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COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–RADIOIMMUNOASSAY FOR THE CHARACTERIZATION AND QUANTITATIVE MEASUREMENT OF NEUROPEPTIDES

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SUMMARY

A method for the extraction, separation, characterization and quantitation of angiotensins, opioid peptides and several other neuropeptides in biological samples is described. Gradient elution in different reversed-phase high-performance liquid chromatographic systems gives high resolution with constant recoveries between 60 and 70%, allows UV detection at 220 nm and is compatible with radioimmunoassay. The method is highly reproducible and applicable to the characterization and quantitative measurement of peptides in the femtomolar range. The practicability of the method is demonstrated for angiotensin peptides in plasma, brain and peripheral organs, as well as for opioid peptides in the heart.

INTRODUCTION

In the last few years many new biologically active peptides have been discovered which participate in important pathophysiological processes such as blood pressure control, volume homeostasis, pain and behaviour[1–4]. The identification and measurement of these active substances which are effective in the picomolar or femtomolar range is still frequently carried out by methods such as bioassay, immunohistochemistry or radioimmunoassay of unpurified material which do not allow unequivocal characterization and precise quantification. These methods are hampered by a number of problems such as insufficient sensitivity and selectivity of the bioassays, lack of specific antisera for immunohistochemistry or radioimmunoassay, impurities within the extracts and absence of synthetic reference standards. This has lead to misinterpretations in the past. High-performance liquid chromatography (HPLC) using different gradient-elution systems combined with specific radioimmunoassay (RIA) offers a powerful method for qualitative and quantitative peptide determination in biological samples[5,6]. The advantages of combining these techniques are specificity and sensitivity nearly comparable to that obtainable with electrochemical detection[7], reproducibility, practicability and speed of analysis of peptides in tissue and plasma. Such methods have been developed by us for angiotensins, opioid peptides and other neuropeptides and are described in this report.

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EXPERIMENTAL

Tissue collection

Animals were decapitated and the tissue was quickly removed and frozen on solid CO_2 or in liquid nitrogen.

Blood collection

Blood from ether anaesthetized rats was collected via puncture of the aorta into polyethylene tubes containing 50 μl of a mixture of 12.5 m*M* ethylenediaminetetraacetate (EDTA) and 2.6 m*M* 1,10-phenanthroline per ml blood. The blood samples were immediately centrifuged for 10 min at 10,000 *g* at 4°C and the plasma was stored at −20°C until used[8].

Peptide extraction from tissue

Small pieces of frozen tissue were boiled for 5 min in 0.1 *M* hydrochloric acid (1:10, w/v), then homogenized by a Polytron (Cinematic Luzerne, Switzerland) and centrifuged for 30 min at 30,000 *g*. The clear supernatant was collected and the pellet was re-extracted in the same volume of 0.1 *M* hydrochloric acid and centrifuged again for 30 min at 30,000 *g*. The supernatants were pooled. The crude hydrochloric acid extracts were purified on octadecasilyl-silica (ODS-silica, Sep-Pak C_{18}) cartridges. In this procedure, the cartridges were first moistened with 3 ml of methanol and then washed with 10 ml of 1% trifluoroacetic acid (TFA) solution in water. In order to minimize non-specific adsorption, the cartridges were next coated with 1 ml of a 1% polypeptide solution and washed again with methanol–water–TFA (80:19:1, v/v/v) and with a 1% TFA solution. The sample was then applied and the cartridges washed twice with 5 ml of 1% TFA–1% sodium chloride (1:1, v/v). The peptides were eluted from the cartridge with 2 × 1 ml methanol–water–TFA (80:19:1, v/v). The organic phase of the eluent was removed under a flow of nitrogen and the remainder then taken to dryness in a vacuum box. The dry residue was dissolved in 4 × 250 μl of 0.001 *M* hydrochloric acid and transferred into polyethylene tubes. This solution was applied directly to the HPLC column. Blank controls comprised the complete procedure but without sample application[8,9]. This "ODS-purification" was used in an identical way for plasma and cerebrospinal fluid (CSF).

Recovery of peptides

For the determination of the recovery of radioactive peptides, 5000 cpm of the ^{125}I-labelled peptides were added to the tissue samples prior to homogenization and the radioactivity was counted in a gammacounter (Gammaszint BF 300, Bertold, F.R.G.) after the "ODS-purification step" and HPLC. To estimate the recovery of synthetic peptides, 1 nmol of each peptide dissolved in 100 μl 0.001 *M* hydrochloric acid was added to the sample before homogenization. The peptide concentrations were measured radioimmunologically after ODS purification and in the HPLC fractions.

