Journal of Chromatography, 327 (1985) 279–292
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 540

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF *IN VITRO* CENTRAL NEUROPEPTIDE PROCESSING

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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) was used to study and characterize the *in vitro* proteolytic processing of β-endorphin by twice-washed membrane homogenates. A high-resolution method, capable of separating over 30 different human β-endorphin-related fragments in a single analysis, was used to study the time course of production of specific, biologically active endorphin fragments by membrane-associated proteases. The results demonstrate that frozen (−37°C), postmortem human and rat brains are viable for processing studies and that metabolism proceeds similarly to that in fresh brain homogenates or slices. Significant differences were noted in the formation rates of putative neuroleptic peptides between sex- and age-matched postmortem brain tissues from controls *versus* postmortem brain tissues from neuropsychiatric patients or drug-treated animals. These data suggest that using HPLC to characterize neuropeptide processing in human or rat membrane-associated enzyme homogenates is both descriptive and quantitative and offers insight into the central regulation of neuropeptide metabolism.

INTRODUCTION

Opioid peptides are derived from one of three known precursor proteins, pro-opiomelanocortin (265 amino acids), proenkephalin A (263 amino acids) and proenkephalin B (256 amino acids)[1]. These three proteins have been shown to serve as the precursors of such biologically active peptides as ACTH, MSH, peptide E, dynorphin and β-endorphin[1–3]. Further proteolytic cleavage of these biologically active peptides has been shown to occur *in vitro*, leading to specific fragments which have distinct central and peripheral activities, quite different from the parent peptide[4–8]. Based on the *in vitro* observations coupled to the biological effects, it has been hypothesized that a specific balance exists both centrally and peripherally in the enzymatic processing of neuropeptides to active fragments[4–9]. In particular, β-endorphin [βE-(1–31)] is processed centrally to specific α- and γ-type endorphins and their non-opioid analogues which lack tyrosine at the amino terminus of the peptide[4–10]. These fragments can produce potent behavioral effects in rats and man. γ-Type endorphins [βE-(2–17) and βE-(6–17)] possess neuroleptic-like antipsychotic activity[6,7,10–12] and

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α-type endorphin [βE-(2–16)] produce effects often found to be opposite to γ-type endorphins, and these effects are similar to those of amphetamines[2–4,8]. An alteration in the balance of these centrally active peptide fragments could underlie specific physiological and/or neuropsychiatric disease states[13–15].

While some biological responses have been well characterized, the use of high-performance liquid chromatography (HPLC) to study and characterize neuropeptide processing is a recent development[5,9,14–18]. The technique is based on the principle that most peptides and peptide fragments vary in their hydrophobicity and, therefore, the more hydrophobic or lipophilic the peptide the stronger it is retained by the hydrocarbonaceous HPLC columns. Therefore, a large volume of organic solvent is necessary to elute the very hydrophobic peptides. Since each amino acid in a given peptide contributes a unique degree of hydrophobicity, we have been able to predict, with a reasonable degree of success, the elution pattern of specific fragments. Based on the work of Molnár and Horváth[19] and others[20,21], retention times for a large group of peptides can be predicted with reasonable accuracy. Reversed-phase HPLC also offers the necessary specificity and selectivity to analyze the large number of peptide fragments produced during a time-course metabolism experiment. Recently, Burbach *et al.*[9] reviewed their studies on peptide processing of ACTH, vasopressin, oxytocin, vasotocin and the endorphins, offering interesting evidence that specific enzymes are responsible for the formation of certain biologically active fragments. Earlier, Koulischer *et al.*[18] had found that cholecystokinin is sequentially processed to active peptide fragments and that some of the cleavages could be inhibited by bestatin and puromycin (aminopeptidase inhibitors) while others were only effected by aprotinin (trypsin-like enzyme inhibitor).

In the present study we have used reversed-phase HPLC to study and characterize central β-endorphin processing to specific α- and γ-type endorphins. The data suggest that a specific balance of the biologically active α- and γ-type endorphins exists centrally that can be disrupted by neuropsychiatric disease states or drug treatment[14,22].

EXPERIMENTAL

Apparatus

The HPLC system employed for analyses included two Model 6000A solvent delivery systems (Waters Assoc., Milford, MA, U.S.A.), Model LC65T variable-wavelength U.V. detector with a temperature-controlled column oven (Perkin-Elmer, Norwalk, CT, U.S.A.), and a Axxiom™ Model 301-99 data saver (Cole Scientific, Calabasses, CA, U.S.A.). A Model 3390A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a strip chart recorder (Linear Instruments, Berkeley, CA, U.S.A.) were utilized for recording and quantitation. Peptides were separated by gradient elution generated by a Model 660 or Model 680 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). Sample injections were made with a Model 610 B WISP™ automatic injector (Waters Assoc., Milford, MA, U.S.A.) or a Model 7125 manual injector (Rheodyne, Cotati, CA, U.S.A.) onto 25 cm × 4 mm (5 μm) Ultrasphere-ODS columns (Beckman Instruments, Berkeley, CA, U.S.A.),

