Journal of Chromatography, 222 (1981) 371—379
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 766

CHARACTERIZATION AND DETERMINATION OF NEUROPEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

J.R. McDERMOTT*, A.I. SMITH, J.A. BIGGINS, M. CHYAD AL-NOAEMI and J.A. EDWARDSON

Medical Research Council Neuroendocrinology Unit, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE (Great Britain)

(Received August 27th, 1980)

SUMMARY

A method is described for the separation and analysis of a variety of neuropeptides using reversed-phase high-performance liquid chromatography coupled with radioimmunoassay. The solvent system (an acetonitrile gradient containing 0.08% trifluoroacetic acid) allows UV detection at 206 nm, gives good resolution and, by being volatile, is readily compatible with radioimmunoassay. Three applications of the method are described: (a) thyrotropin releasing hormone immunoreactivity in the rat brain has been characterized; (b) ACTH immunoreactivity in the rat pituitary pars intermedia has been resolved into its component peptides; (c) degradation of luteinizing hormone releasing hormone in vitro has been followed.

INTRODUCTION

The radioimmunoassay (RIA) of biologically important peptides from the central nervous system and other tissues is complicated in many cases by the lack of specific antisera and subsequent cross-reactivity of different peptides. This is a particular problem for peptides which have amino acid sequences in common, for example brain and pituitary peptides derived from the common macromolecular precursor, pro-opiocortin. Unequivocal identification and analysis have often only been possible after chromatographic separation of the cross-reacting components; and high-performance liquid chromatography (HPLC) using reversed-phase systems has provided a rapid and highly resolutive technique in this respect. A number of methods, most of them using organic

0378-4347/81/0000—0000/$02.50

solvent gradients, have been described for isolating various neuropeptides [1—6]. The combination of HPLC and RIA allows a much greater selectivity and speed in the analysis of peptides than has hitherto been available, and has been applied in the analysis of endorphin peptides [7] and substance P [8].

The purpose of this investigation has been to identify a reversed-phase HPLC separation method which is applicable to a wide spectrum of neuropeptides and the smaller pituitary peptides, and which is readily compatible with RIA. The ideal eluting solvents should be volatile, UV transparent below 215 nm to allow detection of peptides without aromatic amino acids and give good resolution with no tailing of peaks. The most thoroughly investigated methods satisfy the third criterion at the expense of the first or second [1—4]. The starting point for this study was the method described by Hancock et al. [9] which employs a linear gradient of acetonitrile in 0.1% phosphoric acid.

MATERIALS AND METHODS

Equipment

The HPLC apparatus consisted of two Model 6000A pumps, a Model 660 gradient programmer, a U6K injector, a Model 450 variable-wavelength detector and a μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm; Waters Assoc., Northwich, Great Britain). In recent work, an LKB Uvicord-S fitted with an HPLC flow-cell has been used for UV detection at 206 nm. The flow-rate was 1 ml/min at ambient temperature. Fractions were collected using an LKB Ultrorac 2070, controlled by a microprocessor (Acorn) to allow collection of any part of the column effluent defined by its elution time. The fractions were evaporated on a Buchler vortex evaporator, and reconstituted in the appropriate buffer for RIA.

Chemicals

Acetonitrile (S-grade), isopropanol and methanol were obtained from Rathburn Chemicals (Peebles, Great Britain) and were filtered and degassed immediately before use. Trifluoroacetic acid (TFA) (spectroscopic grade), acetic acid (chromatography grade), and phosphoric acid (AnalaR) were obtained from BDH (Poole, Great Britain). Water was deionised, distilled and passed through a Porapak Q column.

Peptides

LHRH, TRH, TRH free acid, His-Pro diketopiperazine, substance P, somatostatin, α-MSH, CCK-8, Leu-enkephalin and Met-enkephalin were obtained from Peninsular Labs. (San Carlos, CA, U.S.A.). Human ACTH and ACTH fragments were gifts from Drs. W. Rittel and P.A. Desaulles (Ciba-Geigy, Basle, Switzerland), γ-LPH, β-LPH, β-endorphin and LPH_{88-91} were gifts from Dr. J. Morley (ICI, Alderley Park, Great Britain) and R. Penny (Department of Dermatology, Royal Victoria Infirmary, Newcastle, Great Britain).

Radioimmunoassay

The procedures used were based on those described previously for TRH [10], and LHRH [11] and ACTH [12]. Details of the antibody specificities are provided in the Results section.

