SPECIFIC PRESSOR ACTIVITY AND STABILITY
OF SYNTHETIC ANGIOTENSINS

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Abstract—Specific pressor activity of Asp¹-Val⁵-angiotensins I and II was determined
by a 4-point assay in rats against various synthetic angiotensins. Specific pressor
activity of various angiotensins was also obtained from the dose-blood pressure-response
(DR) curve, using a single angiotensin sample per rat. Comparison of two values
showed that two angiotensins interacted in the 4-point assay, yielding a potentiation
factor of 0.465-1.373. Therefore, specific pressor activity from DR curve is more
reliable, because two angiotensins are not able to interact. Potency ratio on a molar
basis of Asp¹-Ile⁵-angiotensin I, Asp¹-Val⁵-angiotensins I and II, Asn¹-Val⁵-angiotensin
II (Ciba Lot-094691), and Asp¹-Val⁵-Ser⁹-angiotensin I against Asp¹-Ile⁵-angiotensin
II were 0.76, 0.91, 2.02, 0.50, and 1.39, respectively. Asp¹-Val⁵-angiotensin II had
twice the pressor activity of Asp¹-Ile⁵-angiotensin II. Lyophylized Asp¹-Ile⁵-angio-
tensins I and II kept desiccated at -20°C, were stable for 27 months. Solutions of
100, 10, and 0.8 μg/ml were stable for 12, 8, and 6 months at -20°C, respectively.

In the previous study (1), we re-examined the specific pressor activity of Asp¹-Ile⁵-
angiotensins I and II in the rat by a 4-point assay. The pressor activity of Asp¹-Ile⁵-
angiotensin II was greater than that of Asn¹-Val⁵-angiotensin II. The potency ratio of Asp¹-Ile⁵-
angiotensin I to II was 0.63 on a molar basis. In the present study, we extended the deter-
ninations to Asp¹-Val⁵-angiotensins I and II, since reported values were inconsistent (refer 1).
Using this assay system we also determined stability of Asp¹-Ile⁵-angiotensins I and II.

Specific pressor activity of Asp¹-Val⁵-angiotensins I and II was influenced to various
degrees by other angiotensins used as the standard in the 4-point assay. This was also true
for Asp¹-Val⁵-Ser⁹-angiotensin I (2). The value from a direct comparison was not in ac-
cord with those obtained indirectly using a third angiotensin as the standard. We, therefore,
calculated specific pressor activity from the dose-blood pressure-response (DR) curve,
obtained by injecting a single angiotensin sample per assay rat. This activity was compared
to that obtained by the 4-point assay. We found that the value from DR curves was more
reliable.

The purpose of this paper is to report (i) specific pressor activity of Asp¹-Val⁵-angio-
tensins I and II determined by a 4-point assay, (ii) specific pressor activity of synthetic
angiotensins obtained from DR curves, (iii) comparison of the above two values, and finally
(iv) stability of Asp¹-Ile⁵-angiotensins I and II.
MATERIALS AND METHODS

We synthesized Asp\(^{\prime}\)-Val\(^{5}\)-angiotensins I and II by the classical procedures. Purification and determination of weights were as reported previously (1). Asp\(^{\prime}\)-Ile\(^{5}\)-angiotensins I and II were also treated by the same procedures. Samples in ampoules of Asn\(^{\prime}\)-Val\(^{5}\)-angiotensin II (Ciba Lot-094691) were used without purifying or weighing. They showed ca. 0.80 of pressor activity to the purified sample. Asp\(^{\prime}\)-Val\(^{5}\)-Ser\(^{9}\)-angiotensin I (fowl angiotensin (I)) was synthesized by solid-phase procedure and purified (2).

