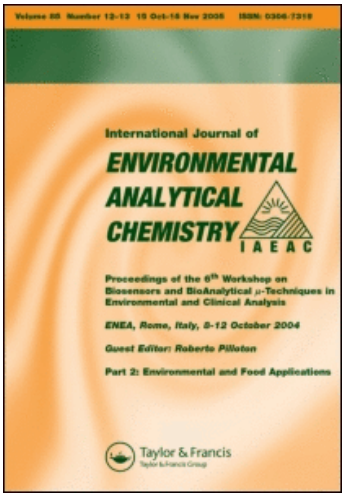

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Octapeptide-Specific and Sensitive Assay for Angiotensin II in Plasma†

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Angiotensin-(1-8)octapeptide (angiotensin II) is the active principle of the renin-angiotensin system. Crossreaction of angiotensin II-antisera with inactive precursors and metabolic fragments prevented the specific quantitation of this hormone in biological fluids. Peptide-extraction on bonded-phase silica followed by peptide-separation using isocratic reverse-phase high performance liquid chromatography and subsequent radioimmunoassay rendered possible the octapeptide-specific measurement of angiotensin II in 2 ml plasma with a detection limit of 0.4 fmol/ml. The coefficient of variation for intra-assay precision was 0.06 and for inter-assay precision 0.13. ^{125}I-angiotensin II was recovered from plasma by solid-phase extraction to 99 ± 2% (mean ± S.D.). The overall recovery of 5, 10 and 20 fmol unlabeled angiotensin II added to plasma was 80 ± 10%. Plasma concentrations in supine normal humans averaged 4.1 ± 1.6 fmol/ml and were suppressed below the detection limit by angiotensin I converting enzyme inhibition.

KEY WORDS: Solid-phase peptide extraction, high performance liquid chromatography, radioimmunoassay, angiotensin metabolites, sensitivity.

INTRODUCTION

The measurement of plasma angiotensin (ANG) II is important since this hormone represents the active principle of the renin-angiotensin

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system. However, the factor most often measured in experimental and clinical studies is plasma renin activity or concentration. This is because the measurement of plasma angiotensin II is hampered by many technical difficulties. In particular, the estimation of low levels is problematic.[1] Thus, during converting enzyme inhibition, existing radioimmunoassays (RIA) measure reduced but clearly not zero plasma immunoreactive "angiotensin II".[2] The lack of specificity of the antisera on one side and recovery- and/or "blank"-problems caused by extraction procedures on the other are probably some of the reasons for this. The present report describes a new method for the measurement of plasma ANG II based on a fast and practically blank-free extraction technique with virtually no recovery losses, a complete separation of the different angiotensins by HPLC and a subsequent direct linkage to a radioimmunoassay using an extremely sensitive antiserum.

METHODS

The following investigations were carried out according to the principles of the Declaration of Helsinki and were approved by the Hospital Ethics Committee. The nature and purpose of the study was explained and informed consent was obtained from every participant.

Reagents

Standard peptides were purchased: human ANG-(1-8)octapeptide, ANG-(4-8)pentapeptide, ANG-(2-8)heptapeptide from Peninsula Labs (San Carlos, CA); ANG-(2-10)nonapeptide, ANG-(1-10)decapeptide from Senn Chemicals (Dielsdorf, Switzerland). ANG-(3-8)hexapeptide was synthetized as previously described.[3] ^{125}I-ANG II was from New England Nuclear (Boston, MA). Phenyl-bonded silica (Bondelut PH) was from Analytichem International (Harbor City, CA). Methanol, ethanol, water, phosphoric acid, hydrochloric acid and acetic acid of highest grades and sodium azide were from Merck (Darmstadt, Germany). Nucleosil-ODS of 10 μm particle size was from Innovativ-Labor (Adliswil, Switzerland). Reagent grade Tris, 1,10-phenanthroline and activated charcoal were from Sigma

(St. Louis, MO) and crystallized bovine plasma albumin (BPA) from Semour Pharmaceutical Company Ltd. (Eastbourne, England). Na_2-EDTA and Dowex 50WX2, 50–100 mesh, H+ were from Fluka (Buchs, Switzerland). Neomycin sulfate was from Syntex (Allschwil, Switzerland). Dextran T70 was from Pharmacia (Uppsala, Sweden).

