Journal of Chromatography, 417 (1987) 27–40
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3599

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–RADIOIMMUNOASSAY METHOD FOR THE MEASUREMENT OF ANGIOTENSIN II PEPTIDES IN HUMAN PLASMA*

MARK E. SOUTHER and RICHARD H. LUMPKIN

Department of Medicine, University of Missouri School of Medicine, Columbia, MO 65212 (U.S.A.)

KENNETH C. KUO

Department of Biochemistry, University of Missouri School of Medicine, Columbia, MO 65212 (U.S.A.)

GARRY P. REAMS and JOHN H. BAUER*

Department of Medicine, University of Missouri School of Medicine, Columbia, MO 65212 (U.S.A.)

and

CHARLES W. GEHRKE

Department of Biochemistry, University of Missouri School of Medicine, Columbia, MO 65212 (U.S.A.)

(First received September 11th, 1986; revised manuscript received January 12th, 1987)

SUMMARY

A highly selective high-performance liquid chromatographic–radioimmunoassay method for the measurement of individual endogenous angiotensin peptides in human plasma is described. This method allows the complete resolution of the immunoreactive angiotensin II peptides. We have also measured the angiotensin peptide levels and compared them in both pooled and individual human plasma. The effects of inhibition of angiotensin-converting enzyme on the angiotensin peptide levels have also been observed in a patient with renovascular hypertension with the plasma angiotensin II level being reduced greater than seven-fold. This new methodology was validated by recovery experiments in plasma over a range of physiological levels using two methods of detection, radioimmunoassay and liquid scintillation counting. Consistent recoveries near 80% have been achieved for each peptide in plasma at concentrations over a physiological range. The described method enables

*Presented at the American Federation for Clinical Research National Meeting, Washington, DC, May 2–4, 1986.

0378-4347/87/$03.50

the direct measurement of the circulating angiotensin peptides and the elucidation of their specific roles in physiological and disease states.

INTRODUCTION

Angiotensin II is the active component of the renin–angiotensin system. It is a potent arteriolar vasoconstrictor hormone [1] and possesses a wide range of biological activities [2–9]. Attempts to measure angiotensin II have been complicated by its low endogenous (femtomolar) concentrations, its close similarity to other immunoreacting metabolites, and its susceptibility to degradation by nonspecific peptidases. Most commonly, angiotensin II has been measured in plasma using radioimmunoassay (RIA) techniques. This was commonly thought to measure active angiotensin II, the octapeptide (A_8). However, due to the cross-reactivity between other (C-terminal) angiotensin peptide fragments (pentapeptide, A_5; hexapeptide, A_6; and heptapeptide, A_7) with the angiotensin II antibody, an immunoreactive angiotensin II was actually measured.

Various analytical techniques such as paper chromatography [10], thin-layer chromatography [11], polyacrylamide gel electrophoresis [12] and isoelectric focusing [12] have been employed to directly measure the individual angiotensin peptides. More recently, reversed-phase high-performance liquid chromatography (HPLC) has been used to separate angiotensin peptides. While many of the angiotensin peptide fragments have been separated, complete resolution of these structurally similar peptides has not been achieved [13–16]. Nussberger et al. [16, 17] recently reported methodology employing HPLC to measure the levels of each individual peptide. However, partial co-elution of the angiotensin peptide fragments (A_5 and A_7) was observed in aqueous solution. This may have resulted in cross-collection of purified individual peptide fractions. Furthermore, resolution at pharmacological and physiological levels of angiotensin peptides in the presence of a plasma matrix was not demonstrated.

Our specific objectives were (1) to develop a quantitative, reproducible, and highly selective HPLC separation of synthetic angiotensin peptides so that their subsequent individual collection could be performed easily; (2) to link a highly sensitive RIA to measure the individual peptides; and (3) to measure the endogenous angiotensin peptide levels in human plasma.

EXPERIMENTAL

Chemicals and reagents

A_8 was obtained from Peninsula Labs. (Belmont, CA, U.S.A.); angiotensin decapeptide (A_{10}) and A_7 were obtained from Vega Biochemicals (Tucson, AZ, U.S.A.); and A_5 and A_6 were obtained from Chemical Dynamics (S. Plainfield, NJ, U.S.A.). The tetrapeptide (A_4) was purchased from Bachem (Torrence, CA, U.S.A.) and the acetate salt of the nonapeptide (A_9) was purchased from Sigma (St. Louis, MO, U.S.A.).

High-purity HPLC-grade solvents (Burdick and Jackson Labs.) were used at

all times. Water was subjected to reverse osmosis, charcoal adsorption, ion exchange, glass distillation, and finally passed through a 0.2-μm nylon membrane filter. The prepared solvents were degassed with helium and stored pressurized in a solvent chamber during HPLC.

