Neuropeptides 1: 429-446, 1981

A SENSITIVE COUPLED HPLC/RIA TECHNIQUE FOR SEPARATION OF ENDORPHINS: MULTIPLE FORMS OF B-ENDORPHIN IN RAT PITUITARY INTERMEDIATE VS. ANTERIOR LOBE

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ABSTRACT

A technique is described which couples High Performance Liquid Chromatography (HPLC) to radioimmuno assays (RIA), thus achieving high sensitivity with good specificity. This method allows the separation and detection of multiple forms of immunoreactive peptides. Using this procedure, we have characterized the B-E-like immunoreactivity in rat pituitary. It had been previously reported that most of the B-E-sized material in both intermediate and anterior lobe of rat pituitary is not true $B\text{-}E_{1-31}$. We now report that N-acetyl $B\text{-}E_{1-27}$, which is inactive at the opiate receptor, is indeed the predominant peptide in rat intermediate lobe, with $B\text{-}E_{1-31}$ representing a low proportion of the immunoreactivity. On the other hand, the opiate active $B\text{-}END_{1-31}$ is the predominant peptide in the anterior lobe. These findings have important implications for the physiological and behavioral roles of the pituitary opioids.

INTRODUCTION

The multiplicity of endogenous opioids is becoming increasingly apparent. Several anatomically distinct opioid systems have been described in brain, pituitary and peripheral tissues. These include methionine and leucine enkephalin containing pathways (1,2), the pro-opiocortin networks in the brain and pituitary (3,4,5) and the recently discovered peptides dynorphin and alpha-neo-endorphin which contain at their N-terminal the structure of leucine enkephalin (6). The picture is further complicated by the presence of stored and potentially releaseable precursors, such as beta-Lipotropin (B-LPH) the precursor of B-Endorphin (B-E) (7,8), and of biologically active product, such as alpha or gamma Endorphin (9). Moreover, the work of Smyth and Zakarian (10) has shown the existence of N-acetylated forms of B-E, which exhibit little or no opiate activity but cross react with B-E antisera. Swann and Li (11) have also described B-E like immunoreactivity in brain with an amino-acid composition different from B-E (47% discordance) and little opiate activity. This multiplicity renders it difficult to carry out meaningful physiological/biochemical studies using only bioassays since

several of these substances occur in the same structures and interact with opiate receptors. It is equally difficult to exclusively rely on radioimmunoassays since cross-reactivity, even if minimal, can become a significant issue if behavioral manipulations affect one or the other cross-reacting peptide differentially. It is therefore critical to couple such procedures as radioreceptor assays (RRA) or radioimmunoassays (RIA) with reliable chromotographic steps which separate the various peptides.

At a more theoretical level, it is critical to determine the relative concentrations of "true" B-E in various tissues, since this would greatly affect interpretation of physiological and behavioral studies. According to Zacharian and Smyth (10) little authentic B-E is present in rat pituitary, suggesting that material released upon stress along with ACTH (12) is not opiate active.

We therefore sought to establish a procedure which would reliably separate the various species of opioid and related peptides, and which could be employed easily in extracts from single animals in order to permit assay of specific levels after physiological, pharmacological or behavioral manipulations. The criteria were as follows: a) precision and power to separate the critical form of opioid peptide, especially those similar in molecular weight and in amino acid sequences. b) reliability to permit comparison between small groups of animals. c) very high sensitivity since the concentration of these peptides in brain can be very low - e.g. less than 100 pmoles of B-E in whole rat brain, and less than 100 fmoles/ml in normal rat plasma. d) good recovery across a wide range of levels, since experimental manipulation could cause alterations by several orders of magnitude. e) speed in order to process numerous experimental samples.

The use of high performance liquid chromatography (HPLC) offered the advantages of precision and power, speed and reliability. This technique had been used for purification of synthetic peptides, as well as for large scale extraction, purification and identification of peptides (11,13,14,15). However, in most of these procedures extremely high sensitivity was not an important issue nor was high recovery of very small quantities. Thus, Gentleman et al (16) have described a rapid purification method for B-E but good recovery is not seen below 10 nmoles. In his excellent paper on the use of triethylamine-containing phosphate buffer to improve recovery, Rivier (17) reports a detection limit by UV of 50ng - which would be equivalent to about 15 pmoles of B-E. The use of fluorescence detection (14,15) does improve the sensitivity to the low pmole range. However, these detection limits are far below those possible with RIA which can detect in the low fmole range - i.e. at least one hundred fold better (18). It seemed desirable to couple HPLC precision with RIA sensitivity in an attempt to achieve both of the above-specified criteria. Aside from being potentially more sensitive, this approach would permit the detection of multiple species of peptides for which there are no commercially available

standards - such as the acetylated forms of rat B-E, or the authentic form of dynorphin (not yet completely sequenced). A few other investigators have employed such an approach in the study of opioids and related peptides with good success - e.g. to identify and quantitate multiple forms of a-MSH (19,20), or to measure the metabolites of B-E (9). We have extended these sepration and quantitation methods to include the coupling of HPLC with opiate radio-receptor assay (RRA) to determine whether an immunoreactive peak also possesses opiate activity.