Preparation of rats, assay procedures, and statistical analyses were the same as in the previous report (1). Female rats of the Donryu/HOS strain, weighing 200±20 g, were used. Angiotensins were dissolved in 0.3 ml of 9 mg/ml NaCl containing 10 nl/ml Tween 20, and injected intravenously. A 4-point assay was performed on combinations of angiotensins in 6 rats each. Low and high doses were 0.5 and 5.0 mg for Asp\(^{\prime}\)-Val\(^{5}\)-angiotensin II, 0.1 and 10 ng for Asp\(^{\prime}\)-Ile\(^{5}\)-angiotensin II and Asp\(^{\prime}\)-Val\(^{5}\)-Ser\(^{9}\)-angiotensin I, and 0.2 and 20 ng for the rest of angiotensins. Tachyphylaxis did not occur with these low doses. In fact, pressor responses usually became larger during the course of repeated injections in 20 times. PR\(_{w}\) and PR\(_{m}\) are designated to potency ratio on a weight and a molar basis, respectively. The 4-point assay was used throughout the determination of stability of angiotensins.

DR curves were obtained by injecting 0.2 to 40 ng of angiotensins in each 6 rats. Tachyphylaxis should be slight when injecting angiotensins in the increasing doses up to 40 ng. Specific pressor activity was also calculated from DR curves. A dose of angiotensin to elicit blood pressure rise by 30 mmHg was determined from each DR curve. PR\(_{w}\) and PR\(_{m}\) were obtained as the ratio of these doses. The 95% confidence interval was calculated by Filler's equation (3).

RESULTS

Specific pressor activity of 5-valine-angiotensins I and II by 4-point assay

Results are shown in Table 1. PR\(_{m}\) of Asp\(^{\prime}\)-Val\(^{5}\)-angiotensin I against Asp\(^{\prime}\)-Val\(^{5}\)-angiotensin II by the direct comparison was 0.615, while the values calculated from PR\(_{m}\) against the third angiotensins were 0.431, 0.543, and 0.640, respectively. The first two figures were less than the 95% confidence limit of PR\(_{m}\) obtained by the direct comparison, indicating that two angiotensins may interact in 4-point assay. The same discrepancy was observed during 4-point assay of Asp\(^{\prime}\)-Val\(^{5}\)-Ser\(^{9}\)-angiotensin I (2).

Dose-blood pressure-response curves of synthetic angiotensins I and II

DR curves of Asp\(^{\prime}\)-Ile\(^{5}\)-angiotensins I and II, Asp\(^{\prime}\)-Val\(^{5}\)-angiotensins I and II, Asp\(^{\prime}\)-Val\(^{5}\)-Ser\(^{9}\)-angiotensin I (fowl angiotensin (I)), and Asn\(^{\prime}\)-Val\(^{5}\)-angiotensin II were obtained (Fig. 1). Relative potencies of these angiotensins were determined from the DR curves (Table 2).

Comparison of specific pressor activities by 4-point assay and from DR curves

Specific pressor activities of two angiotensins, obtained by direct comparison in the 4-point assay and calculated from DR curves were not always in accord. DR curves made
### Table I  Specific pressor activity of Val\( ^{5}\)-angiotensins I and II in the rat by 4-point assay

| T                  | S                  | PR\( \text{w} \) | PR\( \text{m} \) |
|--------------------|--------------------|-----------------|-----------------|
| Asp\(^{1}\)-Val\(^{5}\)-Ang I | Asp\(^{1}\)-Ile\(^{5}\)-Ang I | 0.942 (0.843–1.053) | 0.932 (0.834–1.042) |
| Asp\(^{1}\)-Ile\(^{5}\)-Ang II | Asp\(^{1}\)-Lis\(^{5}\)-Ang II | 0.505 (0.479–0.532) | 0.619 (0.588–0.652) |
| Asp\(^{1}\)-Val\(^{5}\)-Ang II | Asp\(^{1}\)-Val\(^{5}\)-Ang II (Ciba Lot-094691) | 0.495 (0.453–0.540) | 0.615 (0.563–0.671) |
| Asp\(^{1}\)-Val\(^{5}\)-Ang II | Asp\(^{1}\)-Ile\(^{5}\)-Ang I | 0.974 (0.863–1.098) | 1.211 (1.074–1.366) |