High-performance liquid chromatography (HPLC)

The HPLC system (Waters GmbH, Königstein, F.R.G.) consisted of two pumps (Model 6000 A), a Model 660 solvent programmer, an U6K injection system

with a 2-ml sample loop, a variable wavelength UV detector (Model 450), a one-channel recorder (Servogor 210, BBC Goerz) and a fraction collector (Isco Model 328). All separations were done at room temperature on 300 × 4 mm reversed-phase C_{18} columns (μBondapak C_{18}, 10 μm, Waters) or 7-μm Nucleosil C_{18} (Macherey, Nagel & Co., Düren, F.R.G.). Four different gradient-elution systems were used:

(1) Methanol gradient: 10 m*M* ammonium acetate buffer was adjusted to pH 4.5 or 5.4 with acetic acid, and methanol was increased linearly from 30 to 80% (v/v) within 35 min.

(2) Isopropanol gradient: 10 m*M* triethylammonium phosphate (TEAP) was adjusted to pH 3.0 with concentrated phosphoric acid. In a linear gradient, isopropanol was increased from 5 to 40% (v/v) within 45 min.

(3) Acetonitrile gradient: water was adjusted to pH 2.1 with TFA and acetonitrile was linearly increased from 25 to 45% within 45 min.

(4) Acetonitrile gradient: 10 m*M* ammonium trifluoroacetate was adjusted to pH 2.0 with TFA and acetonitrile was increased linearly from 15 to 30% within 50 min.

The mobile phases were passed through a RC 55 membrane filter with pore size 0.45 μm (Schleicher and Schüll, Dassel, F.R.G.) and degassed by vacuum filtration. Injection volumes varied between 10 and 950 μl. The flow-rate was 1.0 ml/min and the recorder speed was 1 cm/min. The column was calibrated with synthetic standard peptides using UV detection at 220 nm and 0.2 or 0.1 absorbance units full scale (a.u.f.s.). For this purpose, 1 nmol of each peptide was applied to the HPLC column. The eluate was collected in 500- or 1000-μl fractions into polyethylene tubes and subjected directly to RIA. For radioimmunological measurements in the acetonitrile systems, the fractions had to be lyophilized prior to RIA.

Radioimmunoassay (RIA)

All peptides were iodinated with ^{125}I by the chloramine T method and the labelled peptides were purified on DEAE-Sephadex A-25 or Sep-Pak C_{18} cartridges (Waters)[10]. Antibodies were obtained from white New Zealand rabbits immunized against the peptides. Details of the RIA and cross-reactivities of the antibodies are published elsewhere[8,11].

Reagents

All chemicals used were of analytical grade. Isopropanol (LiChrosolv®), water (LiChrosolv®) and TFA (Uvasolv®) were supplied by Merck (Darmstadt, F.R.G.); methanol and acetonitrile for HPLC from Baker (Deventer, The Netherlands). Ammonium acetate was from Fluka (Buchs, Switzerland); acetic acid, phosphoric acid and hydrochloric acid from Merck; (Ile^5)-angiotensin (ANG) I, (IIe^5)-ANG III, kallidin, Met-kallidin, Leu- and Met-enkephalin (Met-Enk) α- and β-neoendorphin. Phe-Met-Arg-Phe-amide (FMRF-NH_2), Met-enkephalin-Arg-Phe, dynorphin 1-8, 1.9, 1-13, 1-17 from Bachem (Genf, Switzerland); (Ile^5)-ANG II-hexapeptide and (Ile^5)-ANG II-pentapeptide from Schwarz/Mann (Orangeburg, U.S.A.). All other peptides were from Beckman (Palo Alto, CA, U.S.A.).

RESULTS

HPLC separation of peptides

The HPLC systems separated angiotensin and its fragment opioids and other peptides in a reproducible way. With the methanol gradient a baseline separation was achieved for (Ile^5)-ANG I, (Ile^5)-ANG II, (Ile^5)-ANG III, (Val^5)-ANG II, (Ile^5)-ANG II-pentapeptide (4–8) (Fig. 1). The same peptides could also be separated in the isopropanol gradient system (Fig. 2). (Ile^5)-ANG II-pentapeptide (4–8), (Val^5)-ANG II, (Ile^5)-ANG II and (Ile^5)-ANG III showed reversed elution orders in the two gradient systems. With the methanol gradient, (Ile^5)-ANG II-pentapeptide (4–8) was eluted before (Val^5)-ANG II and (Ile^5)-ANG III after (Ile^5)-ANG II. With the isopropanol gradient, (Val^5)-ANG II was eluted prior to (Ile^5)-ANG II-pentapeptide (4–8) and (Ile^5)-ANG III prior to (Ile^5)-ANG II.