One difficulty inherent in this technique lies in the interfacing of HPLC with RIA or RRA. Since we shall be dealing with small quantities of peptides, little dilution would occur when moving from chromatography to bioassay. Thus, any non-specific effect, e.g. from the buffer or solvents may alter the RIA or RRA and lead to either false positives or false negatives. The following is a description of a system which meets the criteria set forth above, and minimizes non-specific effects of HPLC on RIA and RRA. This procedure is currently in use for assay of numerous opioid and related peptides. We report here its application to study B-E immunoreactivity in anterior and intermediate lobe of rat pituitary, and suggest that, in anterior lobe, a large proportion of the immunoreactivity is indeed associated with the opiate active form of B-E.

METHODS

Extraction of peptides: The majority of the studies were carried out on rat pituitary and hypothalamus, although other species - such as monkey - and other tissues and samples - such as blood and CSF - have been studied.

Animals are sacrificed by decapitation and the brain or pituitary rapidly extracted and dissected. They are then immediately frozen on dry ice, and stored at $-50^{o}C$.

The extraction procedure consists of homogenizing the tissue in an ice cold mixture of Acetone: 2N HCl (3:1) containing 33 µl/ml of a solution of peptidase inhibitors. The solution of inhibitors consists of 30 mg/ml of phenylmethylsulfonyl fluoride and iodoacetamide dissolved in absolute alcohol. The homogenate is spun down at 35,000 x g x 10 min. The supernatant is collected in plastic tubes and the acetone is evaporated under a stream of N_2. The material remaining in HCl is then subjected to an organic extraction to remove the lipids. Five volumes (v:v) of ethylacetate:ether (3:1) are added to the extract, the mixture is vortexed and spun at low speed (2000 rpm) for 10 minutes. The top lipid-containing phase is discarded, and the peptide-containing phase is evaporated in a Savant Speedvac concentrator, suspended in the appropriate volume of .2N HCI and 10-40 µl are applied onto the HPLC column. Recovery for extraction has been examined by adding both unlabelled and labelled peptides, including B-E, a-MSH, Leucine-enkephalin and methionine-enkephalin. In all cases, it exceeded 85%.

High Performance Liquid Chromatography (HPLC)

The apparatus consists of the Beckman (Altex) programmable system with the 9420 programmer microprocessing unit, two 110A pumps, a Hitachi 100-40 multiwave length detector and a Kipp & Zonen B D 41 chart recorder. All reverse phase chromatography is carried out on Beckman ultrasphere ODS columns.

Several buffer systems, gradient profiles and separation conditions were employed before determining the optional set of conditions for our requirements. The conditions most commonly used consist of a sodium phosphate buffer (50mM, pH 2.7) containing .05% triethylamine (cf 17) as solvent A, and 100% acetonitrite (CH3 CN) as solvent B. The flow rate is typically 1 ml/min. The most commonly employed gradient program is depicted in Fig. 1. Percentage of acetonitrite is raised to 20% in 2 minutes, left constant for 2 minutes, then raised to 45% in 25 minutes, where it remains for another 2 minutes, before returning to 5% in 2 minutes for a total duration of 33 min./run. This gradient profile allows elution of most of the peptides under study. However, it can readily be altered in the interest of better separation or speed when the peptides being run are known to elute at specific points along the gradient.

Other variations include changing the sodium phosphate buffer strength between 30 to 50 mM. While higher concentrations produce sharper resolution, lower concentrations lead to less interference with some of the RIA's, especially when the tissue contains relatively low levels of peptides, and the fractions are not highly diluted. We have also employed potassium phosphate (30-50mM) rather than sodium phosphate buffer as solvent A, when opiate binding assays were to be conducted in the absence of sodium.

In all cases, the buffers and solvents are prepared according to Rivier (17), using HPLC-grade materials, and filtering aqueous buffers through 0.45 Millipore filter. All HPLC procedures are carried out at room temperature.

Pure peptide standards (0.5 - 1.0 nmole) are injected in 10-40 μ liter volume, and monitored at 210 nmeter. The effluent from the Hitachi monitor is collected in 0.5ml fractions and subjected to radioimmunoassay. Radioactive peptides (10,000 - 200,000 DPM) are similarly treated and the fractions counted with beta or gamma scintillation counters as appropriate. Tissue extracts prepared as described above are also injected in a 10-40 μl volume, monitored at 210 nmeter and the fractions evaporated either in a vacuum oven (room temperature) or in a Savant evaporator, resuspended in the appropriate buffer at the proper dilution, and subjected to RIA.