Trifluoroacetic acid and morpholine were purchased from Aldrich (Milwaukee, WI, U.S.A.) and are corrosive as well as severe irritants to skin. They were always used in a well ventilated area.

The angiotensin II antiserum was produced in Dr. J. Alan Johnson's Laboratory (H.S. Truman Memorial Veterans Hospital, Columbia, MO, U.S.A.) by the periodic injection of rabbits with angiotensin II conjugated to bovine serum albumin (BSA); together with Freund's adjuvant, the antiserum was diluted (1:100 000) in 0.1 *M* Tris, 0.25% BSA buffer, pH 7.4.

Blood samples

Whole blood (10 ml) was collected from an antecubital vein and immediately placed into chilled Vacutainer tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.) containing 0.2 ml of an inhibitor solution (200 μg captopril, 15 mg K_3EDTA) preventing the in vitro conversion of A_{10} to A_8. The Vacutainer tubes were immediately chilled in ice, then centrifuged at 1250 *g* for 15 min at 4°C to separate the cellular components from the plasma. The plasma was removed and stored at −20°C.

Extraction, fractionation and radioimmunoassay

The methodology for separation and direct measurement of the angiotensin peptide fragments consisted of three steps: extraction on bonded phase, separation by HPLC, and measurement by RIA.

Solid-phase extraction

Solid-phase extraction was employed to remove non-angiotensin plasma components which may interfere with the separation chromatography or cross-react with the angiotensin II antisera. A solid-phase extraction cartridge (Supelclean LC-Ph, Supelco, Bellefonte, PA, U.S.A.) containing 100 mg of phenyldimethylsilyl packing was used for each plasma sample. The cartridge was placed into a vacuum manifold apparatus (Vac Elut, Analytichem, Harbor City, CA, U.S.A.) to control flow through the cartridge. The cartridges were conditioned by washing with 2 ml HPLC-grade methanol and then equilibrated with 2 ml glass-distilled water. The plasma samples were kept on ice until they were applied to the solid-phase extraction cartridge. This minimized in vitro degradation of the angiotensin peptides by peptidases as well as angiotensin-converting enzyme. The plasma samples were thawed under running tap water, and subsequently, exactly 2.0 ml were applied to the extraction cartridge while a small volume of chromatography water remained above the cartridge packing. The flow-rate through the cartridge can affect the retention of these peptides. Plasma constituents and the inhibitor solution were removed which resulted in the angiotensin peptides being retained on the solid-phase cartridge. The cartridge was then washed with 0.5 ml of 5% methanol to remove additional plasma components. Finally, the angiotensin pep-

tides were eluted from the extraction cartridge with two 0.5-ml aliquots of neat methanol, which was more efficient in eluting angiotensins than using one 1.0-ml aliquot. It was important that all solvents, as well as samples, pass through the extraction cartridge at a flow-rate no greater than 2 ml/min. Following solid-phase extraction, the angiotensin peptides were then either assessed for immunoreactive angiotensin II using RIA or applied to an HPLC column for separation and collection of the individual angiotensin peptides.

HPLC apparatus

The chromatographic apparatus used to perform reversed-phase HPLC was a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 4 liquid chromatograph equipped with a Waters (Milford, MA, U.S.A.) U6K injector. A 25 cm×4.6 mm I.D. (5 μm particle size) column (Supelcosil LC-DP, Supelco) and a 2-cm (5 μm particle size) guard column (Supelguard LC-DP, Supelco) containing diphenylmethylsilyl packing were used to separate the angiotensin peptides using a single-ramp linear gradient solvent program. A column heater (Systec Model CH-1448, Minneapolis, MN, U.S.A.) was employed to maintain the pre-column and column temperatures at 60°C. The solvent system consisted of two solvents: solvent A, water–trifluoroacetic acid–morpholine (100:0.1:0.05); solvent B, acetonitrile–trifluoroacetic acid–morpholine (100:0.1:0.05). An aqueous solution of 10% trifluoroacetic acid and 5% morpholine was passed through a Waters Sep-Pak C_{18} solid-phase extraction cartridge to remove impurities which otherwise resulted in a poor chromatographic baseline when detected at 280 nm. This chromatographically clean solution was then used in preparing the HPLC solvents.

The column was equilibrated for 15 min with solvent A–solvent B (60:40). Upon injection of the plasma extract (equivalent to 2 ml plasma), the solvent system remained isocratic for 5 min following which solvent B increased linearly to 90% in 11 min and remained at 90% for an additional 2 min. Retention times were established initially using microgram quantities of each peptide standard and determining the resulting elution times using ultraviolet (UV) absorption at 280 nm. A wavelength of 280 nm was used because it selectively detects only peptides which contain aromatic amino acids, as do angiotensin peptides.

