at pH 5 (see Table I). When the fractionation was repeated with 50 m*M* ammonium acetate (pH 5), several peptides were unretained by the ion exchangers and were recovered in the neutral pool (not shown). The distribution into the three pools (basic, neutral, and acidic) was very similar to that observed in a

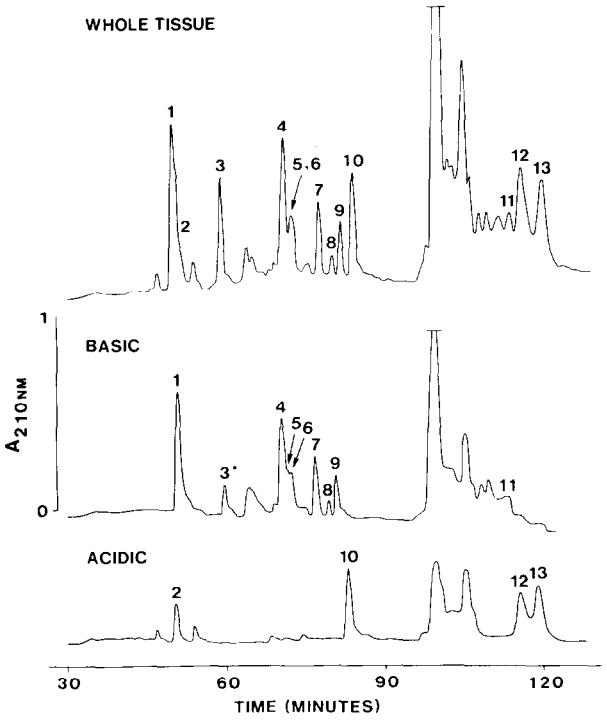

Fig. 1. Analytical RP-HPLC of peptides extracted from bovine posterior pituitaries. The upper panel (whole tissue) shows the elution profile obtained from the RP-HPLC of two posterior pituitaries. A further portion of tissue extract, also corresponding to two posterior pituitaries was dried, taken up in 3 ml of 10 m*M* ammonium acetate (pH 5), and subjected to ion-exchange batch extraction with cation- and anion-exchange Sep-Pak cartridges connected in series, as outlined in Materials and methods. The middle and lower panels show the RP-HPLC profiles obtained for the basic and acidic pools, respectively. The neutral pool was also analyzed (not shown), but only one peak was observed corresponding to oxytocin. Peptides numbered 1–13 were identified by elution position and amino acid composition (see Table I for identification).

TABLE I

SUMMARY OF THE THEORETICAL CHARGES (AT pH 5 AND pH 7) AND THE ACTUAL ION-EXCHANGE PROPERTIES OF PEPTIDES EXTRACTED FROM BOVINE POSTER PITUITARIES

Abbreviations: $POMC_{80-103}$ = the acidic joining peptide of pro-opiomelanocortin, AVP = arginine-vasopression, α-, β- and γ-MSH = α,β- and γ-melanotropin, CLIP = corticotropin-like intermediate lobe peptide (*i.e.* $ACTH_{18-39}$), ($16K_{1-77}$ and $16K_{1-49}$ = amino terminal fragments of pro-opiomelanocortin. Posterior pituitary glycopeptide = the 109–147 sequence of propressophysin. Using the sequence information from refs. 8 and 9, the overall charge for each peptide was determined. The overall charge represents the difference between the number of acidic residues (*i.e.* Asp, Glu and free carboxyl termini) and the number of basic residues (*i.e.* Lys, Arg, His and free amino termini). At pH 5, histidine residues are considered fully charged, while at pH 7, they are considered uncharged. A, B and N represent the acidic, basic or neutral pools, respectively, into which peptides are found to be fractionated. Note that there are two errors in a similar table in ref. 3. In Table I of ref. 3, bovine β-MSH should have an overall charge of 1+ (not 0) and peptide No. 6 should be $Lys^1\gamma_1MSH$ (not $Lys^1\gamma_3MSH$). Note also that, whenever $Lys^1\gamma_3MSH$ is mentioned in ref. 3, the text should read $Lys^1\gamma_1MSH$. Correction of these errors does not alter the general conclusions of the study in ref. 3.