The different retention times in the two gradient systems are shown in Table I. Some peptides were not separated from each other; for example, (D-Ala^2-L-Met^5)-Enk was coeluted with (Ile^5)-ANG II-pentapeptide (4–8) as well as (Val^5)-ANG II and Leu-Enk. The separation between oxytocin, kallidin and (Ile^5)-ANG II was equally insufficient. (Ile^5)-ANG II-hexapeptide, (Ile^5)-ANG III, bradykinin and Met-kallidin were only partially separated. Nevertheless, a definite identification of these peptides was possible by use of an acetonitrile gradient system, where the angiotensin peptides could be separated from the kinins, bradykinin, kallidin and Met-kallidin as well as oxytocin and Leu-Enk (Table I and Fig. 3). The separation of (Ile^5)-ANG II-hexapeptide and (Ile^5)-ANG III in the methanol gradient could be improved using the Nucleosil C_{18} support instead of μBondapak C_{18}, where a baseline separation was achieved for both peptides (see Fig. 1).

For the separation of opioid peptides a second acetonitrile system was established which revealed high resolution between dynorphin 1-17, 1-13, 1-9, 1-8, α- and β-neoendorphin, Met- and Leu-Enk, Met-Enk-Arg-Phe and Phe-Met-Arg-Phe (Fig. 4).

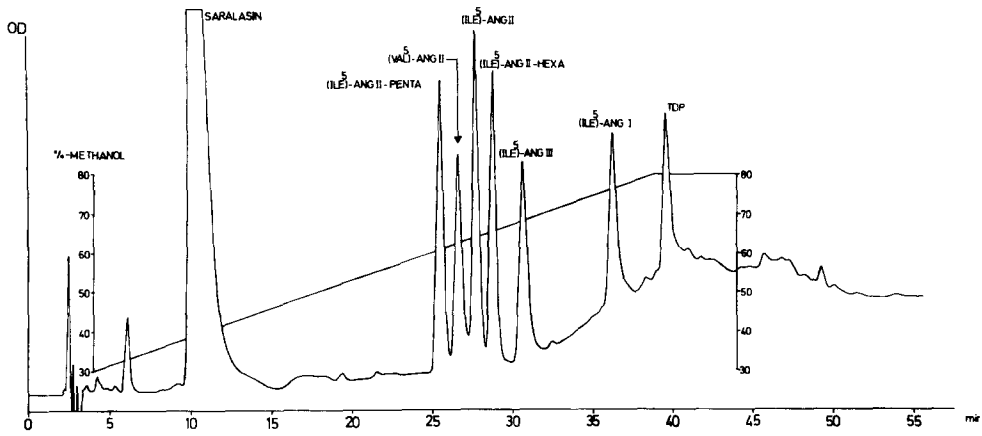

Fig. 1. HPLC separation of synthetic angiotensin peptides on a reversed-phase Nucleosil C_{18} support (300 × 4 mm) with the methanol gradient (pH 5.4). Detection: UV at 220 nm, 0.2. a.u.f.s. For other chromatographic conditions see text. Note the high resolution of (Ile^5)-ANG II and its degradation products (Ile^5)-ANG II-hexapeptide, (Ile^5)-ANG III and (Ile^5)-ANG II-pentapeptide as well as (Val^5)-ANG II.

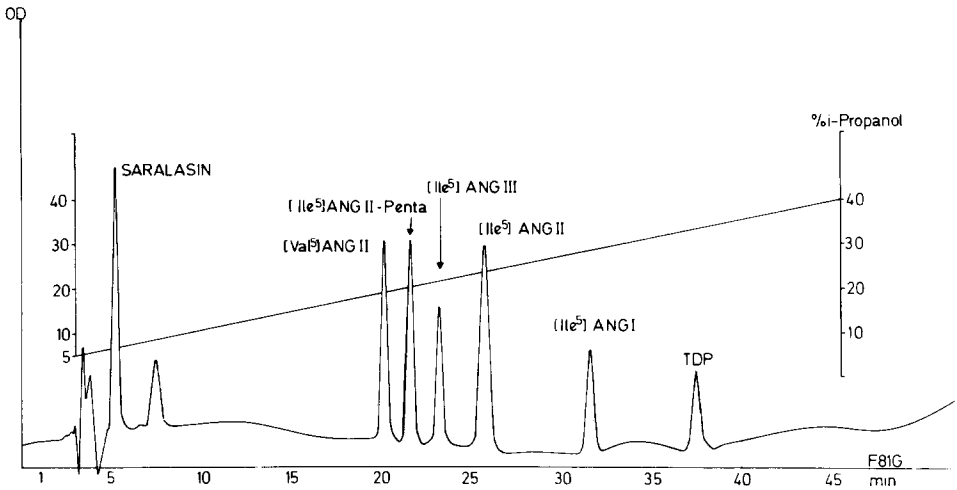

Fig. 2. HPLC separation of synthetic angiotensin peptides on a reversed phase μBondapak C_{18} support (300 × 4 mm) with the isopropanol gradient. Detection: UV at 220 nm, 0.2 a.u.f.s. For other chromatographic conditions see text. Note the reversed elution order of (Ile^5)-ANG II-pentapeptide, (Val^5)-ANG II and (Ile^5)-ANG II and (Ile^5)-ANG III in this system compared with the methanol gradient in Fig. 1.

