Assay of Endopeptidase-24.11 Activity in Plasma Applied to In Vivo Studies of Endopeptidase Inhibitors

Tim Yandle, Mark Richards, Mary Smith, Chris Charles, John Livesey, and Eric Espiner

We developed a fluorometric assay for endopeptidase-24.11 (EC 3.4.24.11) in human plasma. Substrate (gutaryl-Ala-Ala-Phe-amidomethylcoumarin (AMC)) was incubated with plasma (20 μL, 30 min, pH 7.6) with (control) or without the endopeptidase-24.11 inhibitor phosphoramidon. Further incubation with aminopeptidase M released free AMC. Within-assay CVs were 4.5% and 8.6%, respectively, at 3.31 and 0.27 nmol of AMC released per milliliter per minute. The between-assay CV was 10.4% at 0.31 nmol/mL per minute and the detection limit was 0.05 nmol/mL per minute. A highly skewed distribution of endopeptidase-24.11 in 41 normal samples was found, ranging from 0.12 to 6.84 nmol/mL per minute (median = 0.44). Mean endopeptidase-24.11 concentrations were significantly higher in hypertensive subjects (0.68 nmol/mL per minute) than in normotensive subjects (0.34 nmol/mL per minute; P < 0.05). Compared with placebo administration, the oral endopeptidase-24.11 inhibitor UK 79300 significantly inhibited the plasma enzyme at doses of 100 mg (twice daily). Although in normotensive subjects the enzyme was unaffected with doses of 25 mg, the same dose (25 mg) inhibited the plasma enzyme in hypertensive subjects. No activity was detected in sheep plasma, but addition of exogenous endopeptidase-24.11 to sheep plasma in vitro allowed in vivo assessment of the effect of infused endopeptidase-24.11 inhibitor SCH 39370.

Additional Keyphrases: enkephalinase • fluorometry • enzyme inhibition assay • phosphoramidon • aminopeptidase M • hypertension • angiotensin-converting enzyme • atrial natriuretic factor

Endopeptidase-24.11 (EC 3.4.24.11) has long been recognized as an enzyme metabolizing enkephalin in the brain (1), hence its alternative name, enkephalnase. The enzyme also metabolizes many other peptides, including atrial natriuretic factor (2). Because of its diverse activities, several assays of endopeptidase-24.11 have been developed, based on the use of various substrates, including radiolabeled versions of endogenous substrates such as [3H]enkephalin (3) and [3H]-labeled insulin (4), or of the analog [3H](d-Ala²Leu⁵)enkephalin (5). Other assays have been based on the use of artificial substrates with colorimetric (6) or fluorometric (4, 7–10) methods. An immunoradiometric assay (11) has also been established. By far the majority of these studies have been directed to the measurement of endopeptidase-24.11 in tissues, where the enzyme is located on the cell-surface membrane. To our knowledge, two assays have been applied mainly to serum or plasma (6, 7, 12–14). The development and validation of one of these assays (6) has been well documented (6); however, the peptide-2-naphthylamide substrate used may be mutagenic. The more sensitive and simpler fluorometric assay (7) has not been extensively validated for serum or plasma, although kinetic parameters of the enzyme in plasma and cerebrospinal fluid have been published (14).

Investigations of the potential use of oral inhibitors of endopeptidase-24.11 to treat hypertension or heart failure in humans (15) have created a need for assays of endopeptidase-24.11 in plasma, to monitor the in vivo inhibition of endopeptidase-24.11 during treatment. With this in mind we have developed and validated a simple, sensitive fluorometric assay of endopeptidase-24.11 in human plasma and evaluated its use in monitoring the in vivo inhibition of endopeptidase-24.11 upon administration of the oral inhibitor UK 79300. We also studied the effect of an intravenous inhibitor, SCH 39370, in sheep.