Chemicals and solvents

All chemicals used were of the highest quality available (A.C.S.). HPLC-grade "distilled in glass" acetonitrile (Burdick and Jackson, Muskegon, MI, U.S.A.) was used to ensure the lowest possible background absorption. Buffers consisted of reagent-grade monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), or monobasic potassium phosphate (KH_2PO_4) (J. T. Baker, Phillipsburg, NJ, U.S.A.) and special reagent-grade water, produced by deionization, reverse osmosis, and filtration through a 0.2-μm filter (Continental Water Systems, El Paso, TX, U.S.A.). For ion-pairing chromatography, octyl sodium sulfate (Eastman Kodak, Rochester, NY, U.S.A.) was added to our routine 0.1 *M* NaH_2PO_4 buffer. Buffer pH was adjusted with phosphoric acid (J. T. Baker, Phillipsburg, NJ, U.S.A.) or sodium hydroxide (MCB, Gibbstown, NJ, U.S.A.). Buffers were then filtered through 0.2-μm nylon-66 membrane filters (Rainin Instruments, Woburn, MA, U.S.A.), thoroughly degassed by sonication under vacuum, and maintained under constant helium purge during HPLC analyses.

Peptide calibration standards

Human β-endorphin and its related fragments were purchased from Beckman Bioproducts Division (Berkeley, CA, U.S.A.), Vega Biotechnologies (Tucson, AZ, U.S.A.), Peninsula Labs. (Belmont, CA, U.S.A.), or received as gifts from Dr. J. W. Van Nispen (Organon International, Oss, The Netherlands). All peptides were precisely weighed on a Model 29 automatic electrobalance (Cahn Instruments, Whittier, CA, U.S.A.) and all vials, pans, and spatulas were treated with a Zerostat™ antistatic instrument (Discwasher, Columbia, MO, U.S.A.) to prevent static "jump" of peptides during handling. To ensure stability, analytical standards were prepared in 0.01 *M* Ultrex™ acetic acid (J. T. Baker, Phillipsburg, NJ, U.S.A.) and tested by HPLC to assure purity prior to use in standard mixes or in tissue studies.

High-performance liquid chromatographic conditions

The reversed-phase mode of HPLC was chosen for the analysis of human β-endorphin and its related fragments, because the separation is faciliated by the varying degree of hydrophobicity (lipophilicity) of each peptide fragment based on its amino acid composition[19–21].

A series of acetonitrile gradients was developed for the quantitation of different groups of related peptide fragments or for improving the selectivity. Gradient elution with acetonitrile *versus* 0.1 *M* NaH_2PO_4 was used in all cases, with a 25 cm × 4 mm (5 μm) Ultrasphere-ODS column (Beckman Instruments, Berkeley, CA, U.S.A.) at 40°C, operated at a flow rate of 2.0 ml/min. Detection was at 210 nm, where absorption due to peptide bonds is maximized and background absorbance due to the phosphate buffer is minimized.

Membrane-associated enzyme homogenates

To investigate the effect of specific, membrane-associated enzymes on β-endorphin processing, a technique for isolating the membrane-associated peptidases of various tissues was developed.

After careful dissection, all tissues were immediately blotted, weighed, and added to enough ice-cold 1 m*M* Na/K phosphate buffer (pH 7.4) to make a 2% (w/v)

homogenate. Tissues were then homogenized in an Ultra-Turrax Tissuemizer (Tekmar, Cincinnatti, OH, U.S.A.) at 50% speed for 30 sec, then put on ice to facilitate lysing of cells. After 30 min at 0°C, half a volume of cold 50 m*M* Na/K phosphate buffer (pH 7.4) was added for each volume of the initial homogenate, then suspended (10 sec at the 50% setting). The homogenate was centrifuged at 49000 *g* for 45 min in a Model RC2-B superspeed refrigerated centrifuge (Sorvall, Newton, CT, U.S.A.). The supernatant was carefully decanted and discarded, then the pellet was resuspended into a 2% solution with cold 50 m*M* Na/K phosphate buffer and again centrifuged at 49000 *g* for 45 min. The supernatant was decanted, and the pellet was resuspended in an appropriate volume of 50 m*M* Na/K phosphate buffer to yield a homogenate. The resuspended pellet was used for the *in vitro* processing incubations and for the Folin-Lowry protein determinations, performed to normalize all data to mg of protein. Since each tissue varies in the amount of protein per mg wet weight, we varied the percent tissue in order to describe properly the time-course kinetics of β-endorphin processing.

Time-course processing of β-endorphin

Time-course processing was accomplished by incubating the twice-washed membrane-associated enzyme preparation with β-endorphin (1–7 nmol) at 37 ± 0.1°C for time intervals of 5–90 min. β-Endorphin stock solution (0.5 μg/μl) was prepared in sterile water (Abbott Labs, Chicago, IL, U.S.A.) or sterile 50 m*M* Na/K phosphate buffer, pH 7.4 for incubations. Screw-cap microtubes (Walter Sarstedt, Princeton, NJ, U.S.A.) were used for all the incubations including an inactivated tissue control (C_1), a buffer control (C_2), tissue control (C_3), and a dilution control (C_4). For all time points and tissue controls (C_1 and C_3), 200-μl aliquots of homogenate were placed in the tubes. A volume of 200 μl of 50 m*M* Na/K phosphate buffer was used for C_2 and C_4. Prior to incubation, C_1 was capped and placed in a boiling water bath for 15 min to inactive peptidases, then cooled to room temperature. To each tube, 50-μl (10 to 50 μg) aliquots of β-endorphin were added, except to C_2, which received 50 μl of buffer. Tubes were then securely capped, carefully mixed for 1–2 sec in a Vortex mixer, then placed in a 37°C water bath for 5, 10, 20, 40, 60 and 90 min. All controls were incubated for 90 min, except C_4, which was not incubated. After the designated incubation period, tubes were removed and boiled for 20 min, then placed in ice for 30 min. Tubes were centrifuged in a microfuge (Beckman Instruments, Berkeley, CA, U.S.A.), and the supernatant was removed and stored at −37°C until HPLC analyses.