HPLC separation

The solid-phase extraction eluate was evaporated to dryness in a Savant (Farmingdale, NY, U.S.A.) Speed Vac concentrator equipped with a vacuum system and a −50°C cold-finger trap. After reconstituting the dried peptides in 600 μl of HPLC starting solvent [solvent A–solvent B (60:40)], the sample was vortexed and injected onto the diphenyl HPLC column. Following separation, the individual angiotensin peptides were collected in single eluate fractions and then evaporated to dryness (about 60 min) in the Savant concentrator. The level of angiotensin peptide in each fraction was then directly measured using RIA.

Both the guard and analytical columns were subjected to substantial amounts of proteinaceous material, which may accumulate over the course of many injections. We employed an *n*-propanol wash, consisting of a linear gradient from 25 to 90% *n*-propanol over 15 min, after inverting the columns. By employing the

propanol wash, the life of the column was extended considerably (from analysis of 20 plasma samples to greater than 100). Pharmacological levels of angiotensin peptides are not applied to the chromatographic system on a regular basis. However, periodically it is necessary to inject 3–5 μg of angiotensin peptides and to follow spectrophotometrically the chromatographic separation to ensure continued resolution. Following addition of pharmacological levels, an extended *n*-propanol gradient wash is performed to eliminate any residual peptide material remaining in the chromatographic system which may interfere with the measurement of very low endogenous (pg/ml) levels. This wash involved programming the HPLC pump to run ten times consecutively a 30-min gradient from 25 to 90% *n*-propanol (column temperature 40°C, flow-rate 1.0 ml/min). Following this, a set of performance blanks was collected using the solvent program for the separation of angiotensin peptides and subjected to RIA. If levels of angiotensin peptides were detected, the procedure was repeated.

The retention time of A_8 can be verified at physiological levels by using 10–15 pg [^{3}H]angiotensin II. Since [^{3}H]angiotensin II has the same retention time as angiotensin II, this provides a quick and easy reference to ensure that the retention time of A_8 has not changed without subjecting the chromatographic system to pharmacological levels that require a lengthy wash.

Radioimmunoassay

A standard of angiotensin II was serially diluted from 200 to 0.75 pg in a final volume of 1 ml of 0.25% BSA and analyzed in triplicate at nine different levels with each RIA sample set. From this standard curve, measurements of the amount of angiotensin in samples following HPLC separation were performed. A known excess of radiolabeled trace [^{125}I]angiotensin II antigen (New England Nuclear, Boston, MA, U.S.A.) was diluted in BSA buffer until there was approximately 2000 cpm per 0.5 ml buffer. Equal volumes of trace and angiotensin II antiserum (0.50 ml each) were added to the samples being assayed.

A 48-h incubation at 4°C was terminated by addition of 1.0 ml of 1.6% charcoal–0.16% dextran suspension (in barbital buffer, pH 7.4). After 5 min, the samples were centrifuged for 25 min at 1250 *g* and the supernatant containing the antibody-bound angiotensins was aspirated and discarded, leaving the free angiotensins bound to the charcoal pellet. The charcoal (containing free angiotensin) was counted in a multi well-type gamma counter (Packard Instruments, Downer's Grove, IL, U.S.A.). The results were calculated from a log–logit transformation of the concentration of angiotensin standards versus the ratio of the bound counts of angiotensin for each standard to the bound counts of angiotensin for the zero angiotensin standard. The above method is a modification of the RIA reported previously from our laboratory for immunoreactive angiotensin II [18].

Quantitative analysis of plasma peptide levels

Correction factors for (a) cross-reactivity of the peptides with the angiotensin II antibody, (b) recovery through the total method, and (c) the purity of angiotensin II (A_8) used in the standard curve need to be considered in calculating the concentrations of the angiotensin peptides in plasma. A molar concentration of

the peptide in the sample is derived from the standard curve of the octapeptide which is measured with each RIA of a set of samples. The equation illustrating the calculation of angiotensin peptide level in plasma is shown below:

$$C_{A_p}\,(\text{pg/ml}) = \frac{C_{A_{RIA}}\,(\text{fmol/ml})}{\text{Purity } A_8} \times \frac{MW_{A_p}}{1} \times \frac{1}{Rec_{A_p}} \times \frac{1}{CR_{A_p}} \times \frac{1\text{ pg}}{1000\text{ fg}}$$

where: C_{A_p} = angiotensin peptide level being measured; $C_{A_{RIA}}$ = angiotensin level reported by RIA; Purity A_8 = purity of A_8 used in standard curve (=0.945); CR_{A_p} = cross-reactivity of angiotensin peptide with angiotensin II antibody; Rec_{A_p} = total recovery of angiotensin peptide as determined in Results; MW_{A_p} = molecular weight of angiotensin peptide.

Since the RIA standard curve is based upon the measurement of angiotensin II (A_8), the computed results are in units of molar concentration of the peptides. The data are first corrected for the purity of the A_8 standard, and then converted to units of weight multiplying by the molecular weight of the peptide being measured. This result is then corrected for recovery and for the appropriate cross-reactivity with the angiotensin II antibody.