Peptide Materials Used for HPLC

These include synthetic human B-E (Bachem) alpha MSH (Organon, Ciba-Geigy) des Acetyl MSH (Organon, Ciba Geigy), methionine, and Leucine enkephalin (Bachem), oxytocin, vasopressin, dynorphin$_{1-13}$ (Peninsula Labs), beta Lipotropin (courtesy of Dr. C. H. Li, U.C. San Francisco). The camel forms of B-E, N-acetylated B-E$_{1-31}$ (NA$_c$-B-E$_{1-31}$), B-E$_{1-27}$, NA$_c$-B-E$_{1-27}$ (Peninsula Labs) and human B-E$_{1-31}$ (National Institute of Mental Health), as well as human NA$_c$-B-E$_{1-31}$ (in collaboration with Dr. David Coy). Radioactive peptides include ^{3}H-B-E (from Dr C.H. Li), ^{3}H met- and Leu-Enkephalin (New England Nuclear), as well as iodinated peptides labelled within our laboratory.

Radioimmunoassays:

Several RIA's were used to validate the method and quantitate the tissue extracts. These include assays to Leucine-Enkephalin, methionine-enkephalin, (Final antibody dilution 1:1000) and B-E (final dilutions ranging from 1:3000 to 1:50,000) These sera have been raised by us.

The general conditions for the enkephalin assays (21) and the B-E assay (18) are similar to conditions previously described. We have also employed Dr. Vaudry's a-MSH antisera (cf 22) and Dr. A. Goldstein's dynorphin RIA (23) and followed their suggested conditions. In some cases we have found it necessary to alter the strength or pH of the RIA buffer in order to permit interfacing with HPLC, as will be described below. All assays were carried out in 0.25ml volumes. Labelled peptides were added in the range of 8-20,000 dpm/tube, and incubations carried for 24 hours to 72 hours at 4°C.

RESULTS

Two aspects of the results will be presented here. The first relates to the characterization of the HPLC/RIA coupled approach to endorphins and related peptides, and the second focuses on the multiplicity of B-E-like immunoreactivity in rat pituitary.

1. Characteristics of this HPLC/RIA Procedure:

At the simplest level, Figure 1 depicts the known power of HPLC for separating the peptides which vary slightly in their structure but have the same molecular weight. Thus, human B-E is clearly more hydrophobic than camel B-E (which is thought to have the same structure as rat B-E). The same gradient profile also separates a-MSH from des-acetyl a-MSH, an important capability since both forms are present in brain (19,20), and are centrally active (24). Such a separation can greatly enhance the speci-

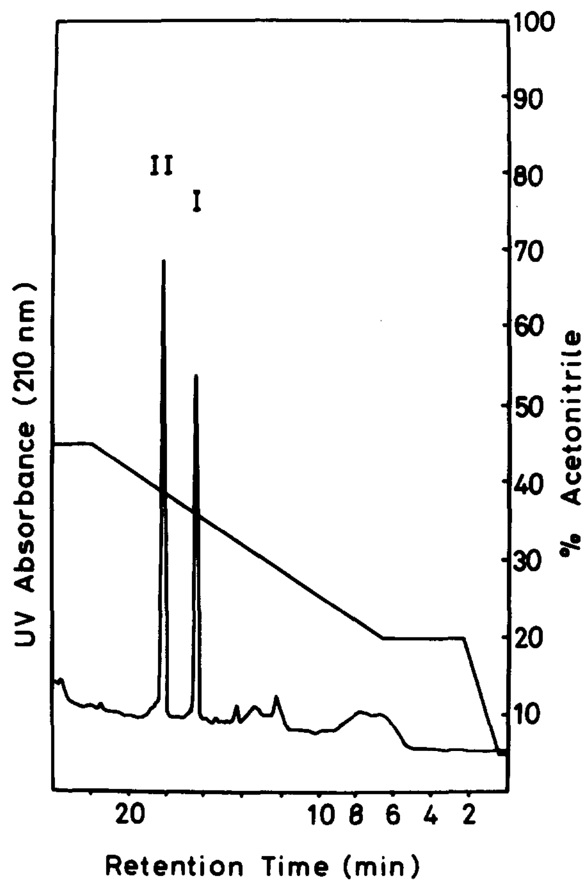

Figure 1: HPLC separation of a-MSH (N-acetyl $ACTH_{1-13}$ amide) from des-acetyl-a-MSH ($ACTH_{1-13}$ amide). Peak 1: des-acetyl-a-MSH. Peak 2: a-MSH. Gradients begins at right of figure, at 5% acetonitrite and rises in stages, to 45% acetonitrite. 1nmole of peptide injected.

ficity of radioimmunoassays and radio-receptor assay and would permit the detection of opiate activity in other parts of the profile not clearly associated with a fully sequenced opioid.