Postmortem human brain tissue. For human brain studies, postmortem putamen and cortex, previously stored at −80°C for 4–5 years, were obtained from the MRC Neurochemical Pharmacology Unit (Cambridge, U.K.). Control tissues were from patients with no known neurological or mental disease, and sex- and age-matched tissues were obtained from patients diagnosed as having confirmed schizophrenia. A complete sample history accompanied each sample attesting to its integrity[14].

Postmortem rat brain tissue. Male Sprague-Dawley rats (134–185 g) were used in a drug treatment study to investigate the effects of several centrally acting drugs on brain peptidases[22]. Each rat was anesthetized with diethyl ether and implanted subcutaneously with an Alzet Model 2001 osmotic minipump (Alza, Palo Alto, CA,

U.S.A.) in the neck region. Wound clips were utilized to close the wound. Animals were treated with haloperidol "HL" (McNeil Labs., Ft. Washington, PA, U.S.A.), chlorpromazine "CP" (Elkins-Sinn, Cherry Hill, NJ, U.S.A.), promethazine "PR" (Elkins-Sinn, Cherry Hill, NJ, U.S.A.), or phenobarbital "PN" (Sigma, St. Louis, MO, U.S.A.), diluted in sterile 0.9% saline. (For each control and drug treatment group, $n = 5$). To facilitate a chronic treatment, drugs were administered over eight days by the minipumps, which delivered 1.00 ± 0.05 μl/h, providing for the following dosages: haloperidol, 3 mg/kg per day; chlorpromazine, 4.2 mg/kg per day; promethazine, 5 mg/kg per day, and phenobarbital, 20 mg/kg per day. Following the treatment period, the animals were sacrificed by cervical dislocation, and the whole brain minus cerebellum was removed and maintained on ice until homogenization, as described above, was performed.

Quantitation

All peptides were quantitated by the external standard method of analysis. β-Endorphin and all fragments were precisely weighed and diluted in 0.01 *M* Ultrex™ acetic acid, which stabilizes these peptides during storage. All peptides are checked for purity by HPLC prior to the assignment of a relative-weight-response factor. Response factors for each peptide were derived by making repetitive 50-μl (250 ng) injections of the single compound, then verifying that value with repetitive injections of a standard mix, composed of all fragments (100–300 ng each) of interest. Linearity of response was also verified, as was reproducibility of injection in a series of injections over a six-month period. Both variables had less than a 5% coefficient of variation throughout the six-month period.

Peak identification

Identification of sample peaks was based on comparison of retention times from five different columns with simultaneous elution of standards in the standard mix when similar HPLC gradient profiles were used. Additionally, peaks were verified on various other gradient profiles, for example, the verification of βE-(6–17), -(7–17), and -(8–17) required development of a novel ion-pairing–acetonitrile separation *versus* a phosphate buffer–acetonitrile separation in order to resolve these fragments, which differ by only a single amino acid. Fast atom bombardment–mass spectrometry (FAB–MS) is another technique we have used to verify the identity of fragments[23]. Molecular ions for all fragments less than 1800 molecular weight can be obtained. Prior to analysis and quantitation, all peptides are also hydrolyzed and amino acid analyses are performed. The resultant amino acid values are compared to the proposed sequence. Only peptides with confirmed sequences are used in our studies. Assigning peptide fragment identity through their elution position is not entirely unambiguous but by using different chromatographic systems as shown for βE-(6–17), FAB–MS and peptide hydrolysis–amino acid analysis, our ability to assign fragment identities is enhanced.

RESULTS AND DISCUSSION

High-performance liquid chromatography

The amino acid sequence of β-endorphin, a β-lipotropin (β-LPH)-related pep-

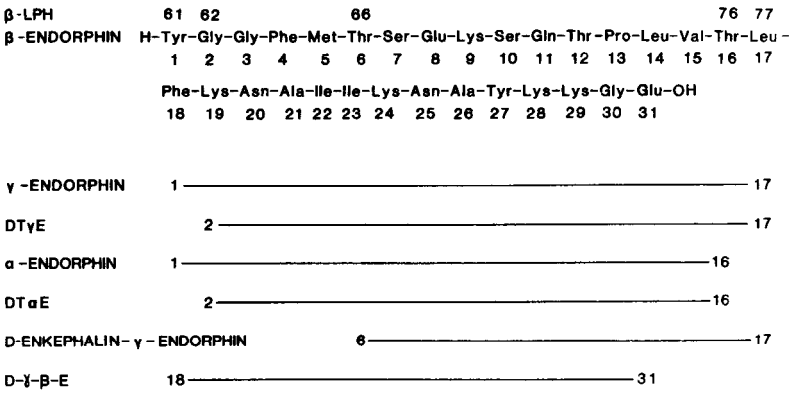

Fig. 1. Amino acid sequence of β-endorphin and related peptide fragments.