TABLE I

RETENTION TIMES (min) OF SOME SYNTHETIC PEPTIDES ON A REVERSED-PHASE C_{18} COLUMN IN FOUR DIFFERENT GRADIENT SYSTEMS

Peptide	*Methanol*	*Isopropanol*	*25–45% Acetonitrile*	*15–30% Acetonitrile*
Tyr-Gly-Gly	3.25	3.35	3.05	
Saralasin	7.35	5.25	4.85	
Vasotocin (AVT)	13.90	13.00	6.10	
Arg-Vasopressin (AVP)	19.15	17.85	9.50	
Met-Enk	19.75	24.40	9.30	35.80
(Ile^5)-ANG II-pentapeptide	22.65	21.75	21.75	
(Val^5)-ANG II	23.40	20.30	20.05	
Leu-Enk	23.60	30.20	14.15	46.70
Oxytocin (OT)	25.35	25.78	17.50	
Kallidin (Kall)	25.50	18.40	13.95	
(Ile^5)-ANG II	25.70	26.00	27.05	46.10
(Ile^5)-ANG II-hexapeptide	26.40	25.80	26.03	
(Ile^5)-ANG III	26.90	23.79	26.85	
Bradykinin (BK)	26.85	21.35	17.20	
Met-kallidin (Met-Kall)	27.25	20.75	18.10	
(Ile^5)-ANG I	33.85	31.85	35.70	57.90
Substance P	36.40	—	—	
TDP (Tetradecapeptide)	37.70	37.75	45.05	
β-Endorphin	45.75	43.30	46.30	
α-Neoendorphin				34.50
β-Neoendorphin				40.90
Phe-Met-Arg-Phe-amide (FMRF-NH_2)				43.40
Dynorphin 1–9				44.60
Dynorphin 1–8				49.90
Dynorphin 1–13				50.80
Met-Enk-Arg-Phe				57.70
Dynorphin 1–17				60.20

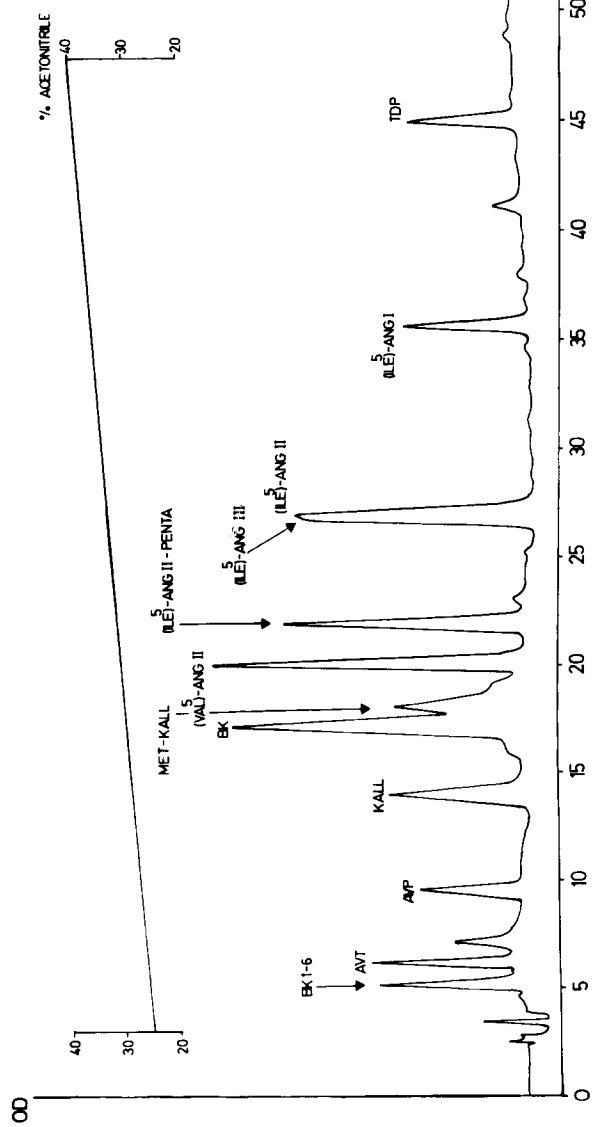

Fig. 3. HPLC separation of a variety of synthetic peptides on a reversed-phase C_{18} support with the acetonitrile gradient (pH 2.1). Chromatographic conditions as in Fig. 2, except 0.1 a.u.f.s. The kinins, AVT and AVP which were poorly separated from the ANG II peptides with the methanol gradient (Table I) were eluted earlier in this system.

