True Versus Immunoreactive Angiotensin II in Human Plasma

JURG NUSSBERGER, DORETTE B BRUNNER, BERNARD WAEBER, AND HANS R. BRUNNER

SUMMARY To measure specifically angiotensin-(1-8)octapeptide, peptides were extracted from 2 ml of plasma by reversible adsorption to bonded-phase silica. The angiotensin-(1-8)octapeptide was then isolated by isocratic reversed-phase high-performance liquid chromatography and quantified by radioimmunoassay. The extraction recovery of $^{125}$I-angiotensin II added to 2 ml of plasma was 99 ± 2% (mean ± SD). The overall recovery of 5, 10, and 20 fmol unlabeled angiotensin II added to 1 ml of plasma was 80 ± 10%. The coefficient of variation for within-assay precision was 0.06 and for between-assay precision 0.13. The detection limit was 0.4 fmol/ml. Buffer and plasma blanks were below the detection limit. Normal subjects on a free diet in supine position averaged 4.2 ± 1.7 fmol/ml angiotensin-(1-8)octapeptide. Furosemide (40 mg p.o.) and standing increased these values to 22 ± 7.6 fmol/ml. In four volunteers, immunoreactive "angiotensin II" (more or less angiotensinlike material) was measured serially before and after converting-enzyme inhibition (Hoe 498) with conventional Dowex extraction. At peak inhibition, plasma immunoreactive "angiotensin II" levels decreased by only 44%. In contrast, angiotensin-(1-8)-octapeptide isolated by high-performance liquid chromatography completely disappeared. In hypertensive patients receiving long-term treatment with enalapril, plasma levels of angiotensin-(1-8)octapeptide fell from 2.7 ± 0.9 to 0.9 ± 0.3 fmol/ml (mean ± SEM) 2 hours after the morning dose, whereas levels of immunoreactive "angiotensin II" were not significantly changed. We found that this sensitive method specifically measured angiotensin-(1-8)octapeptide and demonstrated that true angiotensin II virtually disappears during converting-enzyme inhibition. (Hypertension 7 [Suppl I]: I-1-I-7, 1985)

KEY WORDS • peptide extraction • reversed-phase high-performance liquid chromatography • radioimmunoassay • cross-reactivity of angiotensin II antisera • angiotensin fragments • sensitivity • converting-enzyme inhibition

THE need for an accurate measurement of very low plasma angiotensin II (ANG II) levels has become apparent because of the introduction of new therapeutic means to treat hypertension and cardiac failure (i.e., drugs that block the renin-angiotensin system by inhibiting the conversion of angiotensin I [ANG I] to ANG II). The measurement of low plasma ANG II levels is hampered by several technical difficulties.1-3 During converting-enzyme inhibition, currently used radioimmunoassays measure reduced but clearly not zero plasma levels of immunoreactive material (herein called "ANG II") that might be more or less angiotensinlike, since antisera do not sufficiently discriminate between angiotensin (ANG)-(1-8)octapeptide and its metabolites, and since extraction procedures or plasma proteins may produce additional pseudo-ANG II. Therefore, we devised a new method for the specific determination of ANG-(1-8)octapeptide that is based on a fast and practically blank-free extraction technique with virtually no recovery losses, a complete isolation of ANG-(1-8)octapeptide by high-performance liquid chromatography (HPLC), and a subsequent very sensitive radioimmunoassay. We used this method to compare plasma levels of ANG-(1-8)octapeptide and immunoreactive "ANG II" in normotensive and hypertensive subjects after furosemide stimulation and during converting-enzyme inhibition.

Materials and Methods
Immunization
A New Zealand White rabbit was immunized with ANG II that had been unidirectionally coupled to bovine serum albumin. Amino-terminal protected (N-α-trifluoracetyl-p-nitrobenzyl-Asp)'-ANG II was synthesized and coupled to the carrier protein by the carbodiimide method as described elsewhere.4 Deprotection was effected by alkaline treatment followed by
dialysis. Amino acid analysis showed a peptide-to-carrier ratio of 5. Two mg of conjugate was suspended in 0.5 ml of saline, emulsified with 0.5 ml of Freund's complete adjuvant, and injected intracutaneously. An intradermal booster injection of 0.5 mg of antigen was given 8 weeks after the primary immunization. Serum obtained in 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, Boston, MA)

Blood Sampling

Ten milliliters of blood was collected into prechilled glass tubes containing 0.5 ml of inhibitor solution (2% ethanol, 0.025 M phenanthroline, 0.125 M Na₂EDTA, 2 g/L neomycin). The blood was immediately chilled in ice and centrifuged at 4°C. The plasma was stored at -20°C until analyzed. Figure 1 shows the analytical procedure for measurement of ANG-(1-8)octapeptide.