For example, if the A_5 concentration was determined by RIA to be 3.0 fmol/ml and then the correction factors below were applied:

$$C_{A_5} = \frac{3.0}{0.945} \times \frac{678.6}{1} \times \frac{1}{0.85} \times \frac{1}{0.96} \times \frac{1\text{ pg}}{1000\text{ fg}} = 2.64\text{ pg ml}$$

where C_{A_5} = actual concentration of A_5; 3.0 = concentration (fmol/ml) of A_5 determined by RIA; 678.6 = (g/mol) molecular weight of A_5; 1/0.85 = correction for 85% recovery; 1/0.96 = correction for 96% cross-reactivity of A_5 with the angiotensin II antibody. The actual concentration of A_5 would be 2.64 pg/ml plasma.

RESULTS

Peptide separation

In order to establish optimal chromatographic separation and reproducible retention times, pharmacological concentrations of the angiotensin peptides were measured in an aqueous solution and detected by UV absorption at 280 nm. Fig. 1 is a chromatogram illustrating the separation of pharmacological levels (3–5 μg) of each synthetic peptide fragment (A_5, A_6, A_7, A_8, A_{10}) in an aqueous solution. There is a distinct separation of A_5 and A_6 from A_8, and of A_8 from A_7, and a late elution of A_{10}.

After achieving a separation of pharmacological levels of the angiotensin peptides in aqueous solution, physiological concentrations (pg/ml) of the synthetic peptides were separated by HPLC and measured in an aqueous solution by RIA. Fig. 2 is a chromatogram illustrating the separation of physiological levels (5–15 pg/ml) of aqueous angiotensin peptides (A_5, A_6, A_7, A_8). Since UV absorption is not sufficiently sensitive for the detection of endogenous levels of angiotensin peptides, the separated angiotensin peptides were collected in "blind cuts" before

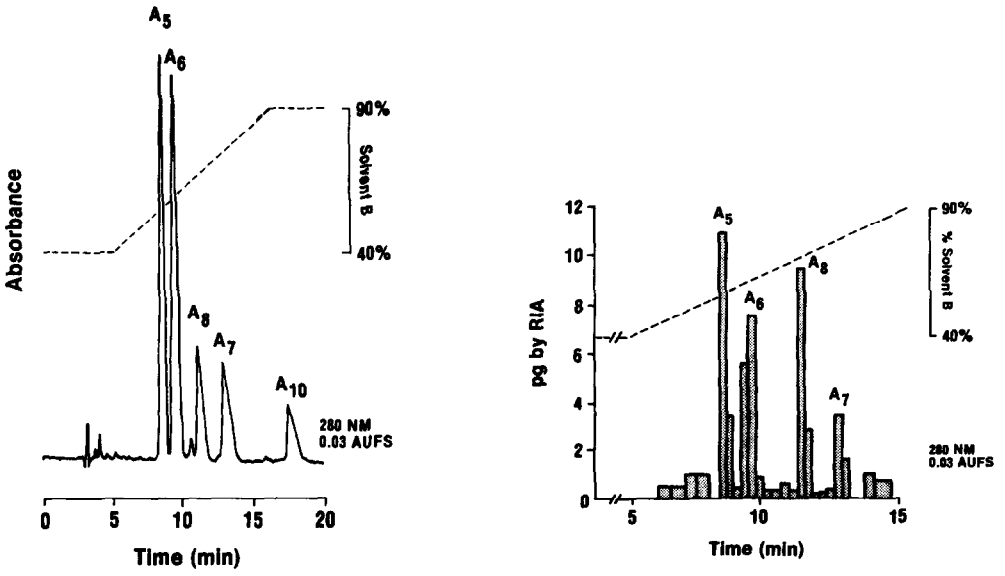

Fig. 1. Separation of pharmacological levels (3–5 μg) of aqueous angiotensin standards by HPLC. Solvent A: water-trifluoroacetic acid-morpholine (100:0.1:0.05); solvent B: acetonitrile-trifluoroacetic acid-morpholine (100:0.1:0.05); flow-rate: 1 ml/min; column temperature: 60°C. Peaks: A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide (angiotensin III); A_8 = octapeptide (angiotensin II); A_{10} = decapeptide (angiotensin I).

Fig. 2. HPLC-RIA separation of physiological concentrations (5–15 pg/ml) of angiotensin peptides in an aqueous solution. Solvent A: water-trifluoroacetic acid-morpholine (100:0.1:0.05); solvent B: acetonitrile-trifluoroacetic acid-morpholine (100:0.1:0.05); flow-rate: 1 ml/min; column temperature: 60°C. Peaks: A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide (angiotensin III); A_8 = octapeptide (angiotensin II).

being subjected to RIA. Eluate fractions were collected in time windows determined from previously established retention times using pharmacological levels of angiotensin peptides. The intra-assay (within-day) and inter-assay (day-to-day) reproducibility of retention times of angiotensin peptides in aqueous solution are shown in Table I. The relative standard deviation (R.S.D.) at no time was greater than 1%.