Materials and Methods

Apparatus

We measured fluorescence with an LS5B fluorometer (Perkin-Elmer, Norwalk, CT) calibrated with a 500 nmol/L solution of 7-amidomethylcoumarin (AMC) in assay buffer.

Reagents

We prepared the substrate solution (5 mmol/L glutaryl-Ala-Ala-Phe-AMC; Enzyme Systems Products, Livermore, CA) by dissolving 4.34 mg of substrate in 150 μL of dimethylformamide and then adding 1350 μL of assay buffer (0.1 mol/L Tris·HCl buffer, pH 7.6). We centrifuged (7000 × g, 3 min) 200 μL of aminopeptidase M suspension (Boehringer Mannheim, Mannheim, FRG) and dissolved the sediment in 1 mL of assay buffer to give a 1 g/L solution of aminopeptidase M. This solution was diluted with an equal volume of 10 mmol/L EDTA just before use. Phosphoramidon (Peninsula Labs Inc., Belmont, CA) was dissolved in assay buffer to give a 0.1 mmol/L solution. SCH 39370 was supplied by Schering-Plough Research (Bloomfield, NJ), and UK 79300 was supplied by Pfizer Central Research, Sandwich, UK.

Assay of Human Endopeptidase-24.11

Blood was collected into chilled heparin-containing tubes and rapidly centrifuged (1500 × g, 10 min); the plasma was stored at −20 °C. We performed the assay in...
two separate stages, first incubating the substrate glutaryl-Ala-Ala-Phe-AMC with endopeptidase-24.11 in the plasma sample to produce Phe-AMC, and then incubating the product with aminopeptidase M to release free AMC, which we measured by fluorometry. In the first stage, we incubated duplicate 20-μL samples of heparinized plasma, 10 μL of substrate (final concentration 0.6 mmol/L), and 50 μL of assay buffer (80 μL total volume) at 37 °C for 30 min. We stopped the reaction by adding 10 μL of phosphoramidon and transferred the tubes to an ice bath. In the second stage, we added 10 μL of aminopeptidase M reagent and incubated the sample for 30 min at 37 °C. After diluting the product with 3 mL of assay buffer, we measured fluorescence at 440 nm with excitation at 360 nm. We processed a control incubation in an identical manner except that phosphoramidon was added before the first incubation at 37 °C. We calculated endopeptidase-24.11 activity from the difference in fluorescence between the sample (S) and control (C) incubations by the equation below.

$$\text{Endopeptidase activity} = \frac{3.1 \times 1000 \times 1}{(S - C) \times 20 \times 30} = \frac{(S - C)}{194}$$

The constant (194) converts the concentration difference in the final 3.1 mL volume derived from a 30-min incubation of 20 μL of plasma to the rate of AMC produced (nmol/mL per minute).

During assay development, and for most results reported here, we also processed a sample incubated for 0 min and its corresponding control. These zero-time incubations were later omitted, because the sample and control were processed in a nearly identical manner, and their results were identical over many samples. The assay detection limit was determined by measuring 10 replicates of one plasma sample in the presence of SCH 39370 (final concentration in the incubation, 1 mmol/L). This concentration of endopeptidase-24.11 inhibitor was greater than that required to completely inhibit the enzyme. The detection limit was taken as 2 SD above the mean of these replicates.

Interference Studies

We drew 10 mL of blood from a normal subject into heparin and centrifuged it at 1500 × g for 10 min to prepare plasma. We retained the plasma fraction and washed the erythrocyte sediment four times with isotonic saline (NaCl 9 g/L), centrifuging as above. We then lysed the washed erythrocytes by adding 1 mL of distilled water and sedimented any unlysed cells at 1500 × g for 15 min. To produce mild (light pink) to heavy (red) hemolysis, we added increasing amounts of the lysed cell preparation to the original plasma. To measure interference by bilirubin, we prepared a stock 25 mmol/L solution of bilirubin in dimethyl sulfoxide and mixed this with plasma to give a 1 mmol/L concentration of added bilirubin. We diluted this preparation with plasma to give 800, 400, and 200 μmol/L concentrations of added bilirubin in plasma. We added Intraplur (Kabi Vitrum, Stockholm, Sweden) to plasma to give concentrations of 10 and 30 g/L to assess lipid interference. We assayed all of the above plasma preparations plus the untreated plasma, adjusting the results for dilution by the added reagents. To assess any quenching of AMC fluorescence by plasma components, we added 20 μL of plasma to 3 mL of 500 nmol/L AMC solution and compared the fluorescence produced with that produced in the absence of plasma.