Extraction

For each plasma sample, one cartridge (Bond Elut, Analytichem, Harbor City, CA) containing 100 mg of phenylsilyl-silica was inserted into a vacuum device (Vac Elut, Analytichem). The column was conditioned with 1 ml of methanol and then with 1 ml of water. Subsequently, 2 ml of cold plasma was rapidly passed through the cartridge followed by a wash with 1 ml of water. Adsorbed angiotensins were then eluted with 0.5 ml of methanol into conical polypropylene tubes coated with albumin buffer (0.1 M Tris buffer, pH 7.5, 5 g/L bovine plasma albumin, 0.2 g/L Na₃). The methanol was evaporated under air stream in a 40°C water bath.

Chromatography

The extraction residue was redissolved in 140 μL of 0.1 M acetic acid, and 100 μL of this solution was submitted to isocratic reversed-phase HPLC; a liquid chromatograph was used (Model HP-1090A, Hewlett Packard, Waldbronn, Germany) with a Rheodyne 7010 manual injection valve. The system was completed by an electronic fraction collector (Model 202, Gilson, Villiers Le Bel, France). A 250 × 4.6-mm column containing octadecasylsilica (Nucleosil-10, Innovativ-Labor, Adliswil, Switzerland) was used together with a methanol/0.085% phosphoric acid (33.5:66.5) mobile phase, thus applying a modification of the method proposed by Hearn et al. The glass fiber filters of the column had been replaced by steel sieves. The column compartment was kept at 45°C. The flow was 1 ml/minute. At the corresponding retention time (established previously with 100 ng of standard ANG II), ANG-(1-8)octapeptide was collected in 10 fractions of 105 μL directly into 0.5 ml of albumin buffer. All fractions were subsequently assayed individually. As an alternative, pooling of two fractions is possible without compromising the sensitivity of the system.

Radioimmunoassay

The ¹²⁵I-ANG II (2000 counts per minute [cpm] or 0.6 fmol; New England Nuclear, Boston, MA) in 0.05 ml of buffer and 0.5 ml of antiserum (1:26,000 diluted in buffer) was added to the collected fractions. For standards, increasing amounts of ANG II (0.16–20 fmol; Peninsula Labs, San Carlos, CA) were diluted in 0.5 ml of albumin buffer and 105 μL of mobile phase; antiserum and label were added as to the unknown samples. A 2-day incubation at 4°C was terminated by adding 0.3 ml of charcoal-dextran suspension (2% in water) followed by centrifugation and separation of bound and free hormone by decanting. Radioactivity was measured in a gammacounter to a counting error of less than 1% (±10 minutes). A standard curve was plotted relating the percent of bound tracer ANG II versus unlabeled added hormone. The hormone concentration of the HPLC fractions was read from this standard curve.

Other Assays

Immunoreactive "ANG II" was measured by the Dowex extraction method, and ANG I was measured by the ethanol extraction procedure, as previously described in detail. Plasma renin activity was estimated with the use of ANG I trapping antibodies, as proposed by Poulsen and Joergensen. Converting-enzyme activity was determined with a commercially available kit (Ventrex Corporation, Portland, ME). Aldosterone was measured by a direct microradioimmunoassay.
Human Studies
For the measurement of ANG II and immunoreactive "ANG II," blood samples were obtained from the cubital vein of 25 normal volunteers on an unrestricted diet. They were in supine position for 30 minutes before sampling. Six of the volunteers then received 40 mg of oral furosemide and had another blood sample taken 90 minutes later while in the upright position. Seven of the volunteers received a single oral dose of 10 or 20 mg of the converting-enzyme inhibitor Hoe 498 (Hoechst AG, Frankfurt, Germany), and a second blood sample was analyzed at the time of peak converting-enzyme inhibition (i.e., 1 or 2 hours after administration of the drug).