Solutions

Buffer Tris 0.1 M; BPA 5 gm/L; sodium azide 0.2 gm/L; hydrochloric acid (for adjustment to pH 7.5 at room temperature).

Inhibitors Ethanol/water 2%; phenanthroline 0.025 M; Na_2-EDTA 0.125 M; neomycin 2 gm/L.

Label ^{125}I-ANG II was diluted in buffer and used at a final concentration of 1 fmol per assay tube (2,000 counts per minute).

Charcoal In 1,000 ml water, 20 gm charcoal and 2 gm dextran were suspended.

Antiserum The antiserum was diluted in buffer and used in a final dilution of 1:60,000.

Equipment

A VacElut® vacuum manifold from Analytichem was used for simultaneous extraction of 10 plasma samples. A Hewlett Packard (HP) liquid chromatograph, model 1090A, was used: it consisted of an HP-31 binary solvent delivery system, a Rheodyne 7010 manual injection valve with loop-filler port and position-sensing switch, an HP-81 filter photometric detector and an HP-3390 integrator. The system was completed by a fraction collector, type Gilson 202. An HP-85B microcomputer was used as controller. Radioactivity was measured in an Autogamma® 800 counter from Packard Instruments (Downers Grove, IL).

Immunization

A New Zealand White rabbit was immunized with ANG II that had been unidirectional coupled to BPA. Amino-terminal protected (N-alpha-trifluoracetyl-para-nitrobenzyl-Asp^1)-ANG II was synthetized

and coupled to the carrier protein by the carbodiimide method as described elsewhere.[3] Deprotection was effected by alkaline treatment followed by dialysis. Amino acid analysis showed a peptide to carrier ratio of 5. Two mg of conjugate were suspended in 0.5 ml saline, emulsified with 0.5 ml complete Freund's adjuvant and injected intracutaneously. An intradermal booster injection of 0.5 mg antigen was given 8 weeks after the primary immunization. Blood was collected from the central ear artery at weekly intervals. Serum obtained at the 12th week was used in the assay. (The antiserum was produced by one of us [J. N.] in the Cellular and Molecular Research Laboratory, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.)

Blood sampling

Samples (10 ml) of blood were collected into prechilled glass tubes containing 0.5 ml inhibitor solution. The blood samples were immediately chilled in ice and centrifuged at 4°C. The plasma was stored at −20°C until analyzed.

Extraction

One Bondelut-cartridge containing 100 mg phenylsilyl-silica for each sample was inserted into the VacElut apparatus which was attached to a vacuum source. With the vacuum on, the cartridges were washed with 1 ml methanol and primed with 1 ml water. Subsequently, 2 ml cold plasma was rapidly passed through the cartridge followed by another wash with 3 ml water. The angiotensins were retained at the matrix of the cartridges and eluted with 0.5 ml methanol into buffer-coated conical polypropylene tubes. The methanol was evaporated under air stream in a 40°C waterbath.

HPLC-Separation

The residue was redissolved in 140 μl 0.1 M acetic acid and 100 μl of this solution was analysed by isocratic reverse-phase HPLC using a 250 × 4.6 Nucleosil column and a methanol/0.1% phosphoric acid (33.5/66.5) mobile phase, i.e. a modification of the method proposed by Hearn.[4] The flow was 1 ml/min, the pH 2.5 at a temperature of

45°C. Five or 10 fractions of 200 or 100 μl eluate were collected at the retention time for ANG-(1-8)octapeptide (previously established with 100 ng of standard ANG II) directly into 0.5 ml buffer for the subsequent RIA.