The question of whether retention time is altered in the presence of tissue extract was addressed by injecting on several occasions, 3H-B-E (from Dr. C.H. Li) in the presence and absence of such extracts. The retention time for the radioactive peptide remained constant regardless of the condition. Furthermore, 3H-B-E was added to tissue at the onset of extraction, and at the end of the procedure chromatographed on HPLC. The retention time was unaltered compared to untreated 3H-B-E. Thus, there was no evidence of oxidation, acetylation or non-specific binding taking place throughout the experimental protocol.

Recovery was also estimated using various radioactive peptides. Without TEA, recovery was poor and highly variable. However, in the presence of TEA the recovery of 3H-B-E varied between 95 and 100%. The full range of peptide concentrations could be assessed by running 3H-B-E, or iodinated B-E (or other peptides) alone, in the presene of millimolar concentration of synthetic B-E, or in the presence of tissue extracts. Here again, recovery remained excellent across all conditions even when we employed as little as 20,000 dpm of 3H-B-E, or 100,000 DPM of ^{125}I-B-E. Given the high specific activity of the iodinated material (500-1000 ci/mmole), the amount used corresponds to 50 fmoles of B-E. In spite of the excellent recovery, the small amount of unrecovered peptides can produce a significant artifact if not completely eluted. For example, if one employs 1 nmole of authentic unlabelled peptide (e.g. B-E) as a standard and recovers 99% of it, the adsorbed 1% still represents 10 pmoles of peptides remaining on the column, which can be eluted upon later runs. This amount is equivalent to the total immunoreactive content of a rat hypothalamus, and can easily lead to artifactual quantitation in biological samples. If such high amounts of standard peptides are used for calibration, we recommend extensive washing of the column and a "blank" run with no sample added, in order to ascertain the absence of any contamination. Alternatively, small quantities of radioactive peptides can be used prior to injection of biological samples. We now inject a radioactive marker prior to loading the sample, and run our peptide standards after the biological samples have been eluted, in order to avoid any possible contamination.

Finally the reliability of the systems was tested by estimating the B-E-like immunoreactivity (HPLC/RIA) in rat neurointermediate lobe or anterior lobe on at least 5 different occasions. Our estimates of total B-E-like immunoreactivity varied by less than 10%

In interfacing the HPLC generated fractions with RIA's we required that the solvents *per se*, throughout the gradient, would not produce any non-specific effects on the antigen-antibody

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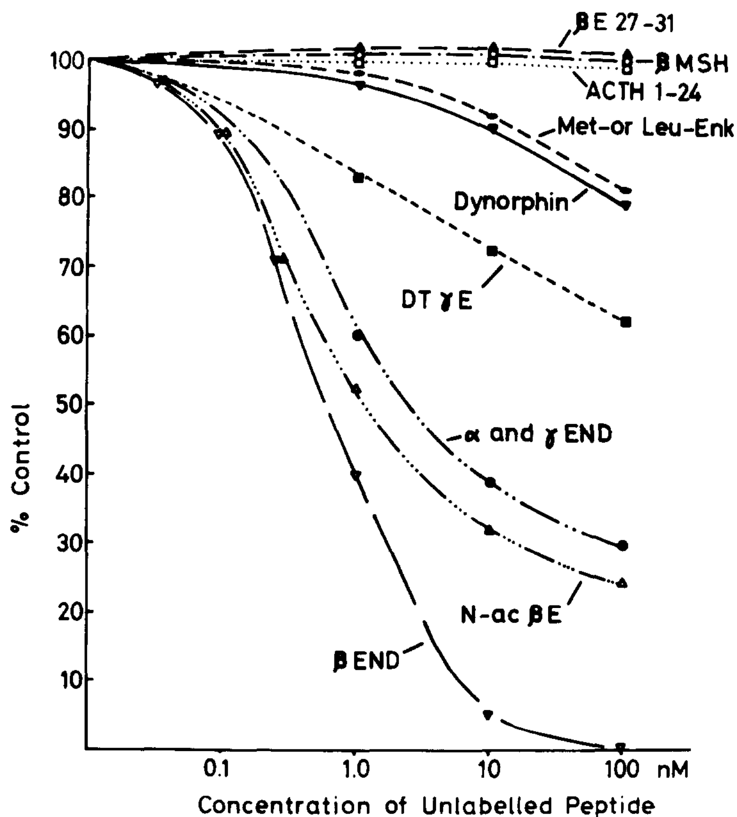

Figure 2: Radioimmunoassay comparing immunoreactive potencies of the acetylated and non-acetylated forms of the human B-E, and other opioid peptides. Note close correspondence at low concentrations, and non-parallelism at high concentration, suggesting N-terminus directed low affinity population of antibodies. The absence of a C-terminus-directed population is indicated by the lack of inhibition of binding when the C-terminus tetrapeptide (B-E_{27-31}) is employed. Thus, B-E_{1-27} shows the same cross reactivity as B-E_{1-31} - (not shown but confirmed).

