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COMBINATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY IS A POWERFUL TOOL FOR THE SPECIFIC AND QUANTITATIVE DETERMINATION OF ENDORPHINS AND RELATED PEPTIDES

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SUMMARY

A method for the separation and subsequent quantification of endorphins and related peptides was developed. Separation of the peptides was achieved by high pressure liquid chromatography on a reversed phase column. By virtue of the high resolving capacity of this system peptides differing in only one amino acid residue could be separated easily. For quantification of the isolated peptides specific radioimmunoassay systems were used. The combination of the two techniques was applied to determine specifically a number of endorphin-like peptides in rat pituitary gland. For the first time the presence of des-tyrosine-endorphins in addition to known endorphin fragments is demonstrated.

INTRODUCTION

Recently, peptides derived from the C-terminal part of β-LPH, e.g. β-endorphin (β-LPH 61-91), γ-endorphin (β-LPH 61-77) and α-endorphin (β-LPH 61-76) have been isolated from the pituitary gland and the brain (1-4). These peptides elicit morphinomimetic activities following intracerebroventricular injection (see for review, ref. 5). Also, the endorphins and their des-tyrosine analogs have been implicated in the control of adaptive behaviour (6).

A new technique was developed by which the majority of endorphin-like peptides could be measured in biological samples without the interference of closely resembling peptides. By the development of specific radioimmunoassays it was possible to measure the concentration of groups of peptides, e.g. peptides of the α-endorphin type, β-endorphin type, γ-endorphin type and so on, in agreement with the findings of others (7-10). However,

*Abbreviations: β-LPH = β-lipotropin, HPLC = high pressure liquid chromatography.

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within any one of the groups it was impossible to obtain a complete differentiation because of severe cross-reactivity of structurally closely related peptides, e.g. γ-endorphin and des-tyr-γ-endorphin. Consequently, it was necessary to separate the peptides in the sample before performing the radioimmunoassay. The resolving power of classical separation methods as gel filtration or ion exchange chromatography is not sufficient for this purpose. Therefore, the performance of HPLC was investigated and this modern technique with its very high resolving capacity was applied successfully.

We want to emphasize that a combination of HPLC and radioimmunoassay allows the specific and quantitative determination of a large number of endorphin-like peptides. The determination of a number of these peptides in rat pituitary tissue illustrates the potentials of the combined HPLC-radioimmunoassay approach.

MATERIALS

Human β-endorphin and related peptides were synthesized and purified by Dr. H.M. Greven (Organon, Oss). Antiserum to β-endorphin was kindly donated by Dr. A. Herz (München). Radioiodide (IMS-30) was purchased from the Radiochemical Centre, Amersham. Iodogen (1,3,4,6 -tetrachloro-3α,6α-diphenylglycoluril) was obtained from Pierce Chemical Company, Polyethyleneglycol 6000 from Fluka. Bovine serum albumin (fraction V), Bovine thyroglobulin and 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimideHCl were from Sigma and Freund's complete adjuvant from Difco. The methanol used in HPLC was spectrophotometric grade from Baker. All other chemicals were reagent grade.

METHODS

1. High pressure liquid chromatography

The HPLC assembly (Waters Ass.) consisted of two pumps (Model 6000A), a programmer for gradient elution (Model 660) and a universal liquid chromatograph injector (Model U 6K), coupled to a Schoeffel Model 770 variable wavelength UV monitor with an 8 µl flow-through cell and a Kipp BD9 two channel chart recorder. Chromatography was performed using a reversed-phase µBondapak C_{18} column (0.39 x 30 cm; Waters Ass.), packed with octadecyltrichlorosilane chemically bound to 10 µ porous silica particles. Deionized, distilled water was used. All solvents were filtered through a 0.5 µ Millipore filter before use.Gradient elution was obtained with 0.01 M ammonium acetate adjusted to pH 4.15 with glacial acetic acid (A) and methanol (B). The ratio of the concentrations of A and B was 7:3 initially and 1:3 finally. Starting at injection of a peptide sample concave gradient elution (programme 7) was performed during 45 min at ambient temperature; the flow rate was 2 ml/min and the pressure decreased from 2000 to 1100 p.s.i. The retention time of the various peptides was determined by in-line absorption detection at 210 nm. Various fractions were collected in polypropylene tubes, methanol

was evaporated at 60°C using a Büchler vortex-evaporator and the remaining material was lyophilized. The residue was taken up in 1.4 ml water, lyophilized, dissolved in phosphate buffered saline and subjected to radioimmunoassay.

