Specific Measurement of Angiotensin Metabolites and In Vitro Generated Angiotensin II in Plasma

JÜRGEN NUSMBERG, DORETTE B. BRUNNER, BERNARD WAEBER, AND HANS R. BRUNNER

SUMMARY Combining high-performance liquid chromatography with radioimmunoassay enabled the precise measurement of different angiotensins and their metabolites in plasma. Peptides were extracted from 2 ml of plasma by reversible adsorption to phenylsilyl-silica, separated by isocratic high-performance liquid chromatography, and quantitated by radioimmunoassay using a sensitive but suitably cross-reacting angiotensin II antiserum. For the C-terminal angiotensin II metabolites (2-8)heptapeptide, (3-8)hexapeptide, and (4-8)pentapeptide, overall recoveries of 10 fmol peptide added to 1 ml of plasma were (mean ± SD), 74 ± 6, 68 ± 8, and 67 ± 11%, respectively. The detection limit for these peptides in plasma was 0.2 fmol/ml. Blanks were below the detection limits. In eight seated normal subjects treated for 4 days with enalapril, 20 mg p.o., q.d., angiotensin II metabolites tended to decrease during the 4 postdrug hours. However, their cumulated concentration in relation to octapeptide increased from 54 to 163% on Day 1 and from 62 to 103% on Day 4. After 4 hours of converting enzyme inhibition with enalapril there was still a close correlation between plasma renin activity and angiotensin-(1-8)octapeptide level (r = 0.83, p<0.05) and between blood angiotensin I and angiotensin-(1-8)octapeptide levels (r = 0.86, p<0.01). Adding angiotensin I in vitro raised the angiotensin-(1-8)octapeptide levels after incubation at 4°C for 4 hours. Thus, immunoreactive “angiotensin II” does not disappear after converting enzyme inhibition largely because of the cumulated contribution of cross-reacting metabolites and partly because of in vitro generation of true angiotensin II. (Hypertension 8: 476–482, 1986)

KEY WORDS • angiotensin I • angiotensin II • angiotensin III • angiotensin metabolites • peptide extraction • high-performance liquid chromatography • enalapril-induced converting enzyme inhibition • angiotensin antisera cross-reactivity • bonded-phase silica
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(Bondelut-PH, Analytichem, Harbor City, CA, USA), and plasma ANG-(1–8)octapeptide was measured after additional separation from other extracted angiotensins by isocratic reversed-phase HPLC. As shown in Figure 1, the same chromatographic procedure was used to separate plasma ANG-(4–8)pentapeptide, ANG-(3–8)hexapeptide, ANG-(2–8)heptapeptide (Peninsula Laboratories, San Carlos, CA, USA), and ANG-(2–10)nonapeptide (Senn Chemicals, Dielsdorf, Switzerland). A liquid chromatograph was used (Model HP-1090A, Hewlett-Packard, Waldbronn, Germany) with a Rheodyne 7010 manual injection valve (Cotati, CA, USA). Octadecasilyl-silica (Nucleosil; 10 μm particle size) in a 250 × 4.6-mm steel column (Innovative-Labor, Adliswil, Switzerland) served as the stationary phase, and a constant mixture (isocratic) of methanol/0.085% phosphoric acid (33.5:66.5) was used as the mobile phase with a flow of 1 ml/min at 45°C. The glass fiber frits of the column were replaced with steel sieves to prevent peptide recovery losses. At the corresponding retention times (established previously with 100 ng of ultraviolet-detectable standard) each peptide was collected (Model 202 fraction collector, Gilson, Villiers Le Bel, France) in 105-μl fractions directly into 0.5 ml RIA buffer (0.1 M tris(hydroxymethyl)aminomethane buffer [Tris], pH 7.5, 5 g/L bovine serum albumin). The same mixture of 125I-labeled ANG II (0.6 fmol; New England Nuclear, Boston, MA, USA) and antiserum was added to each fraction. The RIA was performed as previously described in detail using dextran-coated charcoal to separate free from antibody-bound hormone after a 2-day incubation at 4°C. The different peptides were quantitated considering their relative cross-reactions with the antiserum. Cross-reactivity of ANG-(1–8)octapeptide was taken as 1.0, and the cross-reactivities were 0.53 for ANG-(2–8)heptapeptide and ANG-(3–8)hexapeptide, 0.52 for ANG-(4–8)pentapeptide, 0.004 for ANG-(2–10)nonapeptide, and 0.001 for ANG-(1–10)decapeptide. Standard curves for the different ANG peptides have been published previously.