Having demonstrated the chromatographic separation of synthetic angiotensins in an aqueous solution, it was then necessary to determine the effect of the plasma matrix on the chromatographic resolution of pharmacological and physiological levels of angiotensin peptides. Fig. 3 is a chromatogram illustrating the separation of pharmacological levels (3–5 μg) of angiotensin peptide standards (A_5, A_6, A_7, A_8, A_{10}) added to 2.0 ml of pooled human plasma. The total sample was subjected to solid-phase extraction followed by HPLC. Similar resolution and reproducibility were achieved using pharmacological amounts of the angiotensin peptide (A_5, A_6, A_7, A_8) added to human plasma as was observed in aqueous solution.

The separation of physiological levels (5–15 pg) of the angiotensin peptides added to 2.0 ml pooled plasma is shown in Fig. 4. A blank eluate fraction was

TABLE I

REPRODUCIBILITY OF HPLC RETENTION TIMES OF THE ANGIOTENSIN PEPTIDES IN AQUEOUS SOLUTION

A_5 = pentapeptide (Tyr-Ile-His-Pro-Phe); A_6 = hexapeptide (Val-Tyr-Ile-His-Pro-Phe); A_7 = heptapeptide (Arg-Val-Tyr-Ile-His-Pro-Phe); A_8 = octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe); A_{10} = decapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu).

	Retention time (min)				
	A_5	A_6	A_7	A_8	A_{10}
Intra-assay reproducibility					
Run 1	9.00	9.94	12.60	11.43	16.06
Run 2	9.03	9.96	12.61	11.45	16.10
Run 3	9.04	9.95	12.59	11.43	16.06
Run 4	9.06	9.97	12.59	11.45	16.06
Mean	9.03	9.95	12.60	11.44	16.07
R.S.D. (%)	0.28	0.13	0.08	0.10	0.12
Inter-assay reproducibility					
Day 1	9.03	9.95	12.60	11.44	16.07
Day 2	9.05	9.90	12.56	11.40	15.98
Day 3	9.08	9.96	12.59	11.46	16.05
Mean	9.05	9.94	12.58	11.43	16.03
R.S.D. (%)	0.28	0.32	0.17	0.27	0.29

collected on each side of the A_8 and A_7 fraction to demonstrate pure isolation; however, in the presence of a plasma matrix, a blank fraction could not be collected between A_5 and A_6. The A_5 and A_6 fractions were collected consecutively and the cross-over of one peptide being collected into the other was found to be less than 10%. This was determined by individual additions of A_5 and A_6 to plasma followed by the collection of their respective HPLC fraction cuts and then calculating the cross-over of the respective peptide. The intra-assay reproducibility of retention times of the angiotensin peptides in plasma is shown in Table II. Despite the complex plasma matrix, a good separation and reproducible retention times were achieved, and were similar to that observed when separating angiotensin peptides added to an aqueous solution.

Calibration and immuno-cross-reactivities

In order to determine the recovery of each of the peptide fragments as well as to achieve accurate measurements, the purity of the peptide standards, as well as the cross-reactivity of each peptide with the angiotensin II antisera was measured. Since RIA results were based on the percentage bound of the angiotensin II (A_8) standard curve to angiotensin II antisera which was measured during each set of RIA analyses, it was necessary to establish the exact purity of the A_8 standard. A_8 was found to be 94.5% pure using HPLC and amino acid analysis. A_{10} and A_7 were determined by amino acid analysis to be 93 and 83% pure, respec-