2. Radioimmunoassay

Preparation of antigens: peptides were conjugated to bovine thyroglobulin with 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide using weight ratios of 1:5:0.23 as described by Skowsky and Fisher (11).

Preparation of antisera: at regular intervals New Zealand white rabbits were injected intramuscularly with 50-200 µg conjugated peptide emulsified in complete Freund's adjuvant. Ten to 14 days after each injection blood samples were taken and the titer determined. When a maximal titer was reached the rabbit was bled and the serum harvested.

Preparation of tracers: all peptides were labelled by incorporation of [^{125}I], by means of the recently developed "iodogen" method (P.J. Lowry, personal communication). In this method a water-insoluble phase of iodogen serves as electron acceptor in the iodination reaction, thus making the presence of damaging reagents as chloramine-T in the peptide-containing solution unnecessary. Briefly, a polypropylene tube was coated with iodogen by evaporating 20-50 µl of a 1 mg/ml solution of iodogen in dichloromethane. Two µg of peptide in 25 µl 0.5 M phosphate and 0.5 mCi Na [^{125}I] solution were added. The tube was tapped gently at one minute intervals. After ten minutes the reaction was terminated by aspiration of the solution. The tracer was then diluted with 0.02 M phosphate buffered saline containing 0.25% bovine serum albumin.

Radioimmunoassay procedure: all dilutions were made in phosphate buffered saline containing bovine serum albumin. Polypropylene tubes (10 x 65 mm) were used. In each tube 100 µl standard or sample, 50 µl diluted antiserum and 50 µl tracer were incubated. Incubation was carried out at 4°C for 4-20 h. Then 50 µl horse serum followed by 1 ml 20% (w/v) Polyethyleneglycol 6000 in phosphate buffered saline was added. After centrifugation the supernatant was siphoned off and the radioactivity in the pellet was counted in an LKB 1280 ultrogamma scintillation counter.

3. Extraction of endorphins from rat pituitary gland

Male Wistar rats (TNO, Zeist) weighing 200-250 g, were used. After decapitation of the animals the pituitary gland was quickly removed and transferred to a tube containing 125 µl 0.2 M HCl. The solution was chilled in ice and 375 µl of cold acetone was added. Homogenization was achieved by sonification for 30 sec with a Branson sonifier at 20 kHz. The homogenate was centrifuged 15 min at 30.000g_{av}. The supernatant was lyophilized, taken up in 750 µl 0.01 M ammonium acetate pH 4.15 and applied on the HPLC column. Fractions with retention times corresponding to the selected reference peptides were collected, lyophilized, dissolved in phosphate buffered saline and subjected to the appropriate radioimmunoassay system.

RESULTS AND DISCUSSION

1. High pressure liquid chromatography

UV detection at 210 nm was selected for two reasons: 1) this wavelength is near the absorption maximum for the peptide bond (187 nm; ref. 12), whereas solvents as water and methanol are much more transparant at 210 nm than at 187 nm; 2) absorbance at 210 nm is independent of the presence of chromophore-containing amino acid residues (Phe, Tyr, Trp) in the peptides.