Radioimmunoassay

To the collected fractions, 0.5 ml anti-serum and 0.05 ml label were added. For standards, depending on the fraction volume, 200 or 100 μl of mobile phase were combined with 0.5 ml buffer containing increasing amounts of ANG II (0.16–20 fmol); antiserum and label were added as for the unknown samples. After a 48 hour incubation at 4°C bound and free hormone were separated by the dextran-coated charcoal method[5] as previously described in detail,[6] using 0.3 ml charcoal-dextran suspension.

Assay criteria

The *extraction recovery* was tested by adding ^{125}I-ANG II (50,000 c.p.m.) to 17 ml of inhibitor containing plasma and extracting 2 ml aliquots of this plasma over phenyl-silyl-silica ($n=8$). The *overall recovery* was tested by adding 5, 10 and 20 fmol ANG II to 2 ml aliquots of inhibitor containing plasma; the aliquots were then processed as unknown samples ($n=12$). Endogenous ANG II (5.6 fmol/ml) was subtracted. The *sensitivity of the antiserum* was defined as the smallest amount of ANG II that could be distinguished from zero (i.e. the amount giving a tracer displacement corresponding to 2 S.D. from the zero point of the standard curve). The amount of cold ANG II needed for a 50% tracer displacement was determined in 10 consecutive standard curves. The *specificity of the antiserum* was assessed by the method of Abraham[7] on a molar basis (after correction for water and salt content of the standards). To evaluate the *within-assay precision*, 8 aliquots of a given plasma pool were processed on one day. The same pool was remeasured on 8 different days to establish the *between-assay precision*. *Blank* tests were performed by processing duplicates of 2 ml aliquots of water or buffer (with and without inhibitors) or of reextracted angiotensin-free plasma containing inhibitors. Plasmas of subjects taking a converting enzyme inhibitor could also be considered as physiological blanks.

Repeatedly, 100 μl acetic acid (0.1 M) were injected prior to and after a day's HPLC runs and fractions were collected as for unknown samples.

Human studies

ANG-(1-8)octapeptide was measured by the new method as well as immunoreactive "ANG II" by the conventional method after Dowex extraction.[6] Blood samples were obtained from the cubital vein of 15 normal volunteers on an unrestricted diet. They were in supine position for 30 minutes prior to the sampling. Six among them received 40 mg furosemide per os. Blood was collected with the subjects in supine position prior to and 90 minutes after furosemide with the subjects in the upright position. In 7 of the normal volunteers, plasma angiotensin II was measured pre- and post-converting enzyme inhibition (Hoechst 498, 10 and 20 mg orally). Venous blood samples were obtained when the subjects had been in supine position for 30 minutes for control values and at the time of peak converting enzyme inhibition, i.e. 1–2 h post drug.[8]

RESULTS

Antiserum

The sensitivity and specificity of the antiserum are demonstrated in Figure 1: At a dilution of 1:60,000, the antiserum bound 39% of 1 fmol ^{125}I-ANG II. Ten fmol ANG II displaced half of this tracer amount (coefficient of variation: 4.6%, $n=10$). The theoretical mean sensitivity of the antiserum was 0.03 ± 0.02 fmol (M $\pm$ S.D.) in 10 RIA. Cross-reaction with various angiotensin peptides were found to be: ANG-(1-8)octapeptide 100%, ANG-(2-8)heptapeptide and ANG-(3-8)hexapeptide 53%, ANG-(4-8)pentapeptide 52%, ANG-(2-10)nonapeptide 0.4% and ANG-(1-10)decapeptide 0.1%.