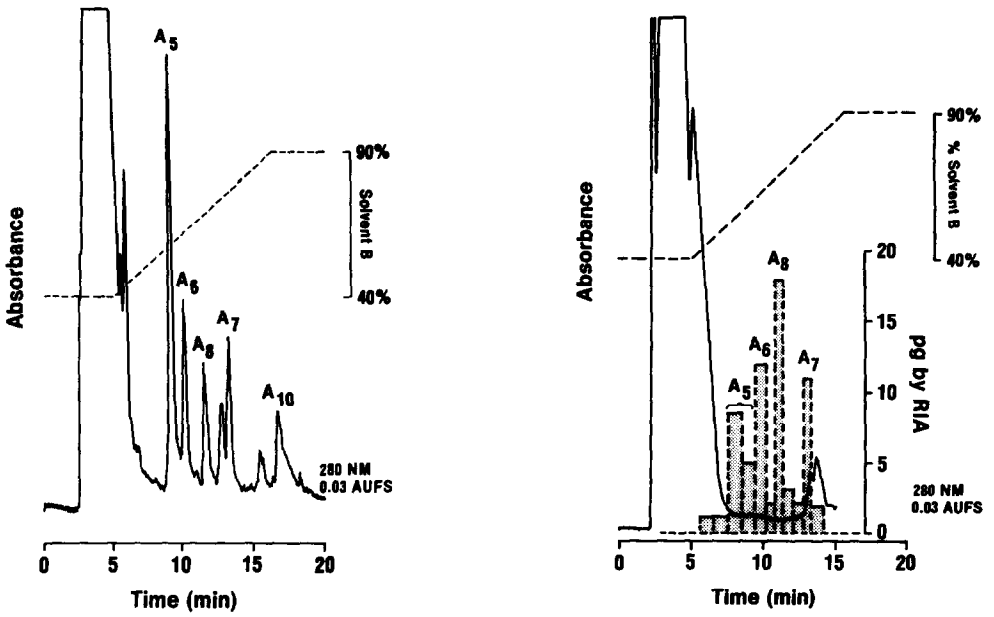

Fig. 3. HPLC separation of pharmacological concentrations (3–5 μg) of angiotensin standards added to 2.0 ml of pooled human plasma. Solvent A: water-trifluoroacetic acid-morpholine (100:0.1:0.05); solvent B: acetonitrile-trifluoroacetic acid-morpholine (100:0.1:0.05); flow-rate: 1 ml/min; column temperature: 60 °C. Peaks: A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide (angiotensin III); A_8 = octapeptide (angiotensin II); A_{10} = decapeptide (angiotensin I).

Fig. 4. HPLC separation of physiological concentrations (5–15 pg) of angiotensin standards added to 2.0 ml of pooled human plasma. Shaded bars represent individual peptide fractions as determined by RIA. Solvent A; water-trifluoroacetic acid-morpholine (100:0.1:0.05); solvent B: acetonitrile-trifluoroacetic acid-morpholine (100:0.1:0.05); flow-rate: 1 ml/min; column temperature: 60 °C. Peaks: A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide (angiotensin III); A_8 = octapeptide (angiotensin II).

tively. A_5 and A_6 were determined by amino acid analysis to be 95 and 83% pure, respectively. The cross-reactivity of the angiotensin peptides with the angiotensin II antibody is illustrated in Table III.

Recoveries

Recoveries were demonstrated employing two different methods of detection, RIA and liquid scintillation counting. The recoveries observed when 20 pg of each angiotensin peptide (A_5, A_6, A_7, A_8) standard were added together to 2.0 ml of pooled human plasma are shown in Table IV, following solid-phase extraction, HPLC separation, and RIA. Table V illustrates the recovery of 30 pg of [^{3}H]A_8 added to 2.0 ml of pooled human plasma detected by liquid scintillation.

Fig. 5 illustrates a linear recovery of A_8 over a range of physiological levels (8–35 pg/ml) added to 2.0 ml plasma. Fig. 6 illustrates a linear recovery of the A_7 over a range of physiological levels (8–28 pg/ml) added to 2.0 ml of pooled human plasma. A_5 and A_6 were also evaluated for recovery in plasma over a range

TABLE II

INTRA-ASSAY REPRODUCIBILITY OF HPLC RETENTION TIMES OF ANGIOTENSIN PEPTIDES IN HUMAN PLASMA

A_5 = pentapeptide (Tyr-Ile-His-Pro-Phe); A_6 = hexapeptide (Val-Tyr-Ile-His-Pro-Phe); A_7 = heptapeptide (Arg-Val-Tyr-Ile-His-Pro-Phe); A_8 = octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe); A_{10} = decapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu).

Run	Retention time (min)				
	A_5	A_6	A_7	A_8	A_{10}
1	9.00	10.06	13.27	11.48	16.68
2	8.86	9.96	13.12	11.47	16.74
3	8.88	9.99	13.17	11.47	16.69
Mean	8.91	10.00	13.17	11.47	16.70
R.S.D. (%)	0.85	0.51	0.60	0.05	0.19

of physiological levels and the recoveries approximated 85 and 76%, respectively.

Concentrations of peptides in plasma

The endogenous angiotensin peptide levels in pooled human plasma are shown in Table VI. The mean concentrations were: for A_5, 1.0 pg/ml; for A_6, 1.1 pg/ml; for A_8, 7.8 pg/ml; and for A_7, 2.3 pg/ml. The octapeptide constituted approximately 64% of the total peptide concentration. The angiotensin peptide levels in a non-hypertensive individual human plasma are shown in Table VI. The individual levels of the angiotensin peptides are similar to the levels of the angiotensin peptides in pooled plasma. Again, the octapeptide represented approximately 62% of the total peptide concentration.