| Asp\(^{1}\)-Val\(^{5}\)-Ang II | Asp\(^{1}\)-Lis\(^{5}\)-Ang II | 2.163 (2.034–2.300) |
| Asp\(^{1}\)-Val\(^{5}\)-Ang II (Ciba Lot-094691) | Asp\(^{1}\)-Ile\(^{5}\)-Ang I | 1.157 (1.061–1.261) | 1.141 (1.047–1.245) |
| Asp\(^{1}\)-Val\(^{5}\)-Ang II (Ciba Lot-094691) | Asp\(^{1}\)-Ile\(^{5}\)-Ang I | 1.892 (1.678–2.133) | 1.893 (1.679–2.135) |

T: test material, S: standard material, PR\( \text{w} \): potency ratio on a wt. basis, PR\( \text{m} \): potency ratio on a molar basis. The 95\% confidence interval is in parentheses.

**Fig. 1** Dose-blood pressure response curves of synthetic angiotensins. Vertical bar represents SEM. No. of assays in parentheses.
TABLE 2 Specific pressor activity of synthetic angiotensins in the rat from DR curves

| Angiotensin                  | PRw | PRm     |
|-----------------------------|-----|---------|
| Asp\(^1\)-Ile\(^8\)-Ang I  | 0.616 (0.536–0.696) | 0.763 (0.664–0.863) |
| Asp\(^1\)-Ile\(^8\)-Ang II | 1.00 (0.856–1.144)  | 1.00 (0.856–1.144)  |
| Asp\(^1\)-Val\(^8\)-Ang I  | 0.738 (0.635–0.841) | 0.905 (0.778–1.031) |
| Asp\(^1\)-Val\(^8\)-Ang II | 2.047 (1.475–2.619) | 2.020 (1.455–2.584) |
| Asn\(^1\)-Val\(^8\)-Ang II | 0.504 (0.443–0.566) | 0.497 (0.437–0.558) |
| (Ciba Lot-094691)            |     |         |
| Asp\(^1\)-Val\(^8\)-Ser\(^8\)-Ang I | 1.145 (0.941–1.349) | 1.389 (1.141–1.636) |

PRw: potency ratio on a wt. basis, PRm: potency ratio on a molar basis. The 95% confidence interval is in parentheses.

TABLE 3 Comparison of specific pressor activities by 4-point assay and from DR curves

|      | S   | PRm(4PA) | PRm(DRC) | 4PA/DRC |
|------|-----|----------|----------|---------|
| Asp\(^1\)-Ile\(^8\)-Ang I | Asp\(^1\)-Ile\(^8\)-Ang II | 0.632     | 0.763     | 0.828   |
|      | Asn\(^1\)-Val\(^8\)-Ang II | 1.125     | 1.536     | 0.732   |
|      | (Ciba Lot-094691)             |           |           |         |
| Asp\(^1\)-Ile\(^8\)-Ang II| Asn\(^1\)-Val\(^8\)-Ang II | 1.636     | 2.012     | 0.813   |
|      | (Ciba Lot-094691)             |           |           |         |
| Asp\(^1\)-Val\(^8\)-Ang I | Asp\(^1\)-Ile\(^8\)-Ang II | 0.932     | 1.185     | 0.786   |
|      | Asp\(^1\)-Ile\(^8\)-Ang II   | 0.619     | 0.905     | 0.684   |
|      | Asp\(^1\)-Val\(^8\)-Ang II   | 0.615     | 0.448     | 1.373   |
|      | (Ciba Lot-094691)             | 1.211     | 1.820     | 0.665   |
| Asp\(^1\)-Val\(^8\)-Ang II| Asp\(^1\)-Ile\(^8\)-Ang II | 2.163     | 2.647     | 0.817   |
|      | Asp\(^1\)-Ile\(^8\)-Ang II   | 1.141     | 2.020     | 0.565   |
|      | Asn\(^1\)-Val\(^8\)-Ang II   | 1.893     | 4.067     | 0.465   |
|      | (Ciba Lot-094691)             |           |           |         |
| Asp\(^1\)-Val\(^8\)-Ser\(^8\)-Ang I | Asp\(^1\)-Ile\(^8\)-Ang II | 1.570     | 1.818     | 0.864   |
|      | Asp\(^1\)-Ile\(^8\)-Ang II   | 1.141     | 1.389     | 0.821   |
|      | Asp\(^1\)-Val\(^8\)-Ang I    | 1.808     | 1.534     | 1.179   |
|      | Asp\(^1\)-Val\(^8\)-Ang II   | 0.851     | 0.687     | 1.239   |