tide, derived from the last 31 amino acids of proopiomelanocortin, is shown in Fig. 1. Although the βE-related fragments differ significantly in their biological activity, they are very similar in amino acid sequence. In fact, α-endorphin differs from γ-endorphin by the amino acid leucine in the 17-position of β-endorphin, yet they are

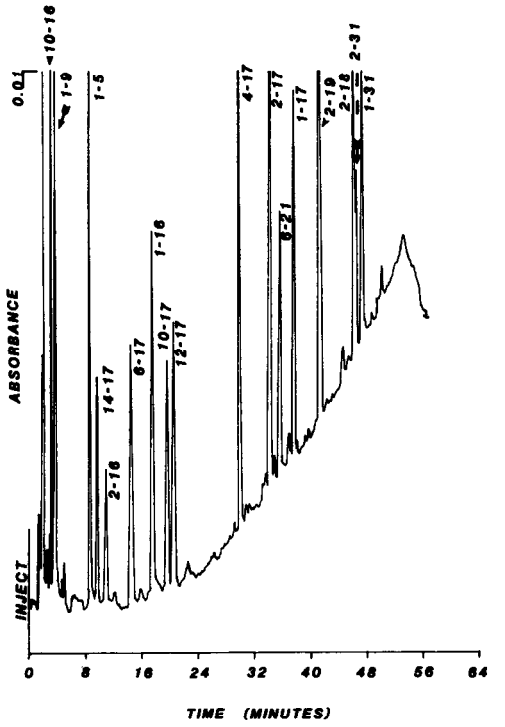

Fig. 2. Reversed-phase HPLC of 17 β-endorphin fragment standards. Peptides were separated on a Beckman Ultrasphere-ODS (5-μm) column (25 cm × 4 mm) with a linear gradient of acetonitrile (16–35%) against 0.1 *M* phosphate buffer for 50 min at 40°C and 2.0 ml/min. Detection was at 210 nm, 0.01 a.u.f.s. Amounts corresponding to 100–150 ng were injected for each peptide.

opposite in their activities in behavior paradigms[3]. Therefore, a specific and selective high-resolution chromatographic procedure was developed for separating and quantitating each fragment produced after β-endorphin incubations.

Initially we would only separate five fragments and the parent β-endorphin[16]. An improved separation was developed to quantitate 17 different fragments in one elution profile. Several of these fragments differed by only one amino acid, but by employing a linear gradient from 16–35% acetonitrile against 0.1 *M* NaH_2PO_4 (pH 2.15) over 50 min we were successful (Fig. 2). Five different pH conditions and gradient profiles were studied for this gradient system (Figs. 3 and 4). Increases in pH from pH 2.1 to 4.0 caused slight changes in the capacity factor (*k'*); for the majority of fragments *k'* decreased slightly, whereas only the more hydrophobic compounds showed an increase in *k'* (Fig. 3). Only the β-endorphin fragment 2–19 changed in its elution order at pH > 2.1 yielding an improved separation from γ-endorphin [βE-(1–17)] (Fig. 3).

Gradient profiles of acetonitrile had a much more dramatic effect on the *k'* of the peptide fragments (Fig. 4). In general, the more convex the curvilinear gradient, the lower the *k'*; and the more concave the gradient, the longer the retention time (Fig. 4). It was noted, that the more polar fragments, such as β-endorphin [βE-(1–9) and -(10–16)], were not affected by changes in the gradient within the 16–35% acetonitrile range. Therefore, for the analysis of the highly polar fragments, another

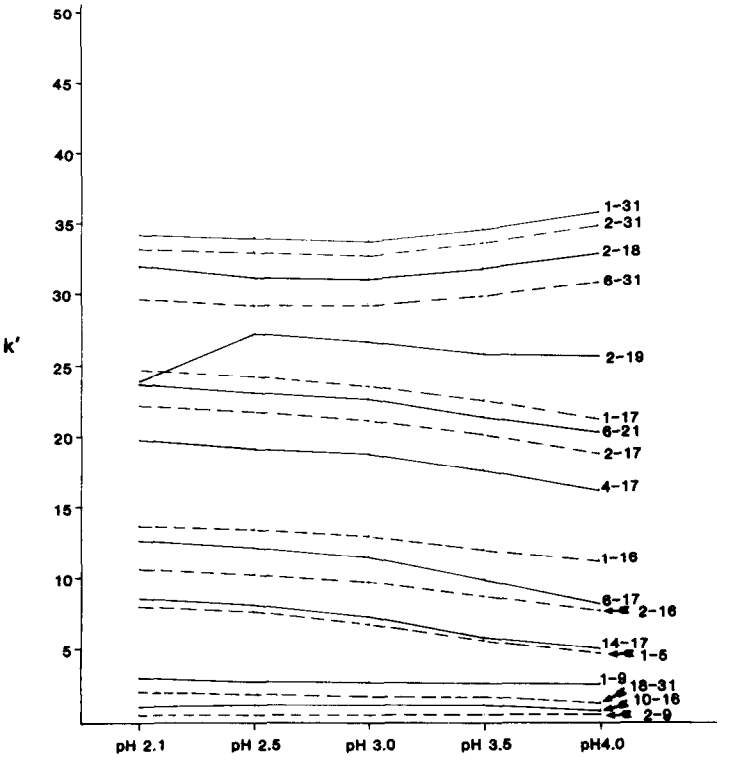

Fig. 3. Effect of phosphate buffer pH on the capacity factor (*k'*), of β-endorphin and related fragments.