The RIA sensitivity for ANG-(1–8)octapeptide was found to be below 0.16 fmol/fraction. The RIA sensitivity for each peptide was calculated by dividing 0.16 by the relative cross-reaction for the given peptide compared with ANG II. Since some of the peaks are contained in a single fraction, the lower limit of detection depends on the minimal amount of peptide detectable in a single fraction. Only 100 μl of the 140 μl of extract solution (in 0.1 M acetic acid) of 2 ml plasma was analyzed by HPLC. The assay detection limits for plasma peptides were therefore established using the following formula: 0.16 × 1/c × 140/100 × 1/2 fmol/ml plasma.

Recoveries were tested by adding individually 10 fmol/ml of each peptide (1250 fmol/ml nonapeptide) to plasma pools and by measuring the corresponding peptide concentration. Endogenous levels of immunoreactive material were subtracted. No correction was made for recoveries except to compare experiments and compare where the relative contribution of each peptide to immunoreactive "ANG II" was determined (see Figures 2 and 3). To exclude cross-contamination and solvent blanks, acetic acid (0.1 M) was analyzed by HPLC and subsequent RIA. Acetic acid was chosen because the evaporated plasma extract was redissolved in this solvent. Blood ANG I levels were measured by the ethanol extraction procedure, as previously described in detail.

Figure 1. Separation of 100 ng of standard angiotensin (ANG) peptides by isocratic reversed-phase high-performance liquid chromatography (upper panel). High reproducibility of retention times enables the recovery of peptides in femtogram quantities from plasma extract in fractions corresponding to peptide peaks, as illustrated here for the ANG-(1–8)octapeptide (lower panel). Stationary phase: Nucleosil C-18, 10 μm, 250 × 4.6 mm. Mobile phase: methanol/phosphoric acid 0.085% (33.5:66.5). Ultraviolet detector setting: 0.008 absorbance units per full scale. (This figure originally appeared with erroneously transposed panels in Nussberger et al.)
Other Assays and Human Studies

Plasma renin activity was estimated with the use of ANG I trapping antibodies, as proposed by Poulsen and Joergensen.13 Plasma converting enzyme activity was determined with a commercially available kit (Ventrex, Portland, ME, USA).14 Plasma aldosterone was measured by direct RIA.13

Eight normal male volunteers (20–27 years old) on an unrestricted diet were given a single daily oral dose of 20 mg of enalapril for 4 days. Procedures followed were in accordance with the guidelines of the hospital ethics committee. Before blood sampling, the subjects were seated in an armchair for 30 minutes and blood pressure and heart rate were measured. Blood samples were collected from the cubital vein before and 4 hours after enalapril intake on Days 1 and 4. For the measurement of the angiotensins, 10 ml of blood was collected into prechilled glass tubes containing 0.1 ml of 2.5 mM chymostatin in dimethylsulfoxide (Sigma Chemical, St Louis, MO, USA), as well as 0.5 ml of 25 mM 1,10-phenanthroline (Sigma), 125 mM Na2 ethylenediaminetetraacetic acid (EDTA), and neomycin (2 g/L) in 2% ethanol. The blood was immediately chilled in ice and centrifuged at 4°C. The plasma was stored at -70°C until analyzed.

Statistical Evaluation

Data are reported as means ± SEM unless stated otherwise. Values below the assay detection limit were taken as if they were at the detection limit. Statistical evaluation of the results was performed using a one-way analysis of variance or, where appropriate, a Student's t test for paired data. Correlation coefficients were calculated by the method of least squares. Significance was set at a p level of less than 0.05.