T: test material, S: standard material, PRm (4PA): potency ratio on a molar basis by 4-point assay, PRm (DRC): PRm from DR curves, 4PA/DRC: ratio of PRm by 4-point assay and from DR curves. Part of PRm (4PA) values were reported previously (1, 2).

*Calculated from the result of Ciba Lot-171671.

from the results in the 4-point assay shifted to the left in various degrees from those obtained above, indicating that potentiation occurred during the assay. Pressor responses usually became larger during the course of the 20 repeated injections of angiotensin. We calculated the ratio of PRm by 4-point assay to that from DR curve (4PA/DRC) (Table 3). The values ranged from 0.465–1.373, indicating that two angiotensins interacted to various degrees.

**Stability of 5-isoleucine-angiotensins I and II**

Angiotensins, 0.5 mg, were lyophylized in vials, and dissolved at concentrations of
Table 4  Stability during the stock as lyophilized material at −20°C

| Duration (months) | S (vial no.) | T (vial no.) | Asp₁-Ile²-Ang I PRw | Asp₁-Ile²-Ang II PRw |
|------------------|-------------|-------------|---------------------|-----------------------|
| 0                | 1           | 1           | 0.840 (0.683–1.030) | 1.414 (1.232–1.627)   |
|                  | 1           | 2           | 0.929 (0.819–1.053) | 1.470 (1.267–1.712)   |
| 27               | 1           | 1           | 0.929 (0.773–1.116) | 1.692 (1.487–1.922)   |
|                  | 2           | 1           | 1.032 (0.808–1.318) | 1.638 (1.386–1.931)   |
|                  | 1           | 2           | 1.065 (0.793–1.431) | 1.828 (1.598–2.089)   |
|                  | 2           | 2           | 0.971 (0.842–1.119) | 1.566 (1.440–1.701)   |

S: Standard material. Asn¹-Val²-Ang II (Ciba Lot 094691) is used. T: Test material. PRw: potency ratio on a wt. basis. The 95% confidence interval is in parentheses.

Table 5  Stability during stock as a solution at −20°C

| Concentration (µg/ml) | Duration (m) | Asp₁-Ile²-Ang I PRw | Asp₁-Ile²-Ang II PRw |
|-----------------------|--------------|---------------------|----------------------|
| 100                   | 4            | 0.988 (0.907–1.097) | 1.049 (0.874–1.260)  |
|                       | 8            | 1.016 (0.873–1.183) | 0.963 (0.817–1.135)  |
|                       | 12           | 0.920 (0.750–1.128) | 1.040 (0.853–1.269)  |

The same angiotensin solution newly dissolved from a vial was used as the standard. PRw: potency ratio on a wt. basis. The 95% confidence interval is in parentheses.