The peptide levels in a patient with documented renovascular hypertension prior to and following administration of an inhibitor of angiotensin-converting enzyme, therapy which blocks the in vitro formation of angiotensin II, are shown in Table VI. The individual peptide levels are markedly higher than those observed in normotensive individuals. The octapeptide represented approximately 57% of the total peptide concentration in the patient with renovascular hypertension. At 4 h following the oral administration of the angiotensin-converting enzyme inhibitor enalapril, the mean levels of all the peptide fragments were greatly reduced. The octapeptide was reduced greater than seven times (from 45.1 to 5.7 pg/ml).

CONCLUSIONS

We have successfully separated and measured those angiotensin peptides (A_5, A_6, A_7, and A_8) which contribute to the immunoreactive measurements of angiotensin II. This was accomplished by developing a quantitative, reproducible, and highly selective HPLC separation which allows isolation and collection of each angiotensin peptide into single eluate fractions. Despite the complex plasma matrix, separation of the angiotensin peptide fragments at both pharmacological

TABLE III

CROSS-REACTIVITIES OF ANGIOTENSIN PEPTIDES WITH ANGIOTENSIN II ANTIBODY

Each level represents the mean of triplicate determinations. A_4 = tetrapeptide (Ile-His-Pro-Phe); A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide; A_9 = nonapeptide (Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu); A_{10} = decapeptide.

Angiotensin peptide	Level (pg)	Percentage cross-reactivity	Mean percentage cross-reactivity
A_4	2000	0.06	0.07
	1000	0.10	
	500	0.06	
A_5	150	93.8	96.0
	75	94.7	
	35	96.2	
	20	99.2	
A_6	130	98.0	97.4
	65	97.8	
	35	97.2	
	15	96.5	
A_7	100	81.5	80.3
	50	81.1	
	25	80.2	
	10	78.2	
A_9	5000	0.13	0.16
	2000	0.15	
	1000	0.20	
A_{10}	5000	0.24	0.25
	1000	0.20	
	700	0.27	

TABLE IV

RECOVERY OF COMBINED ANGIOTENSIN PEPTIDES ADDED TO HUMAN PLASMA DETECTED BY RIA AT THE 20-pg LEVEL

A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide; A_8 = octapeptide.

Run	Recovery (%)			
	A_5	A_6	A_7	A_8
1	76	76	70	79
2	85	85	97	82
Mean	81	81	83	80

TABLE V

RECOVERY OF [^{3}H]ANGIOTENSIN II OCTAPEPTIDE ADDED TO HUMAN PLASMA DETECTED BY LIQUID SCINTILLATION COUNTING AT THE 30-pg LEVEL

Run	Recovery (%)
1	76
2	85
3	80
Mean	80

TABLE VI

ENDOGENOUS PLASMA ANGIOTENSIN PEPTIDE LEVELS

A_5 = pentapeptide; A_6 = hexapeptide; A_7 = heptapeptide; A_8 = octapeptide; ACE = angiotensin-converting enzyme.

Sample	Concentration (mean ± S.D.) (pg/ml)				Percentage* A_8
	A_5	A_6	A_7	A_8	
Pooled human plasma (15 replicates)	1.0 ± 0.31	1.1 ± 0.27	2.3 ± 1.4	7.8 ± 0.62	64
Individual human plasma (n = 5; 3 replicates)	0.9 ± 0.71	1.2 ± 0.60	2.0 ± 1.5	6.6 ± 0.80	62
Renovascular individual					
Pre ACE inhibition (3 replicates)	18.9 ± 1.22	14.0 ± 1.65	8.4 ± 1.27	45.1 ± 0.95	57
Post ACE inhibition** (2 replicates)	2.1 ± 1.00	3.4 ± 0.15	3.6 ± 0.45	5.7 ± 1.7	39

*Percentage A_8 = percent concentration of A_8 to the total peptide concentration (A_5, A_6, A_7, A_8).
**At 4 h following 10 mg enalapril.

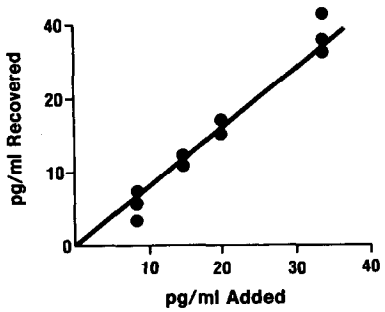

Fig. 5. Recovery of angiotensin II (octapeptide) added to human plasma (recovery ~80% at each level).

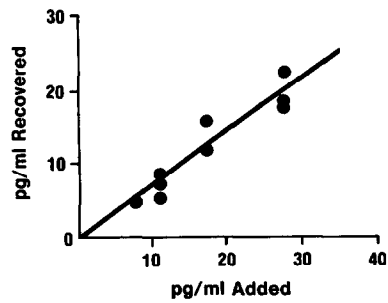

Fig. 6. Recovery of angiotensin III (heptapeptide) added to human plasma (recovery ~74% at each level).

and physiological levels is reproducible. Consistent recoveries near 80% have been achieved for each peptide in plasma at concentrations over a physiological range.