Peptide No.	*Peptide identity*	*Overall charge*		*Pool*	
		pH 5	*pH 7*	*10 mM ammonium acetate, pH 5*	*10 mM Tris, pH 7*
1	$POCM_{80-103}$	7−	7−	A	A
2	AVP	2+	2+	B	N
3	Oxytocin	1+	1+	B/N	N
4	β-MSH	1+	0	B	N
5	$Lys^1\gamma_3MSH$	5+	4+	B	B
6	Desacetyl α-MSH	3+	2+	B	N
7	Monoacetyl α-MSH	2+	1+	B	N
8	$Lys^1\gamma_1MSH$	4+	3+	B	B
9	Diacetyl α-MSH	2+	1+	B	N
10	CLIP	2−	2−	A	A
11	$16K_{1-77}$	0	1−	B	A
12	$16K_{1-49}$	5−	5−	A	A
13	Posterior pituitary glycopeptide	2−	2−	A	A

previous study from this laboratory, in which ion-exchange HPLC columns were used for the fractionation procedure[3].

Fractionation was repeated at pH 7, using 10 m*M* Tris–HCl (Fig. 2). A fractionation pattern markedly different from that shown in Fig. 1 was obtained. The weakly positively charged peptides were not retained by the cation-exchange cartridge (Fig. 2). Thus, vasopressin, oxytocin, β-MSH and the α-MSHs were recovered in the neutral pool. In contrast, all the acidic peptides, whether weakly charged or not, remained in the acidic pool. This behaviour is well correlated with the relative strengths of the ion exchangers (QMA-Accell is a relatively strong exchanger, while CM-Accell is a much weaker exchanger). All peptides having a histidine residue (p*K*

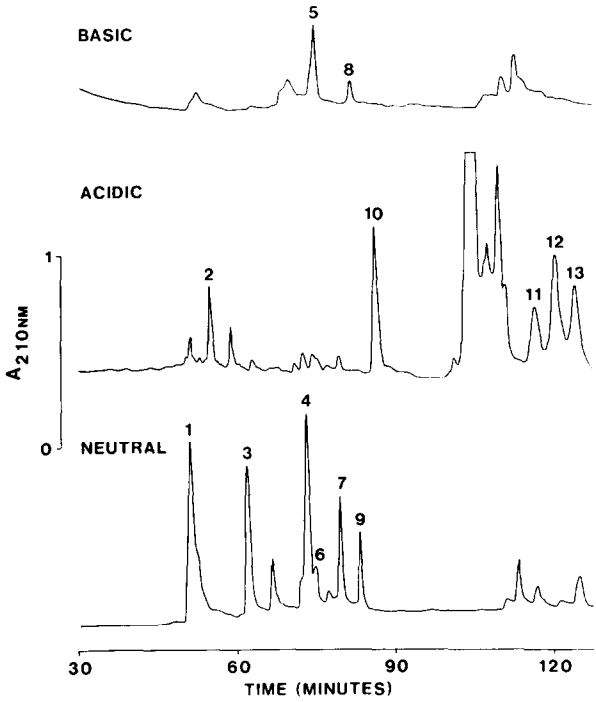

Fig. 2. Analytical RP-HPLC of peptides extracted from bovine posterior pituitaries. A portion of tissue extract, corresponding to two posterior pituitaries, was dried, taken up in 3 ml of 10 m*M* Tris–HCl (pH 7), and subjected to ion-exchange batch extraction with cation- and anion-exchange Sep-Pak cartridges, connected in series, as outlined in Materials and methods. The upper, middle and lower panel show the RP-HPLC profiles obtained for the basic, acidic, and neutral pools, respectively. Peaks 1–13 are identified in Table I.

6) lose one positive charge when the buffer is changed from pH 5 to pH 7, and this contributes to the change in fractionation behaviour. Indeed, $16K_{1\text{-}77}$ actually switches from the basic pool to the acidic pool upon changing the pH from 5 to 7. This behaviour can be used to prepare $16K_{1\text{-}77}$ of high purity, using a simple ion-exchange fractionation step, followed by a single RP-HPLC step [*i.e.*, load pituitary extract onto ion exchangers in series in 10 m*M* Tris (pH 7), elute anion-exchange Sep-Pak with 10 m*M* ammonium acetate (pH 5), and then use RP-HPLC].