Fig. 2  Stability of angiotensin solutions (10 µg/ml) kept frozen at −20°C. The same angiotensin solution newly dissolved from the stock solution of higher concentration was used as the standard.
100, 10, and 0.8 \mu g/ml as stock solutions, then diluted further for assay. Angiotensins I or II was recovered equally from 4 different vials, indicating that variation of the amount of angiotensins among vials or of yields by dissolution from vials did not exceed the errors in bioassay. Four samples of 100 \mu g/ml solution were made from a vial, diluted further, and bioassayed. The variations in this step also did not exceed those in the bioassay.

Angiotensins I and II in vials were kept desiccated at -20°C for 27 months. Each 2 vials were compared to Asn^1-Val^5-angiotensin II kept in the same way. PRw of angiotensins I and II were increased, indicating that decay in activity of Asn^1-Val^5-angiotensin II was greater than those of Asp^1-Ile^5-angiotensins I and II (Table 4). Net rises in blood pressure, and comparison of the activity against newly synthesized lots indicated that angiotensins I and II did not lose activity for 27 months under this condition.

Stock solutions of angiotensins I and II were kept at -20°C. A solution of 100 \mu g/ml concentration did not lose the activity for 12 months (Table 5). A solution of 10 \mu g/ml of angiotensin I tended to increase in activity and that of angiotensin II to decrease after 9 and 10 months (Fig. 2). Although the differences did not exceed the errors in bioassay, the stock solutions of 10 \mu g/ml were stable for at least 8 months. A solution of 0.8 \mu g/ml was stable for 6 months at -20°C (Fig. 3). Solutions (0.8 \mu g/ml) frozen and thawed 4 times did not lose the activity.
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**DISCUSSION**

Although the activities of two angiotensins can be compared in an assay preparation by the 4-point assay, the potency ratio deviates if two angiotensins interact. This interaction occurred yielding potentiation factors (4PA/DRC ratio in Table 3) of 0.465–1.373. If the ratio is larger than 1.0, it means that either the test material (T) is potentiated by standard material (S), or S is inhibited by T. If the ratio is smaller than 1.0, either T is inhibited by S or S is potentiated by T.

The specific activity by 4-point assay is correct only for the combination of angiotensins applied to the assay preparation alternatively. The value from a direct comparison was not in accord with those obtained indirectly using a third angiotensin as the standard. Therefore, we concluded that specific activity from DR curves (Table 2) should be used for the general discussion.

The values are expressed as the activity of Asp²-Ile⁵-angiotensin II equal to 1.00, because it is the common active form of mammalian angiotensin (4). Asn⁴-Val⁸-angiotensin II should not be used as the standard because of its relatively unstable shelf life. It has been considered that Asp²-Val⁸-angiotensin II has the same pressor activity as Asp²-Ile⁵-angiotensin II (5, 6, refer also Table I in 1), but such was proved otherwise in our present study. Higher activity is probably due to relative resistance to angiotensinases (7).

Potency ratio of Asp²-Ile⁵-angiotensin I to II was 0.763 on a molar basis, while that of Asp²-Val⁸-angiotensin I to II was 0.448. This may indicate that conversion of Asp²-Val⁸-angiotensin I is slower than that of Asp²-Ile⁵-angiotensin I. PRm of Asp²-Val⁸-Ser⁹-angiotensin I (fowl angiotensin (1)) to Asp²-Val⁸-angiotensin II was 0.688. Conversion of fowl angiotensin (I) may be faster than so-called bovine angiotensin I (Asp²-Val⁸-angiotensin I) in the rat.

The results on stability of Asp²-Ile⁵-angiotensins I and II are: (i) variation in the amounts of lyophylized materials in vials was negligible, (ii) variation in yield from vials when dissolving was negligible, (iii) angiotensin in the vial kept desiccated at −20°C was stable for 27 months, (iv) 100 μg/ml solutions were stable for 12 months at −20°C, and 10 and 0.8 μg/ml solutions were stable for 8 and 6 months, respectively, and (v) 0.8 μg/ml solutions were stable even when freezing and thawing were repeated 4 times.

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