Four normal volunteers took 10 or 20 mg of Hoe 498. Blood samples were analyzed 0, 1, 4, 8, 12, and 24 hours after ingestion for levels of ANG-(1-8)octapeptide (HPLC method), immunoreactive "ANG II" (Dowex method), plasma renin activity, ANG I levels, converting-enzyme activity and aldosterone levels. Blood pressure and heart rate were recorded at the same time.

Nine hypertensive patients who had been treated for 8 months to 4 years (mean, 17 months) with the converting-enzyme inhibitor enalapril were tested before and 2 hours after taking their morning dose of antihypertensive medication. Blood pressure and heart rate were recorded, and blood specimens were obtained to determine the parameters of the renin-angiotensin system. Sex, age, body weight, plasma creatinine levels, and medication of these patients are listed in Table 1. Statistical evaluation of the results was made with a Student's t test for paired data.

Results
Characteristics of the Method
At a dilution of 1:60,000, the antiserum binds 39% of 0.6 fmol 125I-ANG II (Figure 2); 10 fmol ANG II displaced half of this tracer amount in 10 assays (coefficient of variation 0.05). The quantity of peptide that significantly displaced tracer (2 SD from zero binding) was consistently smaller than 0.16 fmol. The specificity of the antiserum was calculated by the method of Abraham from standard curves with different angiotensins (Peninsula Labs, San Carlos, CA, and Senn Chemicals, Dielsdorf, Switzerland). Taking ANG-(1-8)octapeptide as 1.0, the cross-reactivities were:

Table 1: Characteristics of the Nine Hypertensive Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Plasma creatinine (mM)</th>
<th>Enalapril (mg/day)</th>
<th>Extent of treatment (mos)</th>
<th>Blood pressure (mm Hg)</th>
<th>Additional medication</th>
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<td>59</td>
<td>82</td>
<td>80</td>
<td>20</td>
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<td>132/89</td>
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<tr>
<td>2</td>
<td>F</td>
<td>48</td>
<td>51</td>
<td>60</td>
<td>5</td>
<td>16</td>
<td>172/104</td>
<td>162/108</td>
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<tr>
<td>3</td>
<td>M</td>
<td>70</td>
<td>85</td>
<td>180</td>
<td>10</td>
<td>21</td>
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</tr>
<tr>
<td>4</td>
<td>M</td>
<td>37</td>
<td>93</td>
<td>85</td>
<td>10</td>
<td>12</td>
<td>142/92</td>
<td>152/90</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>52</td>
<td>55</td>
<td>60</td>
<td>10</td>
<td>8</td>
<td>122/86</td>
<td>122/84</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>62</td>
<td>68</td>
<td>150</td>
<td>10</td>
<td>12</td>
<td>200/108</td>
<td>114/76</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>72</td>
<td>88</td>
<td>290</td>
<td>10</td>
<td>18</td>
<td>176/106</td>
<td>136/90</td>
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<tr>
<td>8</td>
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<td>60</td>
<td>97</td>
<td>105</td>
<td>10</td>
<td>12</td>
<td>188/98</td>
<td>188/114</td>
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<tr>
<td>9</td>
<td>F</td>
<td>48</td>
<td>42</td>
<td>75</td>
<td>10</td>
<td>10</td>
<td>142/92</td>
<td>132/90</td>
</tr>
</tbody>
</table>

Mean ± SDM: 56.4 ± 3.8  73.4 ± 6.7  120.6 ± 25.2  17.2 ± 3.8  158.9/7 ± 9.3  146.9/4 ± 8.4
ANG-(2-8)heptapeptide and ANG-(3-8)hexapeptide, 0.53; ANG-(4-8)pentapeptide, 0.52; ANG-(2-10)nonapeptide, 0.004; and ANG-(1-10)decapeptide, 0.001.

A total of 99 ± 2% (mean ± sd; n = 8) of 125I-ANG II (6000 cpm) added to 2-ml aliquots of plasma containing inhibitor was recovered after passage over the Bond Elut cartridges and elution with 0.5 ml of methanol. The overall recovery was tested by adding 5, 10, and 20 fmol ANG II to 2-ml aliquots of the plasma containing inhibitors. The aliquots were then extracted and processed by HPLC as unknown samples, and endogenous ANG-(1-8)octapeptide was subtracted (n = 12). The overall recovery was 80 ± 10%.