Table 1 shows the assay detection limits and the overall recoveries for peptides added to a plasma pool. While 100 to 200 amol of ANG II and its metabolites can be detected in a single assay tube, the detection limit for the ANG-(2-10)nonapeptide is substantially higher due to a cross-reactivity that is several orders of magnitude smaller. This nonapeptide also showed a small (34%) though constant recovery after HPLC. Mean recoveries for the other ANG peptides were between 67 and 80%. Solvent blanks were below the RIA detection limit in all fractions corresponding to collected peaks.

Table 2 shows the biochemical data, blood pressure, and heart rate in normal volunteers before and 4 hours after the morning dose of 20 mg of enalapril on the first and the fourth day of medication. Plasma converting enzyme activity was suppressed (<1% of pretreatment value) 4 hours after the first as well as the last enalapril dose. Other assays and human studies showed that plasma renin activity was not affected by enalapril.

### Table 1. Assay Characteristics of the Specific Angiotensin Peptide Measurement

<table>
<thead>
<tr>
<th>Angiotensin peptide</th>
<th>Detection limit (fmol/ml)</th>
<th>Recovery (%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4-8)pentapeptide</td>
<td>0.22</td>
<td>67 ± 11</td>
</tr>
<tr>
<td>(3-8)hexapeptide</td>
<td>0.21</td>
<td>68 ± 8</td>
</tr>
<tr>
<td>(2-8)heptapeptide</td>
<td>0.21</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>(1-8)octapeptide</td>
<td>0.11</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>(2-10)nonapeptide</td>
<td>28</td>
<td>34 ± 2</td>
</tr>
</tbody>
</table>

*Unlabeled standard peptides (10 fmol/ml, nonapeptide 1250 fmol/ml) were added to plasma, extracted on Bondelut, analyzed by high-performance liquid chromatography, and quantified by radioimmunoassay (n = 8; mean ± SD).

### Table 2. Biochemical Data, Blood Pressure and Heart Rate of Eight Normal Volunteers Treated with Enalapril (20 mg p.o., q.d.)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0 hr</th>
<th>4 hr</th>
<th>Day 1</th>
<th>4 hr</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Converting enzyme activity (nmol/ml/hr)</td>
<td>92.6 ± 3.9</td>
<td>0.6 ± 0.2*</td>
<td>12.3 ± 2.0*</td>
<td>0.3 ± 0.3*</td>
<td>0.12 ± 0.3*</td>
</tr>
<tr>
<td>Plasma renin activity (ng/ml/hr)</td>
<td>1.0 ± 0.2</td>
<td>7.1 ± 1.2*</td>
<td>4.9 ± 0.7</td>
<td>12.4 ± 1.5*</td>
<td>12.7 ± 1.2</td>
</tr>
<tr>
<td>Immunoreactive ANG I (fmol/ml)</td>
<td>23.5 ± 3.7</td>
<td>126.9 ± 32.3</td>
<td>62.4 ± 8.1</td>
<td>356.1 ± 43.9</td>
<td>12.7 ± 1.2</td>
</tr>
<tr>
<td>Immunoreactive ANG II (fmol/ml)</td>
<td>12.0 ± 1.6</td>
<td>6.1 ± 0.6*</td>
<td>7.2 ± 1.0</td>
<td>3.0 ± 0.5*</td>
<td>8.0 ± 0.8*</td>
</tr>
<tr>
<td>ANG-(1-8)octapeptide (fmol/ml)</td>
<td>7.3 ± 1.4</td>
<td>1.9 ± 0.3*</td>
<td>7.2 ± 1.0</td>
<td>3.0 ± 0.5*</td>
<td>8.0 ± 0.8*</td>
</tr>
<tr>
<td>ANG-(2-8)heptapeptide (fmol/ml)</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>ANG-(3-8)hexapeptide (fmol/ml)</td>
<td>1.0 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>1.4 ± 0.6</td>
<td>0.9 ± 0.2</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>ANG-(4-8)pentapeptide (fmol/ml)</td>
<td>1.2 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>ANG-(2-10)nonapeptide (fmol/ml)</td>
<td>38.0 ± 7.6</td>
<td>72.7 ± 21.8</td>
<td>30.0 ± 1.9</td>
<td>49.6 ± 12.7</td>
<td>30.0 ± 1.9</td>
</tr>
<tr>
<td>Aldosterone (pg/ml)</td>
<td>89.8 ± 7.2</td>
<td>42.1 ± 4.3*</td>
<td>86.0 ± 9.5</td>
<td>40.0 ± 4.8*</td>
<td>40.0 ± 4.8*</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>114.5 ± 4.1</td>
<td>105.5 ± 4.7</td>
<td>109.0 ± 4.3</td>
<td>100.0 ± 3.5</td>
<td>100.0 ± 3.5</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>77.9 ± 3.1</td>
<td>70.8 ± 3.3</td>
<td>75.8 ± 3.0</td>
<td>67.0 ± 3.0</td>
<td>67.0 ± 3.0</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>66.5 ± 2.7</td>
<td>73.5 ± 5.2</td>
<td>69.5 ± 2.8</td>
<td>70.5 ± 3.7</td>
<td>70.5 ± 3.7</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Peptide concentrations are not corrected for recovery. 0 hours = immediately before treatment; 4 hours = 4 hours after treatment; ANG = angiotensin.