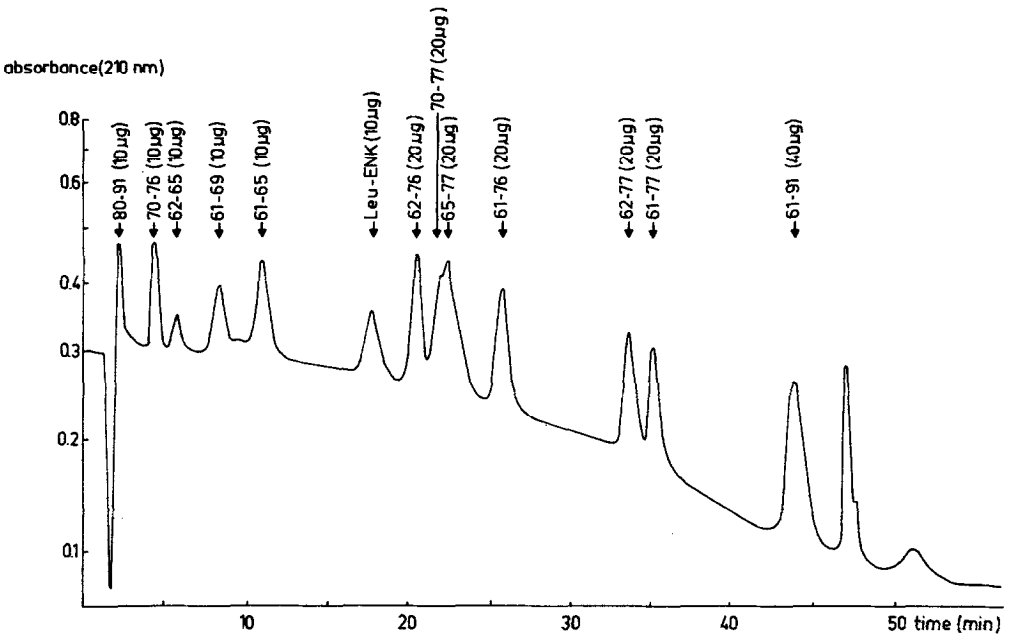

Fig. 1 - Chromatographic profile showing separation of a mixture of human β-LPH 61-91 fragments on a reversed phase µBondapak C_{18} column. UV absorbance was measured at 210 nm. For conditions of separation see "Methods". The last two peaks are caused by impurities in the mobile phase.

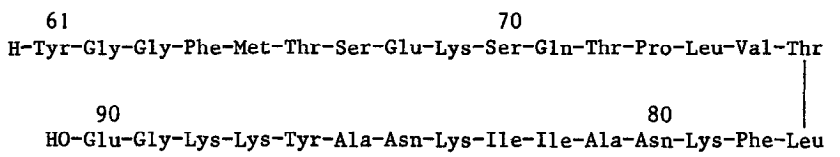

Fig. 2 - The amino acid sequence of human β-LPH 61-91 (β-endorphin; ref.1)

The separation of a mixture of endorphins and related peptides under conditions as described above is shown in Fig. 1 (Compare Fig. 2 for the structures of the various peptides).A complete list of the investigated peptides as well as their retention times is given in Table I. From these data it can be deduced that the retention time of a peptide in this chromatographic system is proportional to its chain length and inversely proportional to its polarity (as indicated by the solubilities of the constituting amino acids

TABLE I

Separation of β-LPH 61-91 fragments by HPLC.

β-LPH 61-91 fragment	Retention time (min)
80-91	2.1
62-69	4.5
70-76	4.5
62-65 (desTyr-Met-enkephalin)	5.5
61-67	8.4
61-69	8.4
61-65 (Met-enkephalin)	11.0
Leu-enkephalin	17.6
66-77	19.0
62-76 (desTyr-α-endorphin)	20.5
70-77	21.8
65-77	22.3
61-76 (α-endorphin)	25.5
62-77 (desTyr-γ-endorphin)	33.5
61-77 (γ-endorphin)	35.0
62-91 (desTyr-β-endorphin)	43.6
61-91 (β-endorphin)	43.6

in water; ref. 13). For instance, by lengthening of β-LPH 61-65 with Thr-Ser or Thr-Ser-Glu-Lys the peptide polarity is increased as well. Consequently, a decrease in retention is observed when β-LPH 61-67 and β-LPH 61-69 are compared to β-LPH 61-65 (Table I). Also, replacement of the methionine residue in β-LPH 61-65 by the more hydrophobic leucine residue (leu-enkephalin) increases the retention time from 11.0 to 17.6 min. Similarly, introduction of a leucine residue at the C-terminus of β-LPH 61-76 (α-endorphin) yielding β-LPH 61-77 (γ-endorphin) enhances the retention time with almost 10 min. Finally, for most endorphins removal of the hydrophobic tyrosine residue at the N-terminus decreases retention, which results in remarkable separations between the β-LPH-peptide pairs 61-65 and 62-65, 61-69 and 62-69, 61-76 and 62-76, 61-77 and 62-77. No resolution, however, could be achieved of β-LPH 61-91 and β-LPH 62-91. Here the effect caused by the removal of the N-terminal tyrosine is subordinate to the effect of the chain length. These examples showing the separation of peptides that differ in only one amino acid residue,illustrate the high resolving power of reversed phase HPLC.