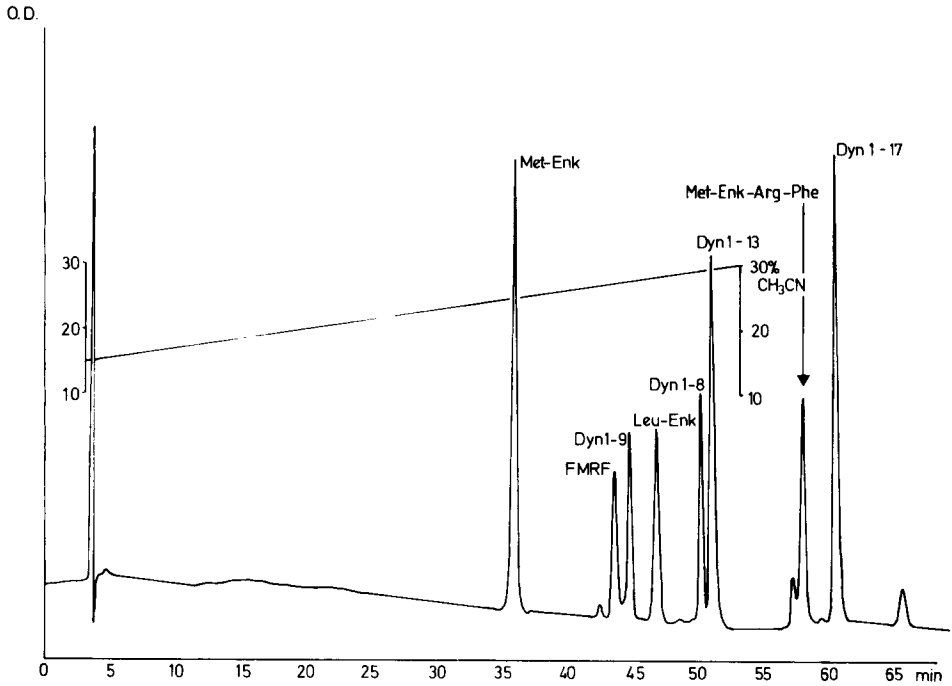

Fig. 4. HPLC separation of synthetic opioid peptides on a reversed-phase C_{18} support with an acetonitrile gradient (pH 2.0). Chromatographic conditions as in Fig. 1, except 0.1 a.u.f.s. and recorder speed was 0.5 cm/min.

UV detection limits with the highest detector sensitivity were about 25 pmol for ANG I and ANG II; 2 fmol ANG I and ANG II, 2 fmol Leu-Enk, 10 fmol Met-Enk, 1 fmol α-neoendorphin and 6 fmol for dynorphin 1-8 per fraction could be detected by radioimmunoassay.

The recovery of ^{125}I-ANG I added to the biological samples was 77 ± 2% after Sep-Pak C_{18} purification and 62 ± 2% (n = 24) after HPLC. The labelled ANG I and ANG II could be well separated from the endogenous peptides, which made it possible to calculate the recovery for each individual sample and for each step of the sample preparation. The chromatogram showed only two peaks of radioactivity indicating that the tracers were intact and not degraded during the extraction (Fig. 5). If the labelled peptides were applied directly to HPLC the recovery was 91 ± 1% (n = 3). The recovery of synthetic (Ile^5)-ANG I and (Ile^5)-ANG II from whole brain extracts was 83 ± 8 and 66 ± 2% (n = 3) after Sep-Pak C_{18} purification; 58 ± 6 and 60 ± 3% (n = 3) of ANG I and ANG II respectively were recovered after the whole extraction procedure including HPLC.

Measurement of peptides in tissue

Characterization of angiotensin peptides in brain extract revealed the presence of (Ile^5)-ANG I, (Ile^5)-ANG II and small amounts of (Ile^5)-ANG III as shown in Fig. 6. The quantitative distribution of ANG I and ANG II in the brain and in different tissues is listed in Table II. A hitherto unidentified substance which was