*p < 0.001; †p < 0.01, compared with Day 1 basal values; †p < 0.05; †p < 0.01, compared with Day 4 basal values.
intake; it had regained 13% of its initial value before
the administration of the final dose. Plasma renin ac-
tivity and blood ANG I level rose considerably 4 hours
post drug on Day 1 (7-fold and 5-fold increases, re-
spectively) and on Day 4 (2.5-fold and 5.7-fold in-
creases, respectively). Plasma renin activity did not
return to pretreatment values before the last dose but
was still elevated five-fold. Levels of immunoreactive
"ANG II" as well as ANG-(1-8)octapeptide were sup-
pressed 4 hours after enalapril treatment on Day 1 (by
49 and 74%) and Day 4 (by 37 and 58%, respectively); both were at pretreatment levels before adminis-
tration of the last dose. Levels of other ANG peptides did not change significantly, but the ANG-(2-10)nonapeptide had a tendency to increase and the smaller metabolites to decrease 4 hours after drug intake. Levels of ANG III were actually correlated with those of ANG II (r = 0.91, p<0.01) 4 hours after medication. Plasma aldosterone concentration followed the same pattern as ANG II: levels decreased 4 hours post drug on both
days and completely recovered within 24 hours after
the third day of medication. Blood pressure and heart
rate did not change significantly during this study.

Figure 2 illustrates the plasma ANG peptide levels
after correction for recovery. Blood ANG I levels were
also corrected for hematocrit to estimate plasma levels.

By taking into account the antiserum cross-reactiv-
ity with the different angiotensins, the contribution of
specifically measured ANG peptides to immunoreac-
tive "ANG II" could be calculated. The sum of all
these calculated immunoreactive ANG II-like materi-
als was compared with "ANG II" levels measured
without HPLC (Figure 3): the gap between calculated
and measured immunoreactive "ANG II" was at most
1.2 fmol/ml. The sum of recovery-corrected nonocta-
peptide immunoreactive "ANG II" before and after
medication was 2.5 and 3.0 fmol/ml on the first day
and 2.5 and 3.1 fmol/ml on the fourth day of treatment;

the corresponding subtotals stemming from ANG II
metabolites (pentapeptide + hexapeptide + hepta-
peptide) not including ANG I and nonapeptide were
1.8 and 1.4 fmol/ml and 1.8 and 1.3 fmol/ml, respec-
tively. In absolute terms, these three ANG II metabo-
lites (also recovery-corrected) taken together averaged
4.9, 3.9 (Day 1) and 5.6, 3.9 (Day 4) fmol/ml, respec-