Assay of Sheep Endopeptidase Inhibitor

This assay was performed in the same way as was the human enzyme assay except that we replaced 20 μL of assay buffer with 20 μL of sheep kidney microvillus preparation (16), diluted 5000-fold in assay buffer.

Inhibitor Studies

In humans. In double-blind, crossover, placebo-controlled studies, we studied two groups of normal volunteers and a group of patients with uncomplicated essential hypertension. The hypertensive subjects had blood pressures consistently exceeding 140/95 mmHg on at least five occasions preceding the study; echocardiography demonstrated increased left ventricular mass and left ventricular hypertrophy in these patients. We excluded subjects with a history of recent cerebrovascular or coronary ischemic events, and those in whom hypertension was complicated by other major systemic disorders. We stopped the antihypertensive medication, and monitored patients for ≥2 months before the study. Group 1 (six normotensive volunteers, ages 19–25 years, weighing 68–92 kg) were given placebo or UK 79300, 25 mg, at 12-h intervals for 4 days. Group 2 (eight normotensive volunteers, ages 19–23 years, weighing 60–83 kg) were given placebo or UK 79300, 100 mg, at 12-h intervals according to an otherwise identical protocol. The hypertensive group (10 patients, ages 18–62 years, weighing 67–88 kg) underwent the same protocol as did group 1 normotensive subjects. For 3 days before and throughout the dosing periods, all patients followed a caffeine- and alcohol-free diet with a constant intake of sodium (150 mmol/d) and potassium (80 mmol/d). We separated the placebo and active study phases by 2 weeks. On the fourth day of dosing (from 0800 on day 7 to 0900 on day 8 of the controlled diets), we studied the subjects in the hospital while they adhered to a carefully regulated protocol with standardized conditions of posture, diet (meals at 0830, 1300, and 1800), activity, and room temperature, throughout the 25 h. We controlled posture by ensuring that the subjects were seated for the first 30 min and stood for the latter 30 min of each hour from 0900 to 2100. They were recumbent from 2100 to 0800 and were then seated for the last hour from 0800 to 0900. Oral doses of UK 79300 were given at 1000 and 2200 on days 4–7. We drew samples for endopeptidase-24.11 assay through a forearm butterfly cannula at 0900, 1000, 1300, 1700, 2100, 2200, 0100, 0500, and 0800, i.e., 1 h predose and 0, 3, 7, 11, and 12 h postdose.
In sheep. We studied the effect of endopeptidase-24.11 inhibition in two sodium-replete merino ewes. A week before experiments began, we inserted a polyethylene catheter into each jugular vein. After taking a baseline blood sample, we injected an intravenous bolus (10 mL) of either SCH 39370 (2.5 mg/kg of body wt.) or vehicle (1 mol/L Tris·HCl, pH 7.4) 2 days apart. We took blood samples 30 and 120 min after the bolus to determine the endopeptidase-24.11 activity in serum.

**Results**

**Assay design and performance.** We assessed the ability of the aminopeptidase M incubation to convert all Phe-AMC (produced by the first incubation with plasma) to free AMC by incubating aminopeptidase M with 1.95 or 7.32 nmol of Phe-AMC for various times. All Phe-AMC was converted to AMC within 30 min when incubated at 37°C (Figure 1) with either amount of substrate. This reaction was not affected by the presence of EDTA, 0.5 mmol/L (added as 5 mmol/L reagent) (Figure 1), which we included to inhibit any endopeptidase-24.11 contamination in the aminopeptidase M reagent. The highest amount of Phe-AMC (7.32 nmol) used was equivalent to that produced by a reaction rate of 12.2 nmol/mL per minute in a plasma sample—twice the maximum rate we have observed in normal plasma to date, and equal to the greatest plasma value we detected in any sample.