RESULTS

Development of a separation

The solvent system described by Hancock et al. [9] gave sharp symmetrical peaks for several peptides (Fig. 1). A 20-min gradient from 5—70% B (i.e. 3.5—49% acetonitrile) in the presence of 0.1% phosphoric acid (pH 2.1) was sufficient to resolve TRH, LHRH, substance P and somatostatin and a variety of other peptides. Slightly inferior resolution was obtained using isopropanol (1.2—40%) in place of acetonitrile. Both solvent systems are transparent at 206 nm but are not volatile and fractions require neutralization before RIA. It was found that the presence of salt interfered with the TRH RIA giving an apparent reduction of 30% in the amount of TRH present.

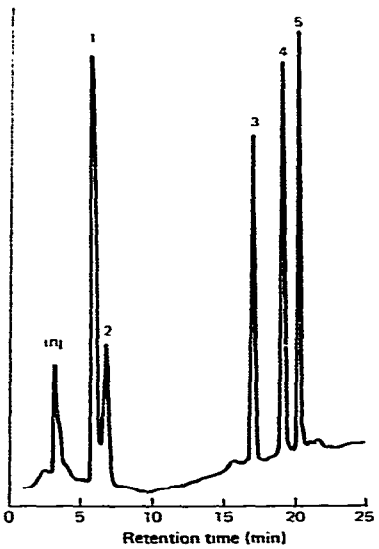

Fig. 1. Separation of peptides using acetonitrile gradient with 0.1% phosphoric acid. Solvent A, 0.1% phosphoric acid; solvent B, 70% acetonitrile in A; 20-min linear gradient from 5—70% B. Peaks: inj = injection; 1-His-Pro-diketopiperazine; 2 = TRH; 3 = LHRH; 4 = substance P; 5 = somatostatin (all 5 μg); 0.4 a.u.f.s. at 206 nm.

Replacing 0.1% phosphoric acid with 0.08% TFA (a volatile acid of the same pH) did not affect the resolution but longer retention times were recorded in the 20-min acetonitrile gradient (Fig. 2a). TFA (1%) has been used in the purification of ACTH peptides from ODS columns [13] and it has been shown that an acetonitrile gradient with 5 m*M* TFA (pH 2.5) was useful for purifying opioid peptides on a μBondapak C_{18} column [14]. We found that a gradient from 3.5—49% acetonitrile containing 0.08% TFA was suitable for the separation of peptides over a wide range of hydrophobicity and molecular weights. Small peptides (e.g. TRH and metabolites such as TRH free acid and His-Pro-diketopiperazine) are resolved in the early part of the chromatogram. β-LPH and several related peptides are also completely resolved (Table I) under these conditions, as were ACTH, α-MSH and CCK-8. No noticeable improvement was detected when sodium chloride (0.9%) was added to the 0.08% TFA (Fig. 2b).

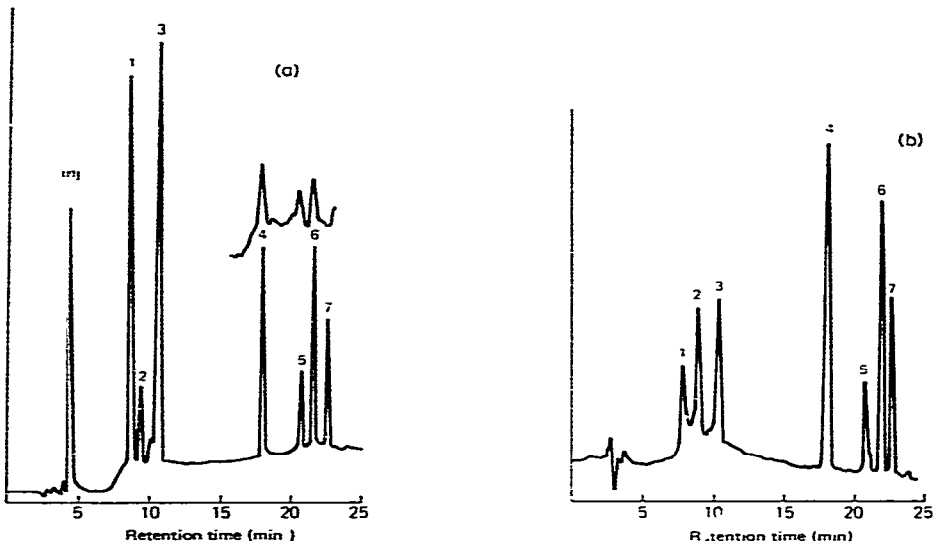

Fig. 2. Separation of peptides using acetonitrile gradient with 0.08% TFA. (a) Solvent A, 0.08% TFA; solvent B, 70% acetonitrile with 0.08% TFA; 20-min linear gradient from 5—70% B. Peaks: inj = injection; 1 = His-Pro-diketopiperazine; 2 = TRH; 3 = TRH-OH; 4 = LHRH; 5 = substance P; 6 = somatostatin; 7 = CCK-8 (all 2.5 μg except 1 and 3 which are 10 μg); 0.2 a.u.f.s. at 206 nm; upper trace, 25 ng using 0.005 a.u.f.s. (b) As (a) except solvent A, 0.09% sodium chloride with 0.08% TFA (all 2.5 μg).