Figure 2. Plasma angiotensin (ANG) peptide levels during
acute and sustained converting enzyme inhibition with enalapril
(20 mg p.o., q.d.) in eight normal volunteers. Peptide concen-
trations have been corrected for recovery (see Table 1). "1-10" rep-
resents plasma immunoreactive ANG I level and was calcu-
lated from blood ANG I level by correcting for hematocrit.
Significant (p<0.01) increase of ANG I and decrease of ANG II
concentrations occurred 4 hours post drug on Days 1 and 4 of
treatment.

Figure 3. Relative contribution of different angiotensin (ANG) peptides and metabolites to
total immunoreactive plasma "ANG II" level after acute and sustained converting enzyme
inhibition with enalapril (20 mg p.o., q.d.) in eight normal volunteers. The sum of individu-
ally measured ANG peptides parallels levels of immunoreactive "ANG II" as measured
after Bondelut-PH extraction alone.
The metabolite concentrations before and after medication were therefore 54 and 163% and 62 and 103%, respectively of the octapeptide concentrations on Day 1 and Day 4 of enalapril treatment, thus increasing threefold (Day 1) and 1.7-fold (Day 4) their ratio to true ANG II. Figure 4 illustrates a close correlation between true ANG II and immunoreactive "ANG II" levels ($r = 0.94$, $p < 0.001$).

A linear relationship was found between plasma renin activity and ANG-(1-8)octapeptide level before treatment ($r = 0.96$, $p < 0.001$; data not shown) as well as 4 hours after the first enalapril dose, when converting enzyme activity was suppressed by more than 99% to $0.6 \pm 0.2$ nmol/ml/min ($r = 0.83$, $p < 0.05$; Figure 5A). A similar relationship was found between levels of ANG I and ANG-(1-8)octapeptide 4 hours post drug on the first day ($r = 0.86$, $p < 0.01$; see Figure 5B) and the fourth day ($r = 0.79$, $p < 0.05$; data not shown) of treatment.

To investigate possible in vitro generation of ANG II in plasma containing EDTA and 1,10-phenanthroline, increasing amounts of ANG I (0, 1000, 2000, and 4000 fmol/ml) were added to a plasma pool and left for 4 hours at 4°C. Conversion of ANG I into ANG II in cold plasma containing 12.5 mM EDTA and 2.5 mM 1,10-phenanthroline could be demonstrated (Figure 6). The respective ANG-(1-8)octapeptide concentrations were 3.2, 3.5, 3.3, and 3.1 fmol/ml before incubation and 5.1, 5.3, 6.4, and 6.5 fmol/ml after 4 hours at 4°C. Similarly, when four different plasma pools with EDTA and 1,10-phenanthroline containing different amounts of the converting enzyme inhibitor enalapril or 1,10-phenanthroline, or both, were spiked with ANG I (0, 1000, 2000, and 4000 fmol/ml) the ANG-(1-8)octapeptide levels in immediately processed plasma remained unchanged at $4.3 \pm 0.8$, $4.0 \pm 0.5$, $4.4 \pm 0.7$, and $3.9 \pm 0.6$ fmol/ml, respectively ($n = 4$). After a 4-hour incubation at 4°C, however, octapeptide concentrations were increased to 4.6, 5.1, 9.6, and 13.1 fmol/ml in the ANG I-spiked pool samples containing 3.6 µM enalapril and to 6.9, 7.6, 13.3, and 19.5 fmol/ml in the ANG I-spiked pool samples containing only 0.8 µM enalapril but 5 mM 1,10-phenanthroline. In the samples containing 3.6 µM enalapril, ANG I levels (percent of expected concentration after spiking) were 42 (100%), 951 (91%), 1993 (98%), and 3686 (91%) fmol/ml in the ANG I-spiked pool samples containing only 0.8 µM enalapril but 5 mM 1,10-phenanthroline. In the samples containing 3.6 µM enalapril, ANG I levels (percent of expected concentration after spiking) were 42 (100%), 951 (91%), 1993 (98%), and 3686 (91%) fmol/ml in immediately processed plasma and 40 (95%), 977 (94%), 1671 (82%), and 3000 (74%) fmol/ml after 4 hours at 4°C.