Extraction of angiotensin

Recovery of ^{125}I-ANG II from plasma was $99 \pm 2\%$ (mean $\pm$ S.D.). No ANG II was detected by RIA when duplicate samples of water or buffer were extracted. With inhibitor solution present, RIA after

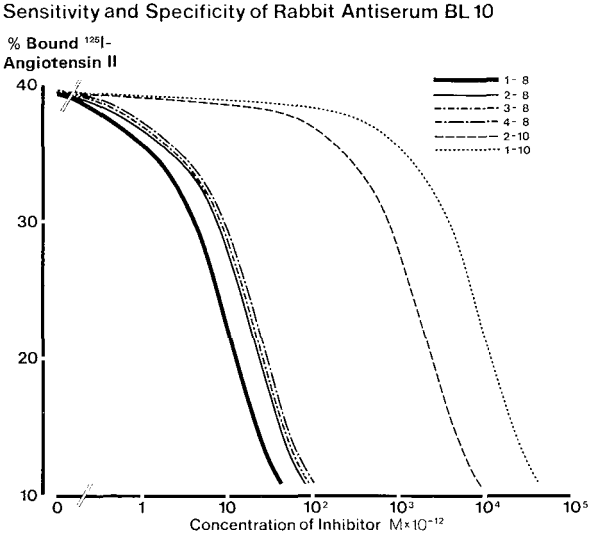

FIGURE 1 Sensitivity of rabbit antiserum directed against angiotensin-(1-8)octapeptide which was carboxyterminal coupled to bovine serum albumin. Significant cross-reaction with angiotensin-(2-8)heptapeptide [53%], (3-8)hexapeptide [53%] and (4-8)pentapeptide [52%] necessitates HPLC-separation for the specific measurement of (1-8)octapeptide.

extraction alone without HPLC measured in water 1.0 fmol/ml (duplicate: 1.0) immunoreactive "ANG II" and in buffer 1.1 fmol/ml (1.3).

Assay criteria

Since 2 ml plasma were extracted, but only 100 out of 140 μl extract solution were analyzed by HPLC, the amount ANG-(1-8)octapeptide found after HPLC had to be multiplied by a factor of 0.7 to obtain fmol/ml concentrations. The *recovery* of 5, 10 and 20 fmol ANG II added to plasma was $80 \pm 10\%$. No correction was made for recovery losses. For routine purposes, the lowest point of the standard curve used was 0.16 fmol ANG II. Four fractions (defining the octapeptide-peak) containing less than 0.16 fmol ANG II provided an assay *detection limit* of $0.64 \times 0.7 = 0.45$ fmol/ml in plasma. The *specificity* of the assay is illustrated in Figure 2: since "giant" peaks of 100 ng

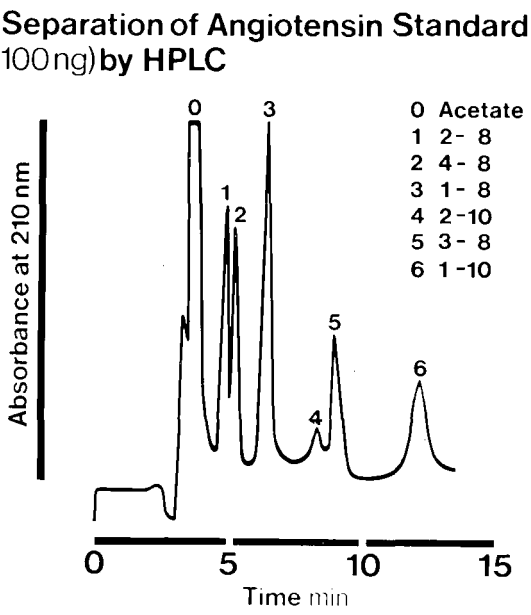

FIGURE 2 Base-line resolution of 100 ng standard angiotensin(1-8)octapeptide (peak 3) from other angiotensin-peptides by isocratic HPLC. Due to high reproducibility of retention times, (1-8)octapeptide in femtogram quantities is recovered in fractions corresponding to peak 3.

amounts of other angiotensins were well separated from ANG-(1-8)octapeptide, it was obvious that the few femtomoles of a plasma sample would not overlap. A reextracted angiotensin-free plasma (plasma blank) and HPLC-runs with acetic acid alone (buffer blank) showed no detectable ANG-(1-8)octapeptide. A *within-assay precision* with a coefficient of variation of 6.2% ($n=8$) and a *between-assay* coefficient of variation of 12.5% ($n=8$) was found.