We used glutaryl-Ala-Ala-Phe-AMC as substrate and performed control incubations with the endopeptidase-24.11 inhibitor phosphoramidon to achieve specificity. Figure 2 shows the effects of varying the substrate concentrations on the reaction rate. We determined the $K_m$ of the human plasma enzyme with this substrate to be 0.37 mmol/L. To conserve substrate, we opted to use a substrate concentration of 0.6 mmol/L. Although this substrate concentration is close to the $K_m$, we found that production of AMC varied linearly with increasing incubation times up to 40 min (Figure 3) and with increasing plasma volume up to 35 μL (Figure 4). With plasma samples >35 μL, we consistently found a marked decrease in apparent activity.

We measured within-assay variation with two plasma samples and obtained CVs of 8.6% (mean 0.27 nmol/mL per minute, $n = 10$) and 4.5% (mean 3.31 nmol/mL per minute, $n = 10$). The between-assay variation was 10.4%, measured with one sample (mean 0.31 nmol/mL per minute) in 32 consecutive assays spread over 94 days.

**Fig. 1.** Production of AMC from added Phe-AMC by aminopeptidase M under assay conditions

Incubation with 7.32 nmol (○, ♦) or 1.95 nmol (▲, △) of Phe-AMC in the presence (○, ▲) or absence (♦, △) of EDTA, 0.5 mmol/L.

**Fig. 2.** Michaelis-Menten plot of reciprocal of velocity (nmol/mL per minute) vs reciprocal of substrate concentration (mmol/L) for a single plasma sample

$K_m = 0.36$ mmol/L.

**Fig. 3.** Linearity of AMC production with time for two plasma samples with endopeptidase-24.11 activities of 4.83 (○) and 0.29 (▲) nmol/mL per minute.

CLINICAL CHEMISTRY, Vol. 38, No. 9, 1992 1787
(Stored at -20 °C, these samples showed no change over the 3 months.) The assay detection limit, determined by measuring 10 samples containing excess endopeptidase-24.11 inhibitor, was 0.05 nmol/mL per minute.

**Inhibitor spectrum.** To plasma samples we added enzyme inhibitors at the final concentrations listed in Table 1 and assayed. Endopeptidase-24.11 activity was inhibited by the inhibitor SCH 39370 (17) and the chelating agents EDTA and o-phenanthroline, as expected (18, 19). Inhibition of endopeptidase-24.11 by dithiothreitol has been ascribed to its ability to chelate metal ions (18). Neither the angiotensin-converting enzyme inhibitors MK422 and captopril nor the protease inhibitors leupeptin and p-mercuribenzoate inhibited the enzyme in this assay. This inhibition spectrum is similar to that for purified endopeptidase-24.11 preparations (18, 19).

**Activities in normal subjects.** We measured samples from 41 apparently healthy men (ages 19–62 years) and 8 apparently healthy women (ages 29–68 years), who were either blood donors or laboratory staff. Data for these samples were highly skewed (Figure 5), ranging from 0.12 to 6.84 nmol/mL per minute with a median of 0.44 nmol/mL per minute, and with 25 and 75 percentiles of 0.24 and 0.78 nmol/mL per minute, respectively.

**Interference.** For convenience we collected blood into heparin, after establishing that heparinized plasma and serum gave identical results. Adding heparin (14 kIU/L) to serum also did not alter measured results for endopeptidase-24.11. Mild hemolysis reduced plasma endopeptidase activity from 8.1 to 7.16 nmol/mL per minute; heavy hemolysis further reduced it to 2.96 nmol/mL per minute. Bilirubin at 200, 400, and 800 μmol/L decreased plasma endopeptidase activity from 8.81 to 7.40, 6.15, and 5.40 nmol/mL per minute, respectively. Intralipid did not affect the assay even at the extremely high concentration of 30 g/L.