To test whether such ANG II generation was blocked by chymostatin, we measured ANG II levels in plasma with and without 25 µM chymostatin, before and after converting enzyme blockade with enalapril.
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The results are summarized in Table 3 (n = 6). The addition of chymostatin to the inhibitor cocktail did not significantly affect the ANG II concentration. Levels of ANG II and its metabolites tended to increase rather than decrease with chymostatin.

In four different samples, plasma storage for 3 days at −70°C yielded more immunoreactive "ANG II" than peptide storage on Bondelut cartridges at 4°C or 25°C after immediate extraction (Table 4).

Discussion

The same chromatography that isolated ANG-(1–8)octapeptide separated the five other angiotensins tested.4 High sensitivity of the antiserum and substantial cross-reactivity with ANG II metabolites and, to a much smaller extent, with the ANG-(2–10)nonapeptide permitted the measurement of these peptides in 2-ml plasma extracts. Recoveries were quite constant. At least part of the recovery losses may have been caused by incomplete filling of the HPLC injection loop,16 since only about 125 μl was available to fill the 100-μl loop.

As expected, the 20-mg dose of enalapril markedly suppressed plasma converting enzyme activity. Levels of ANG I increased while levels of ANG II decreased, but 13% of initial converting enzyme activity was sufficient to return these levels to baseline 24 hours after the last dose. The ANG-(2–10)nonapeptide tended to behave like ANG I, whereas the ANG-(2–8)heptapeptide and the smaller fragments followed the pattern of ANG II. Baseline ANG-(1–8)octapeptide levels accounted for 76% of immunoreactive "ANG II." Others have found values between 65 and 100% in normal plasma17; however, they did not separate ANG I and nonapeptide from ANG II, nor did they individually determine the pentapeptide and hexapeptide levels. The individual contribution of cross-reacting ANG peptides and metabolites to immunoreactive "ANG II" was now determined specifically (see Table 3). The sum of these contributions accounted for most of the difference between immunoreactive "ANG II" and true ANG II — ANG-(1–8)octapeptide — thus confirming previous assumptions.4 A small difference still remains, possibly due to smaller ANG peptides that were not analyzed by HPLC. The ratio between smaller metabolites and ANG-(1–8)octapeptide increased up to threefold with converting enzyme inhibition; this finding is compatible with an alternative pathway of metabolite generation that bypasses ANG II.18

The excellent correlation of ANG-(1–8)octapeptide levels with immunoreactive "ANG II" levels measured after extraction on phenylsilyl-silica (Bondelut-PH) underscores the reliability of this simple and quick extraction method.4 Bondelut-PH extraction yielded all ANG peptides from plasma with little loss (data not shown), while HPLC recovery for the ANG-(1–8)octapeptide was approximately 80%. In Figure 4, the y axis intercept of 4.5 × 0.8 = 3.6 fmol/ml is close to the nonoctapeptide immunoreactive "ANG II," which was found to be 2.5 to 3.1 fmol/ml. As already pointed out, smaller ANG peptides, which were not quantitated after HPLC separation, could explain the remaining gap between calculated and measured immunoreactive "ANG II" (0.3–1.2 fmol/ml).