Recent studies have shown that the glycopeptide derivatives of pro-opiomelanocortin (POMC) found in the rat pituitary are at least partially sulphated[4–6]. We have recently devised a method for inducing mouse pituitary neurointermediate lobes to incorporate ^{35}S-labelled sulphate into glycosylated forms of $Lys^1\gamma_3$-melanotropin, ($Lys^1\gamma_3MSH$), corticotropin-like intermediate lobe peptide (CLIP), and the amino-terminal fragment of POMC ($16K_{1\text{-}74}$). The availability of radiolabelled forms of these peptides will be useful for studies of their biosynthesis and biological action. A rapid means of purifying them in high yield is important, if this is to be a practical method. In the standard RP-HPLC of mouse neurointermediate pituitaries, glycosylated $Lys^1\gamma_3MSH$ tends to be eluted together with desacetyl-α-MSH, and glycosylated CLIP tends to co-elute with monoacetyl-α-MSH while glycosylated $16K_{1\text{-}74}$

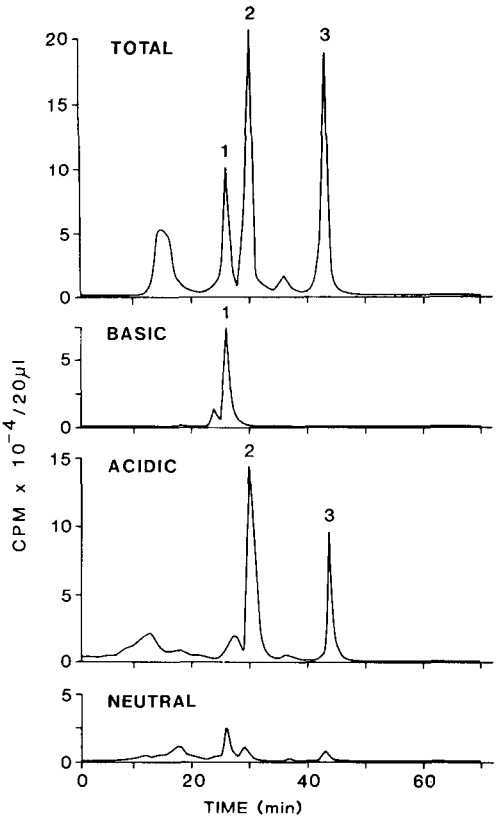

Fig. 3. Analytical RP-HPLC of peptides extracted from five mouse neurointermediate pituitaries that had been incubated with ^{35}S-labelled sulphate for 18 h in explant culture. The tissue and incubation medium were extracted together. The extract was dried, taken up in 3 ml of 10 m*M* Tris–HCl (pH 7), and subjected to ion exchange with Sep-Pak cartridges, connected in series, as outlined in Materials and methods. The upper, middle and lower panel show the RP-HPLC radioactivity profiles, obtained for the basic, acidic, and neutral pools, respectively. Peaks: 1 = ^{35}S-labelled Lys$^1\gamma_3$MSH, 2 = CLIP and 3 = $16K_{1\text{-}74}$.

tends to be eluted with $16K_{1\text{-}49}$[7]. According to our observations in the ion-exchange fractionation of bovine posterior pituitary peptides, it should be possible to fractionate mouse neurointermediate pituitaries in such a way that each of these peptides can be separated from their potential contaminants by batch extraction. Fig. 3 shows that this is indeed the case. Thus, a single RP-HPLC step renders these ^{35}S-labelled sufficiently pure for subsequent biological studies. The rapid nature of the method ensures a high yield throughout the purification procedure.

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REFERENCES

1 H. P. J. Bennett, C. A. Browne and S. Solomon, *Biochemistry*, 20 (1981) 4530.
2 H. P. J. Bennett, *J. Chromatogr.*, 266 (1983) 501.
3 S. James and H. P. J. Bennett, *J. Chromatogr.*, 326 (1985) 329.
4 J. Hoshina, G. Hortin and I. Boime, *Science*, 217 (1982) 64.
5 H. S. Moore, B. Gumbinger and R. B. Kelly, *J. Biol. Chem.*, 97 (1983) 810.
6 Y. Boubonnais and P. Crine, *J. Biol. Chem.*, 260 (1985) 5832.
7 H. P. J. Bennett, *Peptides*, submitted for publication.
8 S. Nakanishi, A. Inoue, T. Kita, M. Nakamura, A. C. Y. Chang, S. N. Cohen and S. Numa, *Nature (London)*, 278 (1979) 423.
9 H. Land, M. Grez, S. Ruppert, H. Schmale, M. Rehbein, D. Richter and G. Schutz, *Nature (London)*, 302 (1983) 342.