We examined the quenching of AMC fluorescence by plasma samples from 11 subjects. Plasma reduced the fluorescence of 500 nmol/L AMC by 3.6% (to 482 nmol/L, SD 6.4 nmol/L). Quenching by two samples of lipemic plasma was similar to that for normal samples (mean = 487 nmol/L), whereas two hemolized samples showed greater quenching (mean = 456 nmol/L).

**Human studies.** Figure 6 illustrates the mean data for human endopeptidase activity from the three studies in which we gave the oral endopeptidase inhibitor UK 79300 or placebo to normal and hypertensive subjects. As in our study of untreated normal subjects, the data in all three of these studies were skewed, so we performed statistical analysis on logarithmically transformed data. For each subject in the hypertensive and normotensive groups, we averaged all the transformed endopeptidase activity values on each subject’s placebo day and compared the resulting means for the normotensive and hypertensive groups by using the Wilcoxon–Mann–Whitney test. We found that the plasma endopeptidase activity (in the absence of endopeptidase inhibitor) in hypertensive subjects differed significantly in location from that in normotensive subjects (P < 0.05). The mean (SE) of untransformed data in hypertensive subjects was 0.68 (0.22) nmol/mL per minute; in the normotensive subjects it was 0.34 (0.09) nmol/mL per minute. Interestingly, the variances of the transformed values in the two groups also differed significantly (F = 4.25, P = 0.005), the hypertensive subjects having the greater variance. Analysis of pooled placebo day data from all subjects showed a small but significant (P = 0.04) variation with time over the 24-h study; peak – trough = 0.07 nmol/mL per minute. We also looked for significant ex vivo inhibition by UK 79300 in these studies by using analysis of variance (ANOVA) with repeated measures. The treatment × time interaction was significant in hypertensive sub-

---

**Table 1. Inhibition of Endopeptidase-24.11 in Plasma**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration, mmol/L</th>
<th>% Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK 422</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Captopril</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>1.0</td>
<td>80</td>
</tr>
<tr>
<td>EDTA</td>
<td>6.25</td>
<td>56</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1.0</td>
<td>76</td>
</tr>
<tr>
<td>p-Mercuribenzoate</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 mg/L</td>
<td>0</td>
</tr>
<tr>
<td>SCH 39370</td>
<td>0.001</td>
<td>37</td>
</tr>
<tr>
<td>SCH 39370</td>
<td>0.001</td>
<td>100</td>
</tr>
</tbody>
</table>

*Uninhibited activity = 0.23 nmol/mL per minute.
jests given 25-mg doses of UK 79300 ($P = 0.002$) and in the normotensive subjects given 100-mg doses ($P = 0.003$), but not in normotensive subjects given 25-mg doses ($P = 0.187$). Thus, UK 79300 inhibited plasma endopeptidase activity in the hypertensive group and in the high-dose normotensive group but not in the low-dose normotensive group (Figure 6).

Finally, we note two other observations from the data in Figure 6: Postdose inhibition of endopeptidase activity appeared to last longer in the second half of the studies when subjects were recumbent. Moreover, in the treatment phases for both normotensive groups, the mean values from samples drawn 11 and 12 h postdose appeared to exceed the corresponding placebo day data. Neither trend was statistically significant (ANOVA).

**SCH 39370 studies in sheep.** We did not detect endopeptidase-24.11 activity in sheep plasma. To monitor the effectiveness of SCH 39370 in circulation, we added exogenous endopeptidase-24.11 (kidney microvillar preparation) to plasma samples and then assessed inhibition of the added enzyme by SCH 39370 in the plasma samples. Figure 7 shows data from plasma samples taken 0, 30, and 120 min after bolus administration of vehicle or SCH 39370 (2.5 mg/kg) in two sheep. In both sheep, the activity of added endopeptidase-24.11 was inhibited in samples drawn postdose, but the values were returning towards baseline values by 120 min. No significant changes were observed on the placebo day.