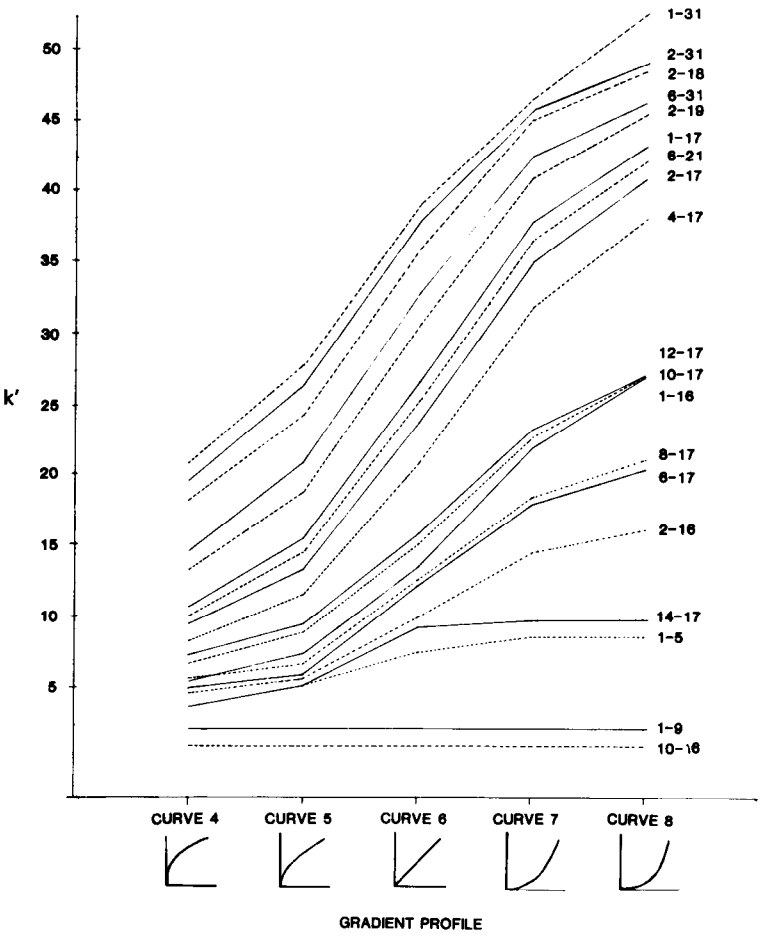

Fig. 4. Effect of acetonitrile gradient profile on the capacity factor (k'), of β-endorphin and related fragments.

separation was developed (Fig. 5). A linear gradient from 10–20% acetonitrile *versus* 0.1 *M* NaH_2PO_4 (pH 2.3) at 40°C over 40 min at a flow-rate of 2.0 ml/min gave excellent resolution of eight additional polar fragments (Fig. 5).

By combining various acetonitrile gradients (linear and concave), formed with 12–35% acetonitrile against 0.1 *M* NaH_2PO_4 over 80 min at 40°C, a separation of over 30 β-endorphin fragments was developed and applied to a study of postmortem tissues (Figs. 9 and 10). Employment of the Axxiom™ data saver in this longer gradient profile provided easy and accurate quantitation by correcting for increased background absorbance due to increasing concentration of acetonitrile as the gradient proceeds. The data saver uses a tape recording of the background absorbance drift and simultaneously subtracts this electronic signal from the detector signal during chromatographic analysis. The consequence is an improved baseline and most sample peaks remain on scale in the latter portion of the chromatogram, and thus quantitation is more accurate and a clean chromatogram results.

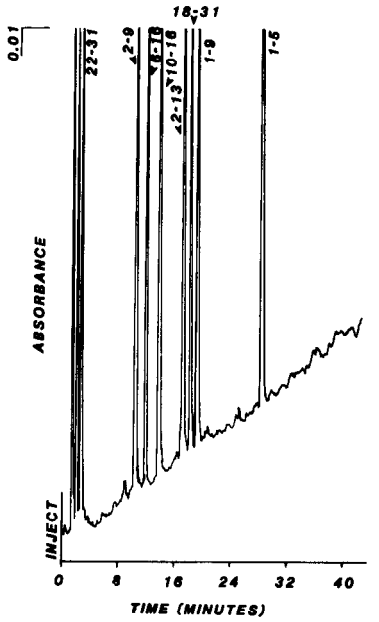

Fig. 5. Reversed-phase HPLC of eight highly polar, closely related β-endorphin fragment standards. Peptides were separated with a linear gradient of acetonitrile (10–20%) against 0.1 *M* phosphate buffer for 40 min at 40°C and 2.0 ml/min. All other conditions were the same as described in Fig. 2.