**Table 3. Angiotensin-(1–8)octapeptide in Plasma of Normal Volunteers With and Without 25 μM Chymostatin as Additional Inhibitor**

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Pre treatment</th>
<th>No chymostatin</th>
<th>Chymostatin</th>
<th>4 hr posttreatment*</th>
<th>No chymostatin</th>
<th>Chymostatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>6.0</td>
<td>7.0</td>
<td>0.7</td>
<td>0.7</td>
<td>1.7</td>
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<tr>
<td>2</td>
<td></td>
<td>5.1</td>
<td>5.8</td>
<td>0.4</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>6.4</td>
<td>8.2</td>
<td>2.9</td>
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<td>3.7</td>
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<tr>
<td>4</td>
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<td>12.8</td>
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<tr>
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<td>6.8</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>9.9</td>
<td>9.1</td>
<td>2.1</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.4±2.4</td>
<td>8.3±2.5</td>
<td>1.5±1.0</td>
<td>1.9±1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Posttreatment with enalapril, 20 mg p.o.

**Table 4. Storage Conditions and Plasma Concentrations of Immunoreactive Angiotensin II**

<table>
<thead>
<tr>
<th>Storage method before RIA</th>
<th>Plasma sample (fmol/ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, −70°C</td>
<td></td>
<td>10.6</td>
<td>5.6</td>
<td>11.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Bondelut, +4°C</td>
<td></td>
<td>7.9</td>
<td>3.7</td>
<td>7.2</td>
<td>8.7</td>
</tr>
<tr>
<td>Bondelut, +25°C</td>
<td></td>
<td>7.1</td>
<td>3.7</td>
<td></td>
<td>7.6</td>
</tr>
</tbody>
</table>

Storage for 64 hours. RIA = radioimmumassay.

*Not done, not enough plasma available.
During acute converting enzyme inhibition, plasma renin activity and ANG I levels were still linearly related to levels of ANG-(1-8)octapeptide. These findings suggest that ANG I is converted to ANG II even in the face of very low plasma converting enzyme activity. These results again raise the question whether converting enzyme inhibition in humans by compounds like captopril, enalapril, or lisinopril is complete.1,3 On the other hand, an alternative pathway18 from high concentrations of decapeptide- and nonapeptide ANG directly to heptapeptide and smaller fragments, remains a possibility, although converting enzyme activity on the nonapeptide substrate appears to be pharmacologically well inhibitable.18 Chymostatin has been reported to be able to block a serine protease of the chymotrypsin family that generates ANG II from ANG I.9 However, the present study showed no changes or rather higher concentrations of the specifically measured angiotensins when chymostatin was added to the inhibitor cocktail. Thus, degradation rather than generation of ANG II may have been blocked by chymostatin. Other enzymes, such as tovin7,8 or cathepsin G,10 also cannot be ruled out as ANG II generators.

We have also demonstrated the in vitro generation of ANG II from ANG I in cold plasma containing EDTA and 1,10-phenanthroline. This finding stresses the need for careful blood sampling and plasma storage when measuring ANG II concentrations. Freezing and thawing might create as well as destroy ANG II in plasma. Whether immediate extraction on bonded-phase silica provides a reliable means for long-term storage without freezing remains to be confirmed. Our findings cast some doubt on the appropriateness of the widely used inhibitor cocktail with EDTA and 1,10-phenanthroline. This finding stresses the findings cast some doubt on the appropriateness of the widely used inhibitor cocktail with EDTA and 1,10-phenanthroline when ANG II levels are to be measured. Cross-reaction with ANG I,20 appears to be less of a problem in the measurement of immunoreactive "ANG II" when antiserum with a thousandfold higher affinity for ANG II are used, than does in vitro generation of ANG II from ANG I when ANG I levels are high during converting enzyme inhibition. Thus, in vitro generation of ANG II may be the most likely explanation for the close correlation between levels of renin or ANG I and ANG-(1-8)octapeptide observed even when plasma converting enzyme was inhibited more than 99%. This would also explain why disappearance of ANG II from the circulation could only be demonstrated at acute peak inhibition at a time when renin and ANG I levels were not yet very high and after immediate processing of the samples.4 Indeed, during converting enzyme inhibition, speed of sample processing and quick freezing may become the single most important factor determining ANG-(1-8)octapeptide levels as long as the perfect in vitro inhibitor of ANG I-processing enzymes has not been identified.

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