**Discussion**

We have developed a simple fluorometric assay for endopeptidase-24.11 in human plasma. During assay development we confirmed that aminopeptidase M was contaminated with endopeptidase-24.11, as described by Almenoff and Orlowski (19). Using two incubation steps in the assay, we were able to inhibit contaminating endopeptidase-24.11 in the aminopeptidase M reagent by adding EDTA to the aminopeptidase M reagent before assay without affecting the measurement of plasma endopeptidase-24.11. This inhibition was maintained in the final assay step by phosphoramidon remaining from the first step. The design also eliminated the requirement for additional control incubations, thereby reducing the assay to a simple incubation of plasma in the presence or absence of phosphoramidon. We did not include controls for plasma quenching of

![Figure 6](image-url)  
Fig. 6. Serial plasma endopeptidase-24.11 activity (mean of untransformed data ± SE) over 24 h in samples drawn on the fourth day of treatment with (△) UK 79300 given at 1000 and 2200 h (arrow) or (●) placebo  
NT, normotensive; HT, hypertensive. Subjects' posture is shown at top.

![Figure 7](image-url)  
Fig. 7. In vitro inhibition of kidney endopeptidase-24.11 preparation by ovine plasma samples containing the inhibitor SCH 39370  
Two sheep were given a 2.5 mg/kg intravenous bolus of SCH 39370 (○, △) or vehicle (●, ▲), and plasma samples were drawn at 0, 30, and 120 min. There being no endogenous endopeptidase-24.11 in ovine plasma, we assessed the potency of SCH 39370 by its inhibition of kidney endopeptidase-24.11 added to the samples.
AMC because our studies showed only a small quenching effect by plasma that was relatively constant between samples. Only hemolyzed plasma stood out as having a greater quenching effect. This agreed with our observation that hemolyzed plasma (and plasma with increased concentrations of bilirubin) is not suitable for assay because of assay interference. The assay results varied linearly with endopeptidase-24.11 activity in plasma incubated as long as 40 min and at plasma volumes up to 35 μL.

We showed specificity of the assay for endopeptidase-24.11 by adding inhibitors to the assay. The resulting inhibition spectrum of the plasma enzyme was similar to that of purified endopeptidase-24.11 (18, 19), except we found that a much higher concentration of SCH 39370 was required for inhibition (1–10 μmol/L) than the 5 nmol/L IC50 value published elsewhere (17). This difference would be expected, given the different assay conditions (substrate concentration and K_m) used here. The K_m of the plasma enzyme determined with this assay was similar to that of purified endopeptidase-24.11 acting on equivalent substrates (18, 19). Ideally, this assay, like other enzyme assays, should use a substrate concentration much greater than the K_m. However, the relatively high cost of reagent prevented this, so we opted for a concentration slightly higher than the K_m, as others have also done (6, 9). The precision of the assay is therefore partly dependent on the precision of substrate addition; however, our results (CVs of 8.6% and 4.5% and linearity studies) show that this is not a problem. Nevertheless, the assay precision probably could be further improved by adding the same amount of substrate in a larger volume (e.g., 50 μL), while decreasing the buffer volume added.