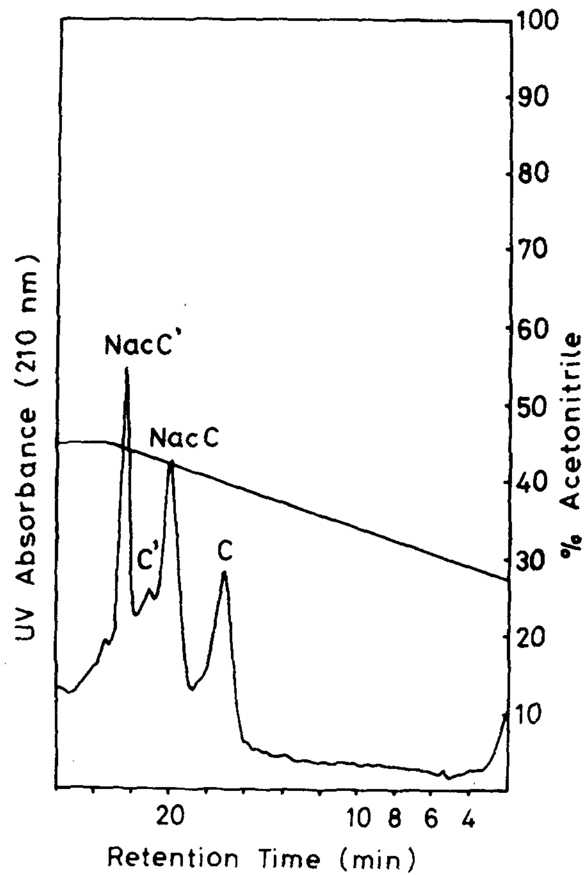

Figure 3: Partial separation of the 4 forms of B-E-like material on HPLC - peptides (500 pmoles each) were premixed and injected in acetic acid.

interactions. When 50mM sodium-phosphate buffer (pH 2.7) was evaporated, resuspended in the B-E RIA buffer (10 mM sodium phosphate, pH 7.6) and assayed, a great deal of non-specific inhibition was observed. This non-specific inhibition was found to be due to the remaining acidity in the HPLC fractions, even after complete evaporation, leading to a decrease in the final pH of the RIA samples. The problem was handled by two changes: a) decreasing the strength of the HPLC sodium-phosphate buffer from 50mM to 30-40 mM. b) increasing the strength of the RIA buffer to 150mM, and the pH of that buffer to 8.2. These changes caused little problem for either HPLC or RIA, and resulted in complete elimination of the non-specific inhibition in the B-E RIA. Similar alterations in alpha-MSH RIA conditions and dynorphin RIA conditions and the opiate radio-receptor assay have led to the same situation. In general, it is necessary to determine a pH "window" for any RIA or receptor assay (i.e. a range which produces good binding), as well as the strongest buffer the assay can tolerate. Then one can use that buffer at the highest acceptable pH for resuspending the samples and maintaining them within the correct pH range.

Thus, the HPLC-RIA system described here yields good separation between peptides, excellent recovery in the fmole range, in the presence or absence of tissue extracts. It appears reliable, and is devoid of the problems of non-specific inhibition, yielding no false positives or negatives.

2. Multiplicity of B-E Immunoreactivity in Pituitary and Brain

Zakarian and Smyth (10) have reported that B-E is stored in both active and inactive forms. They described the existence of "true" B-E or B-LPH_{61-91} which they had termed the C fragment, a C' fragment or B-E_{1-27}, and the N-acetylated forms of these two peptides. All but the C fragment (B-E_{1-31}) exhibit greatly decreased opiate potency. They suggested that the majority of B-E-like immunoreactivity both in intermediate and anterior pituitary is not B-E_{1-31}, whereas in the hypothalamus it is mostly B-E_{1-31}.

We have employed the HPLC/RIA procedure in order to separate multiple B-E-like species in various tissues. It should be noted here that (B-E_{1-27}) and the acetylated form of B-E_{1-31} and of B-E_{1-27} were not available when we began our experiments which has prevented us and others from using them as standards. We have since obtained these compounds, and used them to identify the immunoreactive peaks, as described below. The characteristics of the B-E RIA employed are displayed in Fig. 2. The separation of the multiple forms of B-E is shown in Fig. 3.