One of the most difficult areas of HPLC separation of βE-related fragments is the simultaneous separation of the fragments βE-(5–17), -(6–17), -(7–17), and -(8–17). By using various gradient profiles we have been successful in routinely separating βE-(5–17), -(6–17), and -(8–17) from each other [14–17]. Recently, we have used the sodium octyl sulfate ion-pairing reagent to facilitate the separation of βE-(5–17), -(6–17), -(7–17), -(8–17), -(10–17), -(1–16), and -(2–16) (Figs. 6–8). Using sodium octyl sulfate (100 mg/l), together with the 0.1 *M* sodium phosphate buffer and a linear gradient of 20–30% acetonitrile over 60 min we have been able to separate all fragments (Fig. 6). The separation was then applied to samples of postmortem human cortex to confirm a peak which was eluted together with the putative antipsychotic peptide, βE-(6–17), and to confirm trace amounts of peaks which are eluted together with βE-(7–17) and βE-(8–17) (Figs. 7 and 8). By fortifying a smaller amount of the same postmortem cortex sample with exogenous βE-(6–17) only the peak previously identified as βE-(6–17) increased proportionally. This confirmed that the behaviorally active, β-endorphin-related fragment formed from postmortem human brain was the proposed neuroleptic-like peptide des-enkephalin-γ-endorphin [βE-(6–17)].

In vitro β-endorphin processing studies

Postmortem human brain. Postmortem sex- and age-matched human putamen from control patients and patients diagnosed as having schizophrenia were time-course incubated with β-endorphin (1–7 nmol). After analyzing the chromatographic profiles, a distinct pattern of endorphin catabolism can be demonstrated (Fig. 9). Several βE-related peptides that are known to occur *in vivo* can be identified at 90

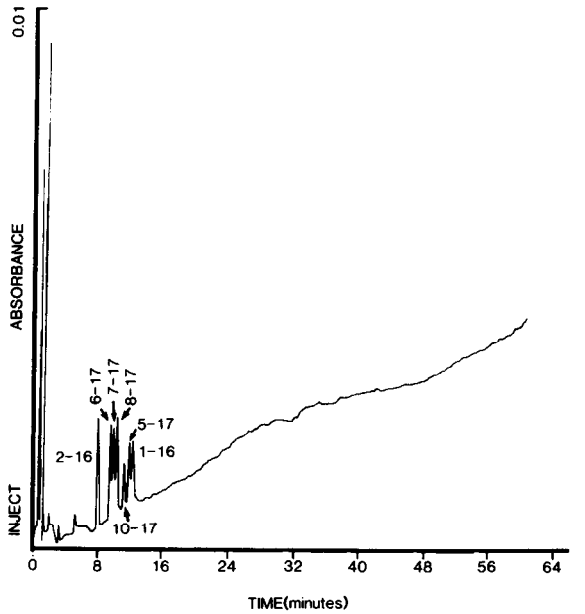

Fig. 6. Reversed-phase ion-pairing HPLC of seven β-endorphin fragment standards. Peptides were separated on a Beckman Ultrasphere-ODS (5-μm) column with a linear gradient of 20–30% acetonitrile against 0.1 *M* phosphate buffer (pH 2.4) containing 100 mg/l of octyl sodium sulfate, for 60 min at 40°C and 2.0 ml/min. Detection was at 210 nm, 0.01 a.u.f.s. Amounts corresponding to 75–100 ng were injected for each peptide.

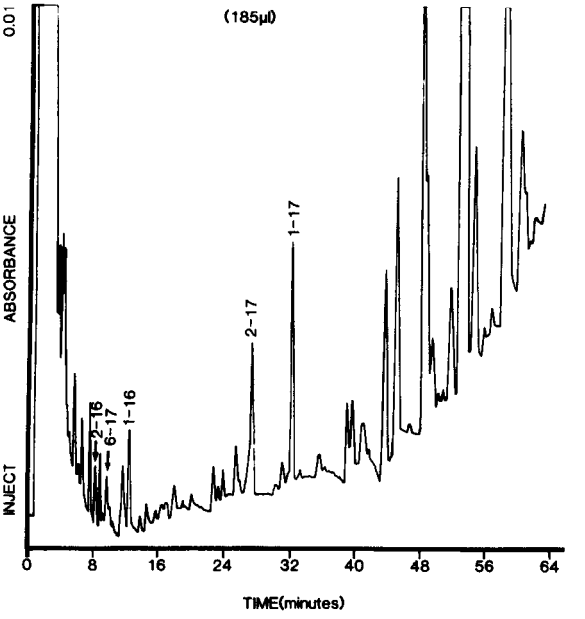

Fig. 7. Reversed-phase ion-pairing HPLC of human cortex, obtained from a combination of specimens. All conditions were the same as in Fig. 6. The 185-μl injection represents approximately 0.9 mg protein.