TABLE I

ELUTION TIMES OF β-LPH/ACTH PEPTIDES ON C_{18} COLUMN

3.5—49% acetonitrile with 0.08% TFA over 20 min; flow-rate, 1 ml/min.

Peptide	Elution time (min)
LPH_{84-91}	3.6
Met-enkephalin	16.9
Leu-enkephalin	18.2
α-MSH	18.9
γ-LPH	19.5
hACTH	20.4
β-LPH	21.4
β-endorphin	22.4

The gradient was less suitable for resolving ACTH from C-terminal ACTH-related peptides ($ACTH_{17-39}$, $ACTH_{25-39}$, $ACTH_{18-39}$). However, a 30-min gradient from 21—35% acetonitrile in the presence of 0.08% TFA gave good resolution of these peptides (Fig. 3).

The presence of TFA was essential for good chromatography of peptides. Chromatography of $ACTH_{17-39}$ and LHRH under isocratic elution conditions showed that peak broadening occurred with the former peptide at concentrations of TFA below 0.07%, while the latter showed no loss of resolution down to 0.02% TFA. However, without TFA, the peptides were not eluted from the column.

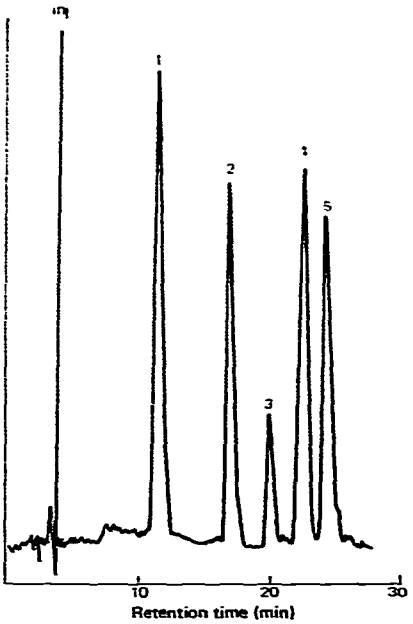

Fig. 3. Separation of ACTH-related peptides on acetonitrile gradient. Solvents A and B as in Fig. 2a. 30-min gradient from 30—50% B. Peaks: inj = injection; 1 = $ACTH_{1-24}$; 2 = α-MSH; 3 = $hACTH_{11-39}$; 4 = $hACTH_{17-39}$; 5 = $hACTH_{1-39}$ (all 2.5 μg); 0.1 a.u.f.s. at 206 nm.

Sensitivity of detection in gradient HPLC is often limited by the sloping baseline caused by the different optical densities of the two gradient components. In the present system we have overcome this problem to some extent by balancing the optical density of solution A with that of solution B by the addition of acetic acid (about 0.02% in solvent A for the reagents in use in our laboratory). The detection limit for LHRH under these conditions at 206 nm is less than 25 ng (see insert, Fig. 2a). Highly reproducible retention times were obtained; in five consecutive runs LHRH was eluted at 17.8, 17.8, 17.9, 17.9 and 17.8 min.

Applications

The acetonitrile—0.08% TFA gradient system has been used in our laboratory for several separations linked to RIA detection in the characterization of brain and pituitary peptides. Three examples are described briefly below.

The identification of extrahypothalamic TRH immunoreactivity. Previous experiments using thin-layer chromatography [15] and gel chromatography [16] have produced conflicting evidence concerning the identity of TRH immunoreactivity (TRH IR) in rat brain. We showed first by RIA that TRH standard (2 ng) could be recovered quantitatively from the column. TRH IR extracted from rat extrahypothalamic brain using methanol was found to have the same retention time as synthetic TRH and TRH from rat hypothalamus on a 0—49% acetonitrile gradient containing 0.08% TFA (Fig. 4) and no other immunoreactive peaks were eluted from the column. This indicates that