When rat neurointermediate/posterior lobes are extracted, applied to HPLC using our standard gradient then subjected to RIA the pattern in Figure 4 emerges. It is apparent that multiple species of END immunoreactivity are present. None of the additional peaks are due to B-LPH, since molecular sieving

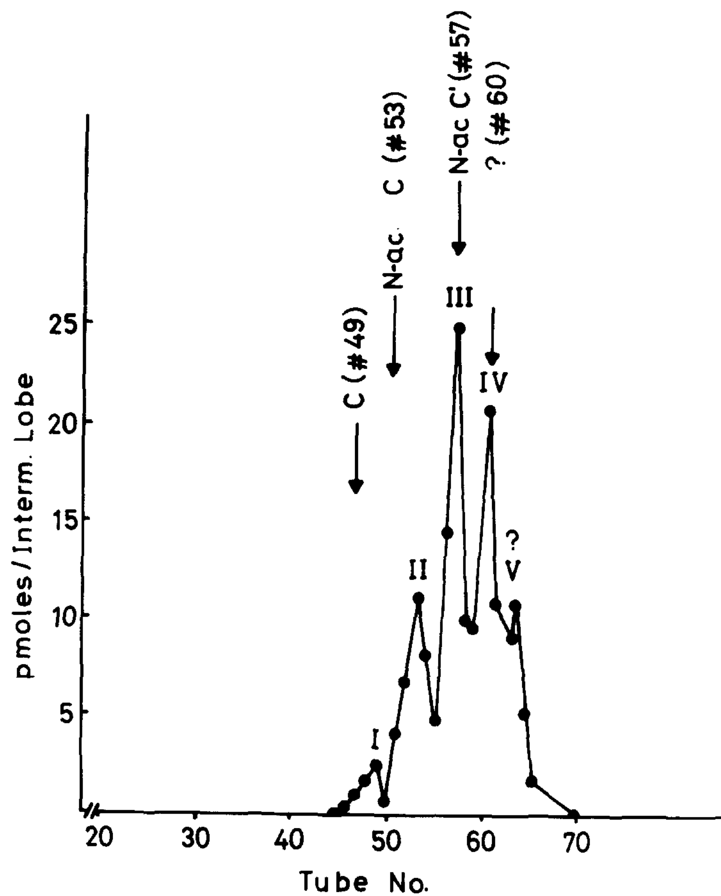

Figure 4: Immunoreactive profile of rat neuro-intermediate lobe after HPLC. Peaks I, II, II are identified as $B\text{-}E_{1-31}$, NAc $B\text{-}E_{1-31}$, and NAc $B\text{-}E_{1-27}$ respectively.

procedures (Biogel P10, 0.9x50 cm) were carried out first and show that only a very small percentage of the intermediate lobe immunoreactivity is due to this peptide. Only B-E-sized material was applied to the chromatograms shown here. Fig. 4 demonstrates that the immunoreactive peak corresponding to authentic $B-E_{1-31}$ constitutes a very small proportion of the immunoreactivity. The major peak (peak 3) is associated with the retention time of $NAc-B-E_{1-27}$ (NAcC'). Peak 2 comprises $NAc-beta-END_{1-31}$ (NAcC) although in this procedure $B-E_{1-27}$ runs very close. However, other work in our laboratory using the ion exchange procedure of Zakarian & Smyth (10) with S-P Sephadex and a NaCl gradient shows that little or no $B-E_{1-27}$ (C') is evident in rat intermediate lobe. Thus, peak 1 is $B-E_{1-31}$, peak 2 is $NAc-B-E_{1-31}$, peak 3 is $NAc-B-E_{1-27}$, peaks 4 and 5 remain unidentified and may represent further processing of the third peak. Peak 4 in particular is a highly reliable finding in posterior/intermediate lobe, and is not seen in anterior lobe extracts. Further work on the HPLC conditions is being carried out to effect a better separation between NAc $B-E_{1-31}$ and $B-E_{1-27}$, in order to confirm our findings that little C' is present in intermediate lobe. The multiple immunoreactive peaks in posterior/neuro-intermediate lobe are not extraction artifacts since other tissues do not exhibit a similar pattern as will be described below. Furthermore, ^3H-B-E added at the start of extraction of this lobe, emerges as a single radioactive peak at the end of the extraction. It should be noted here that the total immunoreactivity here is 600-700 pmoles/intermediate pituitary, a concentration consonant with total levels obtained prior to HPLC. In these assays, only 1/40 of a posterior neurointermediate lobe is needed to measure all the peaks.