Human studies

Plasma ANG-(1-8)octapeptide concentration in 15 normal volunteers in supine position was found to be 4.1 ± 1.6 fmol/ml. Furosemide and orthostasis increased plasma ANG-(1-8)octapeptide levels in 6 normal volunteers from 3.7 ± 1.8 fmol/ml in supine position to 22.4 ± 7.6 fmol/ml (Figure 3). ANG-(1-8)octapeptide in plasma of 7

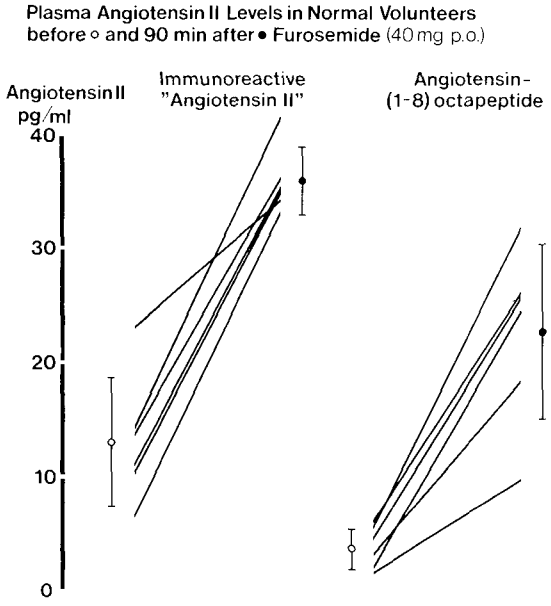

FIGURE 3 Plasma angiotensin II levels in normal human subjects before (○) and 90 minutes after (●) furosemide (40 mg orally) measured by radioimmunoassay after conventional Dowex ion exchange extraction (immunoreactive "angiotensin II") and after extraction on bonded-phase silica followed by reverse-phase HPLC (angiotensin-(1-8)octapeptide): immunoreactive "angiotensin II" increased 2.7 fold, angiotensin-(1-8)octapeptide 6.1 fold.

normal volunteers decreased from 5.2 ± 1.2 to undetectable levels following converting enzyme inhibition (Figure 4). The corresponding plasma concentration for immunoreactive "ANG II" as determined by the Dowex method were 11.3 ± 4.3 for the supine position, an increase from 13.1 ± 5.5 to 35.8 ± 2.9 fmol/ml after orthostasis/furosemide stimulation and a decrease from 9.2 ± 3.0 to 5.0 ± 1.1 fmol/ml after converting enzyme inhibition.

DISCUSSION

The goal of the present work was to develop a new method for the measurement of plasma angiotensin II which is specific for the (1-8)octapeptide. So far no antiserum has been described which is