The individual peptide levels in both pooled and individual human plasma are in agreement with those previously reported by Nussberger et al. [17] using similar methodology. The peptide levels in a patient with renovascular hypertension have been characterized prior to and following angiotensin-converting enzyme inhibition therapy, which blocks the in vivo formation of angiotensin II. The A_8 level was reduced more than seven-fold, while the concentrations of the other angiotensin peptides were reduced greater than 50%.

Validation of our new methodology was demonstrated by: (a) showing that the plasma matrix did not affect the HPLC retention times of the angiotensin peptides, using both UV detection of pharmacological levels and RIA detection of physiological levels; (b) determining the recoveries of the angiotensin peptides added to plasma over a range of physiological levels; (c) measuring the increased angiotensin peptide plasma concentrations in a renovascular hypertensive individual, where angiotensin II levels would be expected to be higher than in a normotensive individual; and (d) observing the expected significant decrease in the angiotensin peptide concentrations following administration of an inhibitor of angiotensin-converting enzyme.

The major advantage of our procedure is the high degree of separation achieved, allowing collection of the individual angiotensin peptides. Other advantages include the ability to apply the total solid-phase plasma extracted sample to the HPLC column, and collection of the isolated peptides into single fractions. These peptide fractions contained high concentrations of a volatile organic solvent (acetonitrile), which evaporated quickly without leaving a residue which may interfere with antigen–antisera equilibria. Evaporation in the Savant concentrator resulted in good recoveries, and the procedural blanks showed no cross-reaction with the angiotensin II antibody. Analyzing femtomolar levels of peptides requires extreme care to avoid contamination. Syringes used for HPLC injection were thoroughly washed between injections with at least 3 ml each of water and of an organic solvent such as propanol. When performing the recovery experiments at different physiological levels, a single injection syringe was used for each different level to reduce syringe contamination.

In conclusion, this methodology allows the direct measurement of the immunoreactive angiotensin peptides in human plasma, specifically the known biologically active angiotensins, the heptapeptide and the octapeptide. The measurement of these circulating angiotensin peptides now provides a means to elucidate their specific roles in physiological and disease states.

ACKNOWLEDGEMENTS

The authors wish to thank Miss Melissa Parisi for her technical assistance, Dr. J. Alan Johnson for his technical advice and supply of the angiotensin II antisera, Dr. Roy Eksteen of Supelco, Inc. for his generous gift of HPLC columns, and Miss Tracey Swope for her help in the preparation of the manuscript.

REFERENCES

1 C.J. Dickenson and R. Yu, J. Physiol., 190 (1967) 91.
2 E. Haker, Circulation, 54 (1976) 849.
3 V.L. Brooks and R.L. Malvin, Fed. Proc., Fed. Am. Soc. Exp. Biol., 38 (1979) 2272.
4 W.H. Cline, Jr., J. Pharmacol. Exp. Ther., 216 (1981) 104.
5 K.U. Malik and A. Madjletti, Circ. Res., 36 (1976) 26.
6 P. Biron, E. Koin, W. Nowachzeynski, J. Brouillet and J. Genest, J. Clin, Invest., 40 (1961) 338.
7 N.M. Kaplan and F.C. Barlter, J. Clin. Invest., 41 (1962) 715.
8 G. Agurlera, R.H. Menard and K.J. Catt, Endocrinology, 107 (1980) 55.
9 J.E. Hall, T.G. Coleman, A.C. Guyton, J.W. Bulfe and H.C. Saldgado, Am. J. Physiol., 236 (1979) F252.
10 P.F. Semple, A.S. Boyd, P.M. Dawes and J.J. Morton, Circ. Res., 39 (1976) 671.
11 S. Cohen, J.M. Taylor, K. Murakami, A.M. Michelakis and T. Inagami, Biochemistry, 11 (1972) 4286.
12 P. Govul, D. Rodbard, J. Drouet, K. Catt and J. Meuard, Biochem. Biophys., 322 (1972) 392.
13 M. Guy, G. Roberson and L. Burnes, Anal. Biochem., 112 (1981) 272.
14 K. Hermann, R.E. Lang, T.H. Unger, C. Bayer and D. Ganten, J. Chromatogr., 312 (1984) 273.
15 J. Tonnaer, Eur. J. Biochem., 131 (1983) 415.
16 J. Nussberger, D. Brunner, B. Waeber and H. Brunner, Hypertension, 7 (Suppl. I) (1985) I-1.
17 J. Nussberger, D. Brunner, B. Waeber and H. Brunner, J. Hypertension, 3 (Suppl. 3) (1985) S269.
18 G.P. Reams and J.H. Bauer, Am. J. Kidney Dis., 7 (1986) 402.