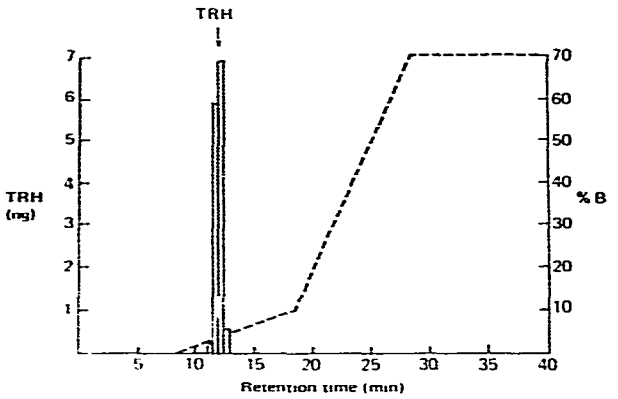

Fig. 4. Elution profile of TRH immunoreactivity from rat extra-hypothalamic brain. Rat brain (less the hypothalamus) was extracted with methanol (10 ml/g tissue). An aliquot was evaporated to dryness, the residue extracted with 1% TFA and the extract applied to the μBondapak C_{18} column. Solvent A = 0.08% TFA; solvent B = 70% acetonitrile with 0.08% TFA; gradients, 0—10% B over 10 min; 10—70% B over 10 min. Fractions were collected, dried and the residue dissolved in buffer (1 ml) for RIA.

TRH IR in rat brain is identical to synthetic TRH and that the antisera used in this study are highly specific for TRH.

The enzymic degradation of LHRH by impurities in bovine albumin preparations. Bovine serum or plasma albumin (BSA or BPA) is used in many radioimmunoassays as a non-specific carrier when the peptide is in low concentra-

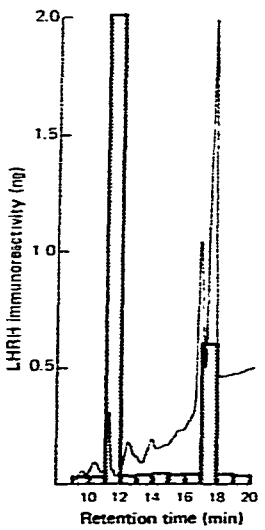

Fig. 5. Elution profile and immunoreactivity of LHRH after incubation with BPA at pH 7. LHRH (1 μg) was incubated at room temperature in Krebs—Ringer solution (1 ml) containing 0.25% BPA for 18 h. Methanol (4 ml) was added, the solution centrifuged and the supernatant evaporated. The residue was dissolved in 0.1% TFA, and applied to the column. Conditions of elution were as in Fig. 2a; UV trace, 0.01 a.u.f.s.; 1-ml fractions were collected and evaporated for RIA (shaded bars).

tion. In experiments to follow the pathway of dopamine-stimulated LHRH degradation by hypothalamic synaptosomes [17] we discovered that LHRH was degraded by BSA (Sigma, St. Louis, MO, U.S.A.) or BPA (Armour, Eastbourne, Great Britain). Although there was no apparent loss of LHRH immunoreactivity in the RIA, little immunoreactive material was found in the HPLC fraction corresponding to LHRH. One major immunoreactive degradation product was seen in the eluate (Fig. 5) and this has been shown to be $LHRH_{6-10}$. The antisera used in this study are known to be directed against the C-terminus of LHRH and this work indicates that the 6—10 sequence cross-reacts on an equimolar basis with $LHRH_{1-10}$.

The separation of carboxy-terminal ACTH fragments from rat pituitary pars intermedia. Corticotropin-like intermediate lobe peptide (CLIP; $ACTH_{18-39}$) and α-MSH ($ACTH_{1-13}$) are thought to be synthesized from ACTH in the pars intermedia. Using an antiserum raised against $ACTH_{17-39}$ we have found three main immunoreactive peaks in acid extracts of rat pars intermedia (Fig. 6). Previous studies using gel filtration have indicated that CLIP was the major C-terminal ACTH-related peptide [18] although recently, there has been evidence to suggest that a glycosylated form of CLIP is present. The antisera used here do not cross-react with glycosylated CLIP [19], being directed against that portion of the molecule where the carbohydrate moiety is attached. The antiserum also fails to recognize porcine ACTH because of the amino-acid substitution of Leu for Ser (position 31) in this region of the molecule. Thus glycosylation is presumably not responsible for the heterogeneity seen on HPLC. The recovery of $hACTH_{1-39}$ and $hACTH_{17-39}$ standards from the column was greater than 90% as demonstrated by RIA (dotted line in Fig. 6).