When anterior lobe is dissected with no particular attention to a clean separation from posterior/intermediate lobe, the identical multiple peaks emerge. However, the total amount of B-E immunoreactivity is approximately tenfold higher in intermediate/posterior lobe than in anterior lobe. Immunohistochemical studies show it to be entirely localized in a thin band of the neurointermediate pituitary, while it is highly heterogeneous in the anterior pituitary (Bloom, 1977). Thus, the extremely high concentration in intermediate lobe makes it easy to contaminate the anterior lobe dissection and lead to spuriously high levels and an erroneous pattern. We then dissected anterior lobe more carefully, and subjected the extracts to sieving on Sephadex G50 or Biogel P10., and the B-E sized material was applied to HPLC then assayed. The B-E immunoreactivity emerged in two peaks, with the major one chromatographing with authentic B-E (Fig. 5). The B-LPH sized material was also collected from the sieving step, and applied to HPLC. A single peak (Fig. 6) emerged which was more hydrophobic than B-E, and which reacted with a B-LPH RIA (5). Unfortunately, no standard is available for this peptide. It is, however, thought to differ from the known structures of B-LPH, and our own results along with others (25) show it to have a smaller molecular weight than human B-LPH. HPLC, however, coupled with sieving and immunoaffinity columns

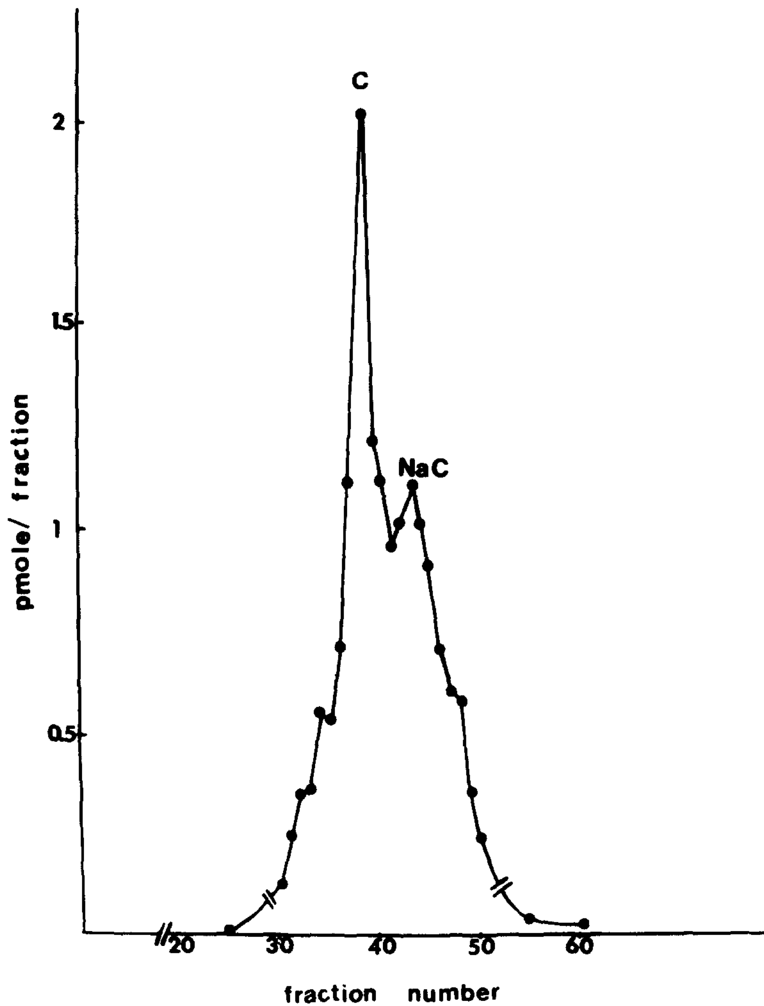

Figure 5: Immunoreactive profile of rat anterior lobe following HPLC. Extracts were first sieved on Biogel P_{10}. The B-E-sized material was then applied to HPLC as described in the text. Peaks are identified as B-E and a small amount of N-acetyl B-E.