Two milliliters of plasma was extracted, and only 100 of 140 μL of extract solution was injected for HPLC. Therefore, the sum of recovered ANG-(1-8)octapeptide after HPLC had to be multiplied by a factor of 0.7 to obtain the femtomoles per milliliter concentrations in plasma. No further correction was made for recovery losses. A minimum of four HPLC fractions defined a small octapeptide peak. As the lowest point of the standard curve was at 0.16 fmol ANG II and 2 ml of plasma was extracted, an assay detection limit of 4 × 0.16 × 0.7 = 0.45 fmol/ml in plasma was established.

The specificity of the assay is demonstrated by the chromatogram of Figure 3. Ultraviolet detectable peaks (0.1 nmol) of different angiotensins are well separated from ANG-(1-8)octapeptide. Obviously, the few invisible femtomoles of a plasma sample can easily be separated. Thus, the addition of 25 fmol ANG-(2-8)heptapeptide, ANG-(3-8)hexapeptide, and ANG-(4-8)pentapeptide left the plasma level of ANG-(1-8)octapeptide unchanged.

The coefficient of variation (0.06) for within-assay precision was established by running in the same radioimmunoassay eight aliquots of a single plasma sample containing 5.7 fmol/ml ANG-(1-8)octapeptide. The coefficient of variation (0.13) for between-assay precision was determined by measuring the ANG-(1-8)octapeptide content of the same plasma on 8 different days.

Without HPLC, no immunoreactive "ANG II" was found when water or buffer was extracted with Bond Elut instead of Dowex; with inhibitors present, water and buffer yielded "ANG II" levels of 1.0 fmol/ml (duplicate 1.0) and 1.1 fmol/ml (1.3) respectively. With HPLC, a reextracted ANG-free plasma (plasma blank) and HPLC runs with acetic acid alone (buffer blank) showed no detectable ANG-(1-8)octapeptide.

Studies in Normal Volunteers

In 25 supine subjects, plasma ANG-(1-8)octapeptide concentration was found to be 4.2 ± 1.7 fmol/ml (mean ± standard deviation) and the plasma level of immunoreactive "ANG II" was 10.1 ± 3.8 fmol/ml Plasma ANG-(1-8)octapeptide levels increased in 6 normal volunteers following furosemide administration and orthostasis from 3.7 ± 0.7 in supine position to 22.4 ± 3.1 fmol/ml (mean ± standard error); the corresponding values for immunoreactive "ANG II" were 13.1 ± 2.2 and 35.8 ± 1.2 fmol/ml.

After converting-enzyme inhibition, ANG-(1-8)octapeptide in plasma of 7 normal volunteers decreased from 5.2 ± 1.2 fmol/ml to undetectable levels (Figure 4). The corresponding plasma concentration for immunoreactive "ANG II" as determined by the Dowex method decreased from 9.2 ± 3.0 fmol/ml to 5.0 ± 1.1 fmol/ml.

Figure 5 shows the biochemical profiles of the renin-angiotensin-aldosterone system before and after converting-enzyme inhibition with HAE 498. While all ANG-(1-8)octapeptide disappeared from plasma after 1 hour (from 3.8 ± 1.0 to < 0.4 fmol/ml), immunoreactive "ANG II" levels only decreased to 5.5 ± 0.4 from 9.9 ± 2.0 fmol/ml (44% reduction) Maximal plasma renin activity and ANG I concentrations were reached after 8 hours when plasma converting-enzyme activity was still extremely low. At that time, ANG-(1-8)octapeptide gradually reappeared. The reduced levels of immunoreactive "ANG II" and aldosterone remained virtually unchanged up to 12 hours and then returned to the initial values. Blood pressure and heart rate were not affected by the converting-enzyme inhibition.