TABLE II

Characteristics of five radioimmunoassay systems for endorphins.

System	β-LPH 61-65 (met-enkephalin)	β-LPH 61-69	β-LPH 61-76 (α-endorphin)	β-LPH 61-77 (γ-endorphin)	β-LPH 61-91 (β-endorphin)
Final antibody dilution	1:800	1:400	1:4000	1:6000	40000
Minimal detectable dose	20 pg	100 pg	20 pg	5 pg	20 pg
		% CROSS REACTION			
β-LPH					
61-65 Met	100	0.06	0.13	0.02	0.02
61-65 Leu	10		< 0.01	0.02	
62-65	< 0.01			< 0.001	
61-67	3	100	0.03	0.07	0.006
61-69	5	100	< 0.01	0.05	
62-69		0.2	< 0.01	0.1	
64-69	< 0.001	< 0.02	0.02	0.23	0.007
65-69	< 0.001	< 0.02	0.2	0.045	0.005
61-76	3	20	100	8	< 0.01
62-76			110	0.6	
61-77	2	14	2	100	< 0.01
62-77		< 0.05	5	120	
62-78			1	60	
62-79				7	
65-77				130	
66-77				110	
70-76			1		
70-77				0.6	
76-77			0.003	0.006	
61-91	1.5	6	1	4	100
62-91				< 0.001	110
78-91				< 0.001	< 0.01

The HPLC fractionation of peptides was found very reproducible. The chromatographic profile of Fig. 1 has been obtained with an injection volume of 110 µl. Volumes up to 1000 µl did not affect the resolution as was found by Krummen and Frei (14) for oxytocin.

2. Radioimmunoassay

The characteristics of the five radioimmunoassay systems currently in use are shown in Table II. The minimum detectable dose is defined as the dose of peptide that gives a 10% decrease of the initial tracer binding. The amount of crossreaction is defined as the ratio of the doses of "standard" and "test" peptide that give a 50% decrease of the initial tracer binding.

From the specificity features of each antiserum as given by the cross-reaction figures it may be concluded that the N-terminal tyrosine is not part of the antigenic site of the larger endorphins (α-, γ- and β-endorphin) since des-tyrosine analogs crossreact completely in the respective systems; in contrast, for met-enkephalin and β-LPH 61-69 the reverse is true, probably because of the much shorter chain length.

3. Endorphins in the rat pituitary gland

As a demonstration of the impact of the use of the presented HPLC-radioimmunoassay method the endorphin levels in the rat pituitary gland were measured. The concentration of the various peptides tested were as follows (ng/mg fresh tissue): β-LPH 61-65: 0.2; β-LPH 61-69: 0.5; β-LPH 61-76: 0.33; β-LPH 62-76: 0.25; β-LPH 61-77: 2.4; β-LPH 62-77: 1.5; β-LPH 61/62-91: 37.5. The figures for β-LPH 61-65, β-LPH 61-76, β-LPH 61-77 and β-LPH 61/62-91 are in accordance to those reported by others (3,9,15). The presence of des-tyr-endorphins in amounts roughly 2/3 of the corresponding parent peptides is remarkable.

CONCLUSIONS

Five radioimmunoassay systems, each one specific for a group of endorphins, were developed. Due to the structural resemblances of the peptides within each one of the groups cross reaction occurred. However, high pressure liquid chromatography enabled effectively the fractionation of a mixture of such peptides. With the combination of HPLC and radioimmunoassay it appeared possible to measure specifically and quantitatively closely related peptides as β-LPH 61-76 and β-LPH 62-76 or β-LPH 61-77 and β-LPH 62-77; in this way crossreaction turned out to be an advantage instead of a drawback.

The HPLC-radioimmunoassay method opens the possibility of investigating the concentrations of a variety of endorphin-like peptides in different parts of the CNS under various experimental or pathological conditions.

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