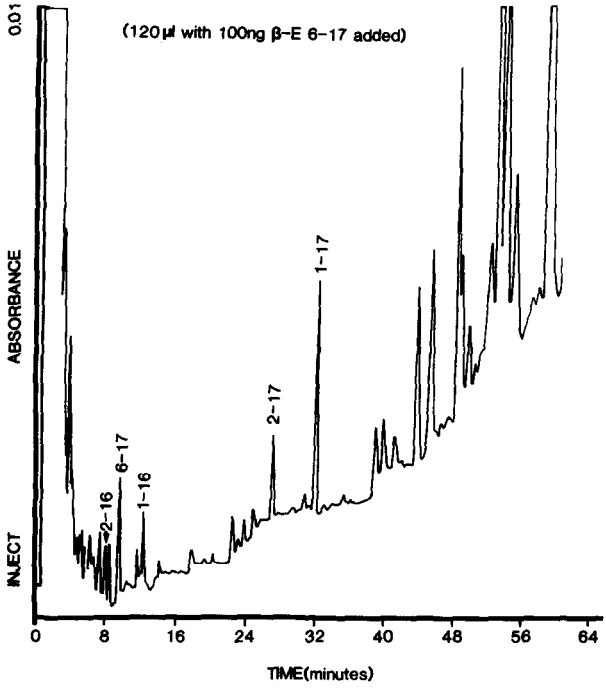

Fig. 8. Reversed-phase ion-pairing HPLC of 120 μl human cortex sample plus βE-(6–17), as in Fig. 7. The sample was fortified with 100 ng of βE-(6–17) to verify peak identity. Sample injected corresponds to 0.6 mg protein. All other conditions were the same as in Fig. 6.

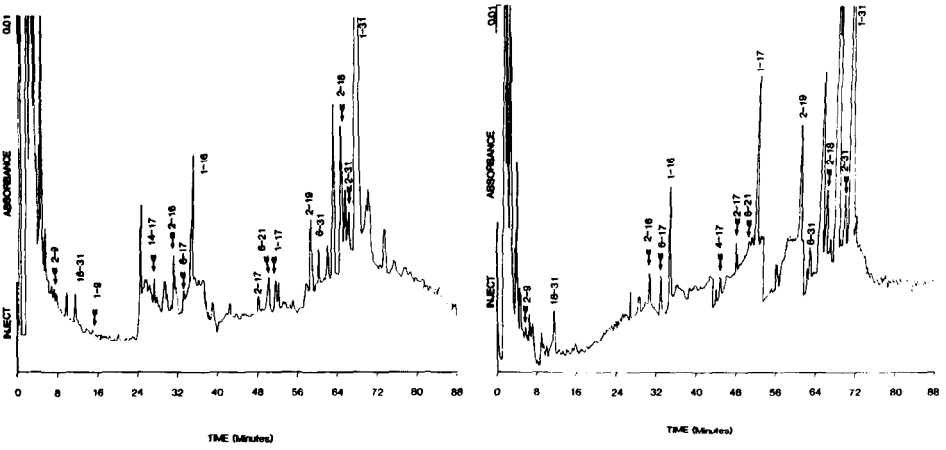

Fig. 9. Representative HPLC separations of a 15% (w/v) membrane-associated enzyme homogenate of postmortem human control (left) and schizophrenic (right) putamen. The homogenates were incubated at 37°C with β-endorphin (7 nmol) for 90 min. Peptides were separated on a Beckman ODS (5-μm) column (25 cm × 4 mm) with a series of curvilinear gradients of acetonitrile (12–35%) against 0.1 *M* phosphate buffer (pH 2.4) over 80 min at 40°C, 2.0 ml/min. Detection was at 210 nm, 0.01 a.u.f.s. Sample injected (175 μl) was equivalent to 0.9 mg protein.

min of incubation with β-endorphin (Fig. 9). Among these are α-endorphin [βE-(1–16)], des-tyrosine-α-endorphin [βE-(2–16)], γ-endorphin [βE-(1–17)], des-tyrosine-γ-endorphin [βE-(2–17)] and des-enkephalin-γ-endorphin [βE-(6–17)]. In addition to these fragments, a peptide [βE-(6–21)] which acts opposite[24] to βE-(6–17) in the nucleus accumbens-administered passive avoidance behavior paradigm of Gaffori and De Wied[25] was found to be produced in significantly lower amounts in the schizophrenic brain samples than in the controls[14], whereas the putative neuroleptic peptide βE-(6–17) was found to be produced at significantly ($p < 0.01$) higher rates (65 *versus* 20 pmol/mg protein) in the schizophrenic samples when compared to controls by repeated measures analysis of variance (Table I).

The validity of this type of peptide analysis was then tested and demonstrated by the fact that HPLC separations, accomplished after incubations of five postmortem brain samples in the absence of exogenously added β-endorphin, did not reveal any UV absorptive materials which coelute with any of our fragment standards above electronic background (results not shown). Moreover, incubation of β-endorphin with tissue homogenates kept at 100°C for 15 min before the start of the incubation did not result in a decrease of β-endorphin levels after 90 min of incubation, indicating that all peptides studied were formed from β-endorphin through membrane-associated enzymatic activity. It was also demonstrated that the time course in previously frozen postmortem human brain homogenate was both quantitatively and qualitatively similar to that in fresh postmortem human tissue or brain slices[17]. Finally, it was demonstrated that the metabolic pattern of β-endorphin fragmentation was not affected by the boiling procedure used to stop enzymatic activity or by the storage conditions of the tissue, because no differences were noted in control specimens.