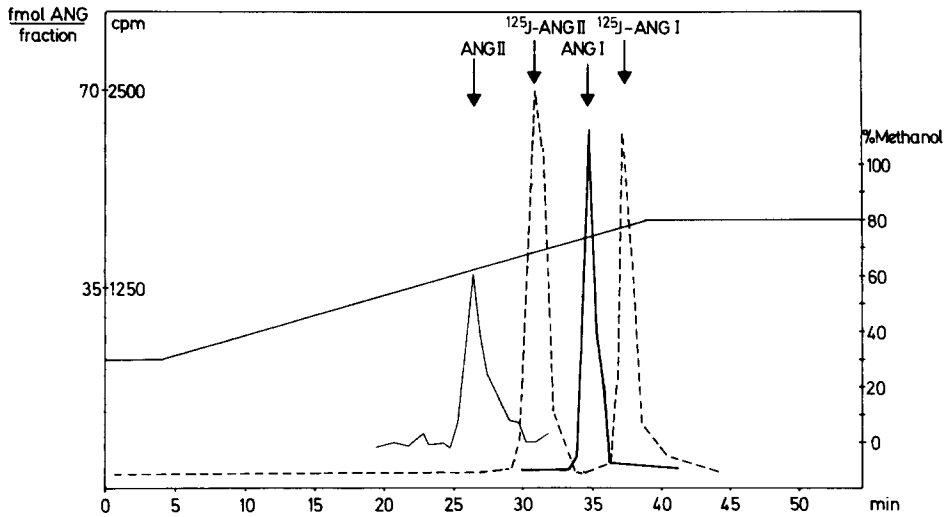

Fig. 5. HPLC separation of ^{125}I-labelled ANG I and ANG II (dashed line) from the endogenous peptides of an adrenal gland extract on a reversed-phase C_{18} column with the methanol gradient. For chromatographic conditions see Fig. 1. Arrows indicate the retention times of synthetic standard peptides.

retained 4 min longer than (Ile^5)-ANG I reacted with the ANG I antibody and produced a decrease in blood pressure in the rat bioassay. Rechromatography of the pooled ANG I- and ANG II-peak fractions from the methanol gradient using the isopropanol system confirmed the results (Fig. 6). (Ile^5)-ANG I and (Ile^5)-ANG II could also be identified in rat and rhesus monkey heart (not shown).

In rat plasma, des-Asp-ANG II (ANG III) was found in addition to (Ile^5)-ANG I and (Ile^5)-ANG II. ANG III is generated in the plasma by aminopeptidases which cleave the N-terminal amino acid aspartic acid from (Ile^5)-ANG II thereby generating (Ile^5)-ANG III. The ratio between ANG I, ANG II and ANG III was 35:44:21 (Fig. 7). It is important to note that the ANG II antibody cannot distinguish between (Ile^5)-ANG II and (Ile^5)-ANG III, but clear identification of both peptides

TABLE II

DISTRIBUTION OF ANGIOTENSIN PEPTIDES IN DIFFERENT TISSUES OF RATS

Tissue from ten rats was pooled. Values represent measurement in five pools, each pool taken from ten rats, and are corrected for recovery in each sample.

Tissue	*ANG I**	*ANG II**
Hypothalamus	121 ± 15	590 ± 106
Brain stem	94 ± 9	69 ± 6
Cerebellum	63 ± 9	57 ± 4
Cortex	45 ± 3	15 ± 1
Adrenal gland	150 ± 12	1043 ± 93
Kidney	24 ± 2	124 ± 7
Plasma	108 ± 4	17 ± 1

* fmol ANG per g wet tissue or fmol ANG per ml.