Studies in Hypertensive Patients

The effect of the morning dose on levels of immunoreactive "ANG II" and ANG-(1-8)octapeptide and converting-enzyme activity in plasma of nine hyper-
Discussion

Methods for the radioimmunological estimation of plasma ANG II levels usually have been based on antisera of limited specificity for the amino terminal of this octapeptide. In addition, nonspecific interference caused by plasma proteins or extraction procedures as well as sometimes insufficient sensitivity of the antisera has prevented an accurate measurement of very low ANG II concentrations. Such low ANG II levels are, however, found in plasma of patients treated with inhibitors that interfere with the synthesis of ANG II.

The method presented here overcomes the problems of specificity and sensitivity with a three-step procedure: a high yield extraction on bonded-phase silica, a timed separation of the various ANG peptides by HPLC, and a direct linkage to a very sensitive radioimmunoassay. This linkage between the HPLC procedure and radioimmunoassay has become possible by
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using an isocratic separation system, a prerequisite to avoiding evaporation procedures and/or blank problems in the radioimmunoassay. The outstanding feature of this method is its specificity for the ANG-(1-8)octapeptide with no interference of either ANG I or the smaller breakdown products. In addition, the method is extremely sensitive because of the quality of the antiserum, the virtual absence of a blank, and the good overall recovery of ANG-(1-8)octapeptide.

Compared to previously described peptide extraction methods, such as ion exchange with Dowex resins, Fuller's earth, ethanol with and without chromatography, ultrafiltration, and glass bead (silica) absorption, the reversible adsorption of the presently used bonded-phase silica provides the advantages of practically complete recovery of ANG II from plasma and of extreme simplicity. In several hundred extractions, three different batches of Bond Elut cartridges as well as the bulk material (Separylate-PH, AnalytiChem, Harbor City, CA) proved to be reliable.

The combination of HPLC 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; the radioimmunoassay is characterized by a high sensitivity but insufficient specificity for the (1-8)octapeptide. A prerequisite for the use of HPLC in this blind fashion, where retention times have been established previously with standards but the samples are collected without any reference other 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. One out of five Nucleosil columns had to be rejected because of obviously bad packing, but otherwise remarkably constant retention times were found even when columns of different filling batches were compared. In addition, in our hands, gradient separation systems were hampered after reconcentration by solvent residue blanks that could hardly be corrected for, and the changing composition of the mobile phase made impossible a peak fractionation without another evaporation step leading to new recovery losses. 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 before the radioimmunoassay. Quantitative collection into tubes containing coating buffer 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 cross-reacting metabolites even the same antiserum can be used. Needless to say, this principle might be helpful for the measurement of other closely related peptides in plasma.

With this new method, plasma ANG-(1-8)octapeptide levels of normal volunteers were found to be only about 40% of those measured simultaneously as immunoreactive "ANG II." Presumably, the difference reflects in part the presence of other ANG peptides. Furosemide and orthostasis increased the plasma levels of ANG-(1-8)octapeptide approximately sixfold, whereas immunoreactive "ANG II" levels increased only 2.7-fold. Conversely, when the converting-enzyme inhibitor Hoe 498 was administered to normal volunteers, plasma ANG-(1-8)octapeptide levels fell by more than 88% to unmeasurably low levels while immunoreactive "ANG II" levels decreased by only 44%. The greater changes observed with the new method provide some indirect evidence that it measures true physiologically active ANG II rather than a mixture of ANG II breakdown products with some physiologically irrelevant interfering material.

In the hypertensive patients treated for at least 8 months with the converting-enzyme inhibitor enalapril, average ANG-(1-8)octapeptide levels before the morning dose were already below 3 fmol/ml, which reflects persistent, though not complete, inhibition of converting-enzyme activity. The administration of enalapril further reduced these low plasma ANG-(1-8)octapeptide concentrations by 66% to less than 1 fmol/ml. In contrast, the immunoreactive "ANG II" levels decreased by a mere 17% 2 hours after the morning dose of enalapril. Again, the ANG-(1-8)octapeptide measurement better reflected the expected theoretical level following inhibition of converting-enzyme activity (i.e., virtual disappearance of ANG II from the circulation).

In conclusion, ANG II can be extracted from plasma on bonded-phase silica and measured specifically in a sensitive radioimmunoassay after isolation by HPLC. Measurements obtained so far following stimulation by furosemide or converting-enzyme inhibition in normal volunteers or hypertensive patients strongly suggest that this new method provides results that are physiologically more meaningful than those obtained with less specific methods.

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