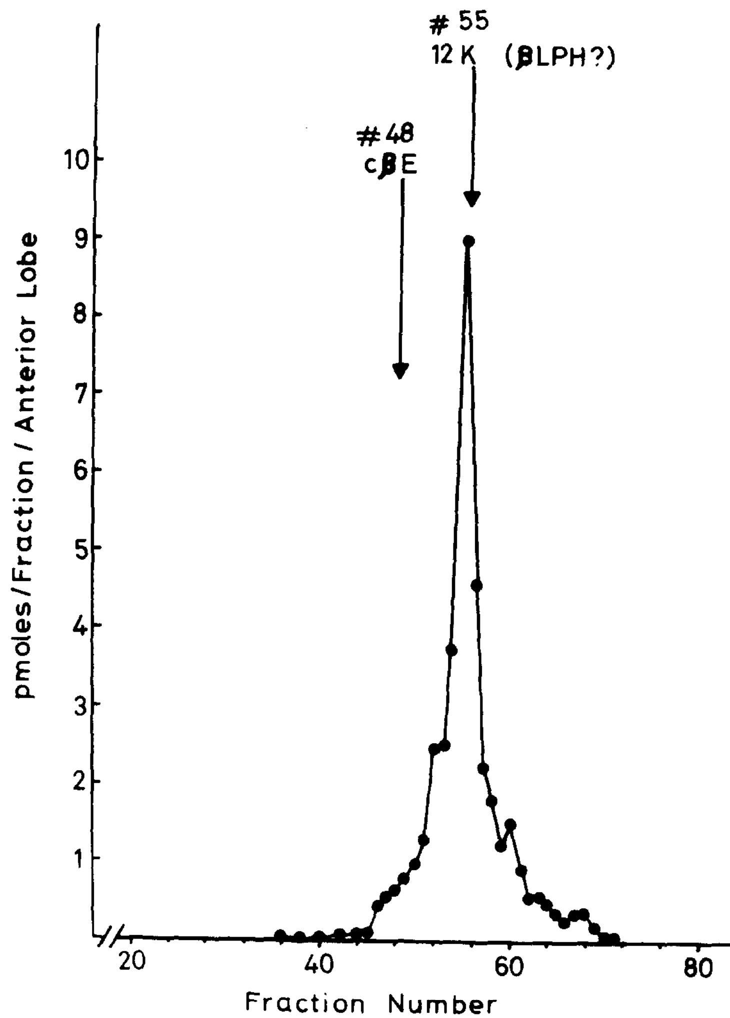

Figure 6: Immunoreactive profile of B-LPH-sized material collected from P_{10} and applied to HPLC.

provides, in our hands a rapid and efficient partial purification of rat B-LPH for marking purposes.

DISCUSSION

The present results support the feasibility of a coupled HPLC/RIA approach to studying peptides in individual animals. They also point to the real need for such an approach since multiple peaks of B-E immunoreactivity emerge in pituitary tissue, several of these possessing some opiate receptor activities. While some of the peaks remain to be completely identified, it is at least possible to single out a specific peak which does co-chromatograph with B-E, its acetylated form or the N-acetyl C' peak and possess equimolar immunoreactivity and partial opiate receptor activity (Akil et al., submitted). This would permit more believable measurement of these peptide under various treatment conditions such as stress, pharmacological or behavioral manipulations.

The present findings are in partial agreement with Smyth and Zakarian's work (10) suggesting that authentic B-E represents only a small proportion of the material in the intermediate lobe - in our hands less than 10%. On the other hand, we find that in rat anterior lobe, a peak which co-chromatographs with B-E represents a substantial proportion of the total immunoreactivity. More recent evidence from Smyth & Zakarian (26) is consistent with this view, suggesting that in another species, the pig, acetylation is rather uncommon in anterior lobe. Mains & Eipper (27) using an ion exchange column also concur in finding a high proportion of B-E in anterior pituitary and not in the intermediate lobe. Another area of disagreement revolves around the nature of the major peaks in intermediate lobe. In our hands, NAc-B-E_{1-27} is the most common product, followed by a more hydrophobic material (peak 4) and NAc-B-E. This is in contradiction with the older findings of Smyth and Zakarian in rat (10) and the more recent ones in pig (26) suggesting B-E_{1-27} to be a major constituent.

Taken together, our results strengthen the notion that processing of pro-opiocortin in different tissues such as intermediate and anterior lobe is quite different. The existence of B-E is anterior pituitary would render it available for co-release with ACTH, suggesting that a biologically active opioid, rather than an inactivated form, would circulate in the bloodstream and find its way to various target tissues. The predominant intermediate lobe material (NAc-B-E_{1-27}), being highly processed, appears to be over 1000 fold less active at the opiate receptor (Akil et al.; submitted), non-opiate functions should be considered for this molecule. Brain, which makes a-MSH from ACTH, may partially process B-E_{1-31} into shorter and deactivated forms. The ratios of these forms may vary under different biological conditions - an area of important investigation further made feasible by techniques such as the one described here.

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ACKNOWLEDGEMENTS

We are very grateful to Dr. D. Coy for supplying human N-Acetyl B-E, to Dr. C.H. Li for his generous gift of ^{3}HB-E and B-LPH antiserum, to Dr. H. Vaudry for a-MSH antibody, and Dr. A. Goldstein for dynorphin antisera. We thank Organon, Ciba-Geigy and the National Institute of Mental Health for their generous gifts of peptides. We thank Pat Erskine for preparing the manuscript. This work was supported by NIDA #DA 02265 and NSF #BNS 800 4512 to H.A. and S.J.W.

Accepted 2 June 1981