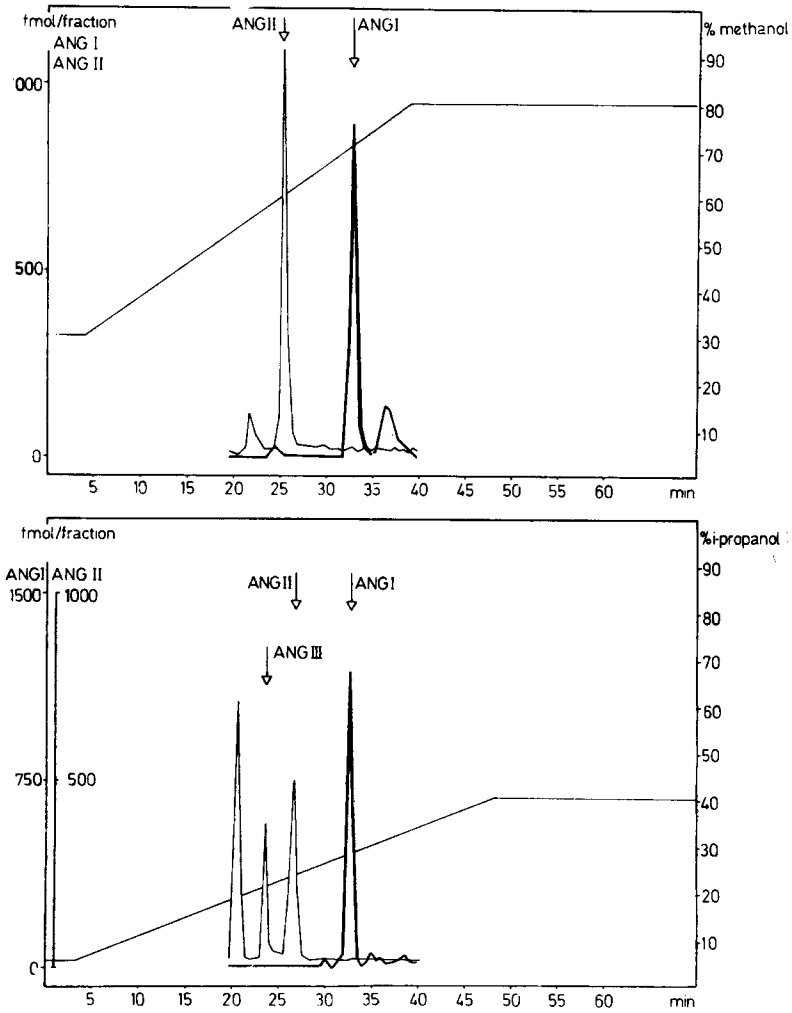

Fig. 6. HPLC separation of a brain stem extract on a reversed-phase μBondapak C_{18} column in the methanol gradient, pH 4.5 (upper), and rechromatography of the ANG I and ANG II peak fractions in the isopropanol gradient (lower). Arrows indicate the retention times of synthetic standard peptides.

was achieved using HPLC. Radioimmunological measurement of ANG II directly in plasma without HPLC separation is therefore not possible.

Opioid peptides were extracted from guinea pig hearts. Characterization by use of HPLC in the methanol as well as in the isopropanol system showed peaks of both Leu- and Met-Enk immunoreactivity corresponding to the position of synthetic Leu- and Met-Enk. The nature of a peptide cross-reacting with the Leu-Enk antibody which was retained 5 min longer than Leu-Enk is unclear. The concentration of Met-Enk was 9.45 pmol/g and that of Leu-Enk 2.57 pmol/g wet tissue and a reduction of about 70% in the Leu-Enk concentration was found following treatment with 6-hydroxydopamine[11]. Dynorphin 1-8, 1-17 and α-neoendorphin could also be iden-

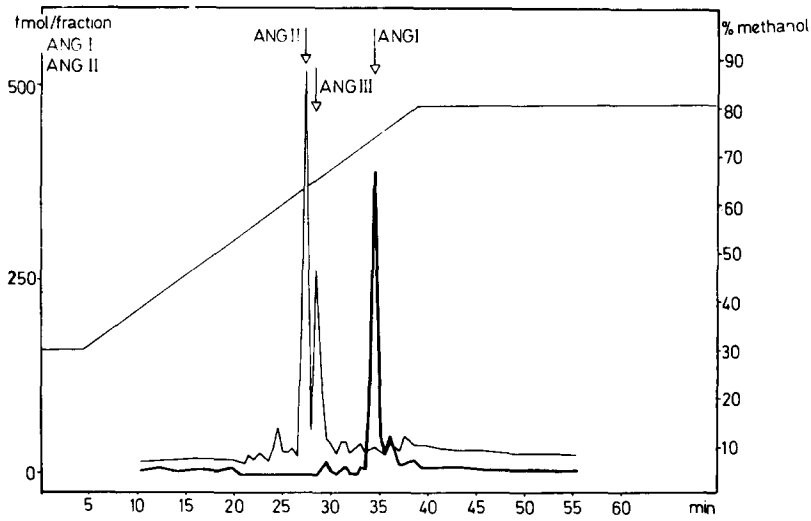

Fig. 7. HPLC separation of rat plasma on a reversed-phase μBondapak C_{18} column in the methanol gradient (pH 5.4). Note the identification of (Ile^5)-ANG III besides (Ile^5)-ANG II and (Ile^5)-ANG I. Arrows indicate the retention times of synthetic standard peptides.

tified in guinea pig hearts following HPLC separation in the acetonitrile gradient system. Additionally, HPLC revealed an unknown peptide cross-reacting with the α-neoendorphin antibody but eluting 8 min prior to α-neoendorphin[12]. It thus appears that all peptides of the pro-enkephalin B-family are present in the heart.

DISCUSSION

HPLC is a fast, versatile and reproducible method for peptide characterization and quantitative measurement. The high resolution is demonstrated by the separation of closely related peptides differing in chain length by only one amino acid, *i.e.*, (Ile^5)-ANG II, (Ile^5)-ANG III and (Val^5)-ANG II, and (Ile^5)-ANG II as well as Leu- and Met-Enk differing in chain sequence by only one amino acid. The different HPLC retention times of (Ile^5)-ANG II pentapeptide, (Val^5)-ANG II, (Ile^5)-ANG II and (Ile^5)-ANG III in the isopropanol and methanol gradients are due to the different degrees of ionization of the peptides which depend on the pH of the mobile phases as well as on pH-independent mechanisms such as the polarity and the character of hydrophobic peptide side chains[13], *e.g.*, valine and isoleucine in position 5 of the angiotensins, and the eluotropic strength of the organic solvent of the mobile phase.