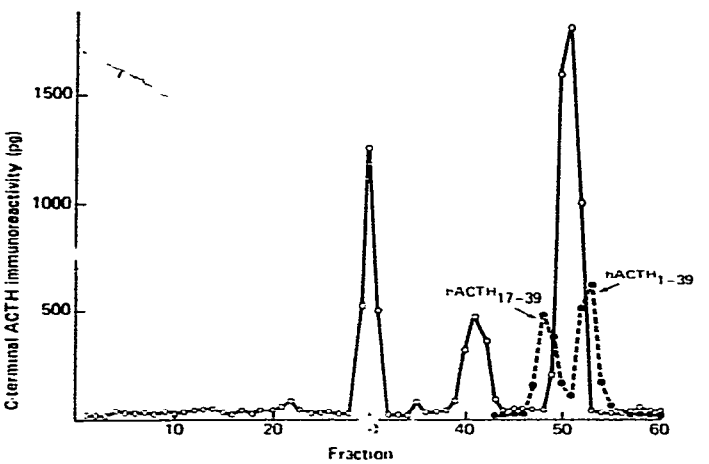

Fig. 6. C-terminal ACTH immunoreactivity in rat pars intermedia. The intermediate lobe of the rat pituitary was extracted with 0.1 *N* hydrochloric acid. The extract was made to 50% methanol, centrifuged and the clear supernatant evaporated. An aliquot (1/20th) was dissolved in 0.1% TFA and applied to the column; 0.5-ml fractions were collected and evaporated for RIA of C-terminal ACTH. $hACTH_{1-39}$ and $hACTH_{17-39}$ (150 ng) were injected in 0.1% TFA (15 μl) and chromatographed as above. Fractions were diluted 1:100 for assay.

DISCUSSION

In this paper we have tried to define the conditions for the reversed-phase HPLC separation of a wide variety of peptides coupled with RIA detection. The most successful and extensively investigated methods for HPLC of peptides have employed an acetonitrile gradient in the presence of ion-pair reagents [1], 0.1 *M* phosphate buffer in pH 2.1 [3], or 0.1% phosphoric acid [2]. An *n*-propanol gradient containing 5% acetic acid has also been described which offers some advantages [4]. While all these methods are potentially compatible with RIA, they suffer from lack of solvent volatility or from not being UV transparent at low wavelengths (below 215 nm). The present method uses volatile solvents (acetonitrile gradient with low concentration of trifluoroacetic acid) and allows the UV detection of peptides at 206 nm. The effect of the gradient on the UV baseline can be overcome by balancing the UV absorbance of solvent A with that of solvent B with acetic acid and this allows UV detection at high sensitivity. In some studies, the presence of salt or buffers has been deemed essential for good resolution [1,3]. However, salt did not improve the resolution obtained with our method.

The potential of HPLC coupled to RIA in neuropeptide analysis has been recognised but applied in only a few specific instances [7,8]. It is particularly useful for the rapid separation of a peptide from the cross-reacting precursors and metabolites often found in tissue extracts and body fluids. The present method is readily compatible with three different radioimmunoassays and is capable of resolving a wide range of peptides. Manipulation of the acetonitrile gradient may prove necessary for optimizing specific separations as we have described for the ACTH-related peptides. An important requirement for the separation of low levels of a number of peptides from a single biological sample (e.g. cerebrospinal fluid) is that elution times are highly reproducible. This is because determinations are made by RIA on specified portions of the column effluent corresponding to the previously determined retention times of peptide standards.

Three examples of the practical application of this method in the characterization of neuropeptides are described briefly and full details will appear elsewhere. The TRH-like immunoreactivity found in extrahypothalamic rat brain has been shown to co-elute with authentic TRH on a highly resolutive HPLC system. This and another study [16] contradict the report that TRH immunoreactivity in rat brain is not due to TRH. We have also confirmed (results not shown) that TRH immunoreactivity in frog (Rana pipiens) brain and skin is identical to synthetic TRH.

The degradation of LHRH by impurities in BSA was discovered unexpectedly and occurred under conditions where the immunoreactivity of the peptide was apparently unimpaired. This result urges caution in the use of serum or plasma albumins as carriers for low concentrations of peptides.

The intermediate lobe is the presumed site of synthesis of α-MSH and CLIP from ACTH. This study demonstrates the presence in significant amounts of other peptides which cross-react in the C-terminal ACTH RIA. The relationship of these peptides to ACTH and CLIP is undergoing further investigation.

ACKNOWLEDGEMENTS

We thank Mrs. J. Coxon and Miss D. Houghton for secretarial assistance.

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