Postmortem rat brain studies. Since it is very difficult to distinguish between etiological factors related to schizophrenia *versus* effects of antemortem drug treatment, we decided to investigate the effect of chronic drug infusion on the *in vitro* processing of β-endorphin to specific α- and γ-type endorphins. This was done to help explain why we observed an increase in a putative neuroleptic peptide βE-(6–

TABLE I

CONCENTRATION OF βE-(6–17) PRODUCED AFTER INCUBATION WITH βE-(1–31) AND POSTMORTEM HUMAN PUTAMEN

Control patients with no known neurological disease (n = 8) βE-(6–17)		*Patients diagnosed as having chronic schizophrenia (n = 8) βE-(6–17)*	
Time (min)	*pmol/mg protein ± S.E.M.***	*Time (min)*	*pmol/mg protein** ± S.E.M.*
30	10.41 ± 2.6	30	26.65 ± 3.1
60	14.42 ± 3.9	60	36.86 ± 4.3
90	19.40 ± 5.2	90	51.93 ± 7.3
120	23.96 ± 5.4	120*	65.27 ± 10.0

* $n = 6$.

** Each time point is significantly different from control ($p < 0.05$) by repeated measures analysis of variance.

*** S.E.M. = Standard error of the mean.

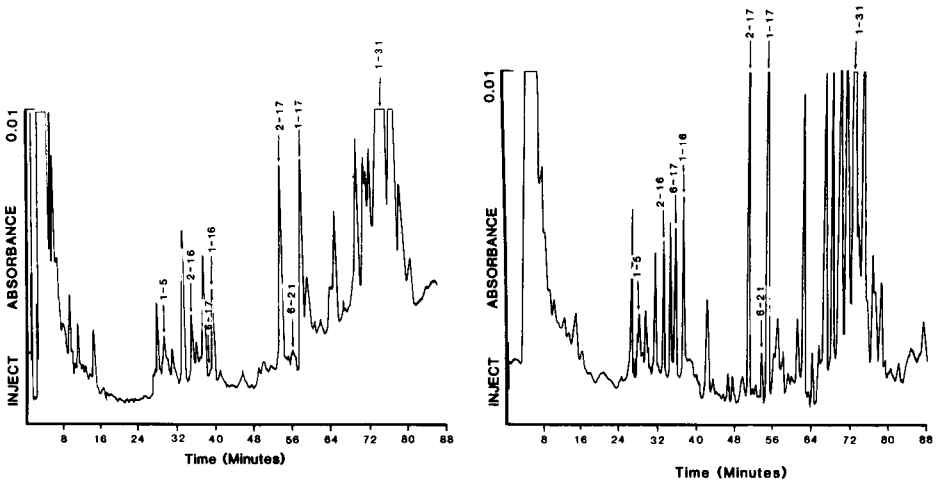

Fig. 10. Representative HPLC separations of a 15% (w/v) membrane-associated enzyme homogenate of control (left) and drug-treated (right) rat brain. Haloperidol was administered for eight days, as described. The homogenates were incubated with β-endorphin (7 nmol) for 40 min. HPLC conditions were the same as described in Fig. 9. The 175-μl injection volume was equivalent to 0.9 mg protein. All other conditions were the same as in Fig. 9.

17) in the postmortem tissue from the patients diagnosed as having schizophrenia *versus* controls.

Fig. 10 shows a representative chromatogram of a 40-min incubation of β-endorphin with a membrane-associated enzyme homogenate of a whole rat brain minus the cerebellum from a rat treated with haloperidol (3 mg/kg per day) *versus* a control sample from an untreated rat. Note the significant increase in the formation of the γ-type endorphins βE-(6–17), -(1–17), and -(2–17). Thorough analysis of all the data points showed that the two neuroleptic drugs, haloperidol and chlorpromazine, caused a significant increase in the formation of γ-type endorphins *versus* the nonantipsychotic central depressants, phenobarbital and promethazine. This raises the possibility that the mechanism of action for certain antipsychotics includes the formation in the brain of γ-type endorphins[22].

In summary, studies presented here offer data that open wider perspectives with respect to using HPLC in studies of neuropeptide involvement in the chemical modulation of CNS function. It is now widely accepted that specific neuropeptides, such as β-endorphin, are derived from protein precursors. As we have shown, a single parent peptide may function as the precursor for several neuroactive peptides that at times have opposing or complementary effects. Given the various pharmacological effects of structurally different neuropeptides, a characterization of their catabolic pathways is very important. The present study shows that HPLC is a powerful tool for studying processing in postmortem human and rat brain, and this type of approach may be applicable to other neuropeptide systems. Since immunological studies are presently capable of only steady-state analyses of those peptides for which a specific antibody has been formed, studies concerning peptide catabolic pathways are of value.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Ann Peterson for her technical assistance and art work, Ms. Rita Wedell for typing the manuscript and Drs. H. M. Greven and J. W. van Nispen (Organon International) for supplying some of the peptide fragments. The authors also wish to thank Dr. Martin Rosser of Cambridge University, U.K., for providing the valuable brain samples for analyses. This work was supported by a Pharmaceutical Manufacturers Association Foundation grant and U.S. Public Health Service Grant AG04439 and CA23074 to Thomas P. Davis.

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