The eluotropic strength of the organic solvent and the buffer composition including pH differences of the mobile phase provide versatile possibilities for optimization of the method. The eluotropic effect of the mobile phases increased from methanol to acetonitrile to isopropanol. Accordingly, (Ile^5)-ANG II was eluted in the methanol gradient at 63%, in the acetonitrile gradient at 33% and in the isopropanol system at 24% of the organic solvent. Based on their pK_a values, the peptides can be ionized depending on the pH of the mobile phase. Charged peptides show less interaction with the C_{18} reversed-phase support and are eluted earlier than uncharged

molecules. This was particularly evident with the basic peptides such as kinins and dynorphins at low pH. The influence of the pH is specially important for peptides with relatively short chain lengths, whereas with increasing chain length and hydrophobicity the pH has less influence. β-Endorphin, for example, was eluted at 46 min in both the acetonitrile and the methanol gradients, but at 43 min in the isopropanol gradient. This indicates that for this long chain peptide the eluotropic strength rather than pH determines the retention time. These findings are in harmony with results from other groups[14]. Knowledge of the characteristics of the mobile phase and the peptides can be used to calculate and predict the retention times of a variety of peptides[15].

All buffers and organic solvents used in this study were chosen to be transparent at 220 nm, compatible with the radioimmunoassay, and volatile in vacuum. The isopropanol system was not useful for preparative purposes because triethylammonium phosphate is not volatile in vacuum. For characterization of peptides using UV detection the purity of solvents and buffers is of particular importance. Twice distilled water still contained volatile substances which interfere with UV detection at low wavelengths and cause baseline instability. Water purified by chromatography was satisfactory for HPLC. UV detection of peptides is performed in the range of 205–220 nm or at 254 nm for peptides with aromatic residues[13]. Since many substances absorb at 220 nm, this detection is less specific but more sensitive than at 254 nm. The monitoring of equimolar concentrations of (Ile^5)-ANG II at 220 nm at 254 nm revealed 8–9-fold increased peak heights at the lower wavelength. The enhanced absorption at 220 nm is due to the peptide bond which has a maximum at 187 nm[16].

The choice of stationary phase is equally important for peptide separation. Poor resolution of very similar peptides, *e.g.*, (Ile^5)-ANG II and (Ile^5)-ANG II-hexapeptide as well as tailing may be due to residual silanol groups in the C_{18} support[17] and to inhomogenity of the particle size. Compact columns with a high number of theoretical plates can be realized only with small particle size supports and a spectrum of narrow corn sizes. In our experience, 7-μm instead of 10-μm particles provided better resolution and smaller elution volumes for angiotensin peptides in both the methanol and the isopropanol gradient systems. The pressure, however, was increased by a factor of two. This is in contrast to results of Wilson *et al.*[14], who found no differences in the separation performance between 10- and 5-μm supports.

Using another reversed-phase support, namely phenylalkyl instead of C_{18} with the acetonitrile gradient system as mobile phase (results not shown), the separation of the angiotensin peptides was less satisfactory. For peptide separations most workers have employed reversed-phase columns, but ion-exchange supports are suitable for this purpose as well[18]. The preference for reversed-phase columns is probably due to their better selectivity and resolution, because both pH-dependent and -independent mechanisms such as polarity and hydrophilicity contribute to the separation power.

Quantification of peptides in tissue is a problem because of their fast degradation by highly active peptidases. Therefore, strict monitoring of peptide recoveries is essential. Fast removal of tissue from the animals, shock freezing on solid CO_2 or liquid nitrogen and subsequent combined use of boiling and acidification was found to destroy peptidase activity completely[9]. For the determination of recovery, syn-

thetic peptides and radioactively labelled peptides can be used. There was no difference in the recovery of synthetic (Ile^5)-ANG I and ^{125}I-labelled ANG I. The labelled peptides were not degraded during sample handling and could be separated from the endogenous peptides. It was thus possible to calculate the recovery for each individual sample and for each step of the sample preparation.

In conclusion, HPLC using different gradient systems yielded high resolution of a variety of different peptides. The combination of HPLC and specific RIAs is generally applicable for the qualitative and quantitative measurement of peptides in the femtomolar range derived from different biological sources. As shown for angiotensin and opioid peptides, the investigation of unknown peptides cross-reacting with the antibodies is possible only using this combined method.

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