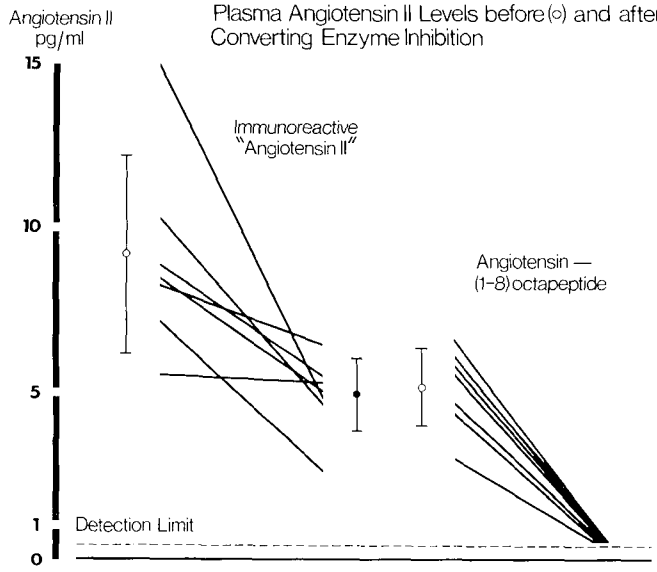

FIGURE 4 Plasma angiotensin II levels in normal human subjects before (○) and after (●) converting enzyme inhibition measured by radioimmunoassay after Dowex ion exchange extraction (immunoreactive "angiotensin II") and after extraction on bonded-phase silica followed by reverse-phase HPLC (antiotensin-(1-8)octapeptide): at peak converting enzyme inhibition, immunoreactive "angiotensin II" has decreased by only 46%; in contrast, angiotensin-(1-8)octapeptide was undetectable.

simultaneously specific for the amino- and the carboxy-terminal of the octapeptide angiotensin II. The problem was solved by a three step procedure: a high yield extraction on bonded-phase silica; a blind separation of the various angiotensin peptides by high performance liquid chromatography; and finally a direct linkage to a very sensitive radioimmunoassay. This linkage between the HPLC procedure and radioimmunoassay has become possible by using an isocratic separation system, a prerequisite to avoid evaporation procedures and/or blank problems in the radioimmunoassay. The features of this method are its specificity for the (1-8)octapeptide, and the high sensitivity made possible by the virtual absence of a blank and also by the high overall recovery. The combination of high performance liquid chromatography with radioimmunoassay overcomes the limitations of the two methods taken individually: HPLC provides a high specificity but the limitations in sensitivity of

the detector systems available so far make the measurement of a few femtomoles or even attomoles impossible, while the radioimmunoassay is characterized by a high sensitivity but insufficient specificity of the (1-8)octapeptide. The need for an accurate measurement of very low plasma angiotensin II concentrations has become apparent with the introduction of converting enzyme inhibition and thus blockade of angiotensin II generation to the treatment of hypertension[9] and congestive heart failure.[10] Indeed, angiotensin II determination in this situation has been hampered by what appeared to be "blank" problems, which presumably reflect also interference of crossreacting metabolic fragments and, at times, the precursor peptides like angiotensin I .[1,2,6,11,12]

Compared to previously described peptide extraction methods, such as ion exchange with Dowex resins,[1,11] Fuller's earth,[13] ethanol with[14] and without[15] chromatography, ultrafiltration[16] and glass bead (silica) adsorption,[17] the presently used bonded-phase silica reversible adsorption provides the advantages of practically complete recovery of angiotensin II from plasma and of extreme simplicity. Nevertheless, it has the disadvantage of higher cost of the material which, however, is partly offset by a gain in time. A prerequisite for the use of HPLC in this blind fashion, where retention times are established previously with standards but the samples are collected without any other reference than time, is an extremely reproducible retention time. In fact, we have injected more than 250 samples into the same system with a variation in retention time of less than 12 seconds. In addition, in our hands, gradient elution systems[18,19] were hampered by solvent residue blanks after reconcentration that could hardly be corrected for, and the changing composition of the mobile phase prevented a peak fractionation without another evaporation step. The unusually high octapeptide-sensitivity of the antiserum rendered the direct linkage of HPLC to radioimmunoassay possible, since the eluted peak could even be diluted rather than concentrated prior to the radioimmunoassay. Quantitative collection into coating-buffer containing tubes prevented any post-column recovery losses. Tests with several C-18 columns revealed the relative importance of pH and temperature resistance of the packing material as well as of suitable filter-frits. Baseline-resolution, especially in the femtomolar range, allows the simultaneous specific measurement of other angiotensins in the same plasma; for the significantly crossreacting metabolites even the same

antiserum can be used.[20] Needless to say, that this principle might be helpful for the measurement of other closely related peptides in plasma.

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