The assay had more than adequate sensitivity to measure endopeptidase-24.11 in human plasma, the enzyme activity in all samples being greater than the assay detection limit. Assay of plasma samples from 49 normal individuals revealed a highly skewed distribution of normal samples, similar in distribution and in absolute values to that published by Almenoff et al. (6). When we analyzed the pooled placebo day data from our human studies, we found a small but significant variation with time over 24 h. This change could be related to posture, food intake, or a circadian rhythm, because these factors, although controlled, were not held constant in this study. We could not detect endopeptidase-24.11 in ovine (sheep) plasma, although we found relatively large amounts in sheep kidney membrane preparations. It is therefore unlikely that differences in substrate specificity between the human and ovine enzymes account for our inability to measure endopeptidase-24.11 in ovine plasma. Whether other species have a soluble endopeptidase-24.11 in plasma remains to be seen, although its presence in mouse blood has been inferred from inhibitor studies (20).

The source of endopeptidase-24.11 in human plasma has not been determined. Unlike angiotensin-converting enzyme, endopeptidase-24.11 is not present in large quantities in vascular tissue (21). However, venous concentrations of endopeptidase-24.11 are greater than arterial concentrations in humans (7), which suggests that the high concentrations of endopeptidase-24.11 in the mammalian kidney, lymph nodes, and gut (11) may be a source of the plasma enzyme. Data are also lacking on whether it is the secretion, or the circulating concentrations, of the plasma enzyme that are regulated, or whether the circulating enzyme merely represents turnover of tissue enzyme and its elution into circulation. This question is directly relevant to our observation of higher concentrations of endopeptidase-24.11 in hypertensive than in normotensive subjects. In addition, we found a greater variance in endopeptidase activity among hypertensive than normotensive subjects. The meaning of this observation is unclear. Perhaps hypertensive subjects have more variable concentrations of the enzyme, or some subgroups of hypertensive subjects may have high or low plasma endopeptidase activities. Further confirmation of these observations is required, particularly because we did not match the normotensive and hypertensive subjects for age or weight and relatively few subjects were studied. However, if these observations are confirmed, further study is needed to assess the many possible factors contributing to the concentration of endopeptidase-24.11 in plasma, including tissue turnover and release of the enzyme and the relationship between plasma enzyme concentrations and tissue content.

Even though plasma endopeptidase-24.11 is a major contributor to atrial natriuretic factor degradation in human plasma in vitro (22), it is unlikely to be important in clearance of atrial natriuretic factor in vivo, given our previously estimated in vitro half-life of atrial natriuretic factor in human plasma of ~30–60 min—much longer than the 3.1-min half-life in vivo (23). Many studies now show that oral, or infused, inhibitors of endopeptidase-24.11 reduce clearance of atrial natriuretic factor (20), including our own studies with the oral inhibitor UK 79300 in humans (24). However, in sheep, where the enzyme is undetectable in plasma, endopeptidase inhibitors induce increases in plasma atrial natriuretic factor similar to those seen in humans (25)—findings consistent with a dominant tissue action of the enzyme. Therefore, although the plasma enzyme may not be important in atrial natriuretic factor clearance in vivo, the tissue enzyme must be involved in clearance.

An important application of this assay is to monitor endopeptidase-24.11 inhibition in vivo after treatment with specific inhibitors. Inhibition of the plasma enzyme may reflect the effectiveness of the drug in vivo, but may not accurately indicate inhibition at the tissue level because of probable differences in access of prodrugs to different tissues and in the rate of prodrug de-esterification in these tissues. We applied the assay to three studies of normotensive and hypertensive volunteers who were taking the oral prodrug UK 79300. The assay demonstrated the expected in vivo inhibition of plasma endopeptidase-24.11 by UK 79300 in hypertensive and normotensive humans. Inhibition was maximum (27–
59% of predrug values) 3 h after ingestion. This degree of inhibition is similar to that observed with 100-mg oral doses of acetylphen (26), where 70% maximum inhibition was achieved 1 h postdose, declining to <50% by 4 h postdose. However, the achieved plasma inhibition will obviously depend on oral availability, clearance, and potency, which will be different for each inhibitor. Although not significant, there was a trend for inhibition by UK 79390 to last longer in the second half of the studies—an observation possibly related to recumbent posture or food intake. Finally, we saw indications (again, not significant) that endopeptidase activity 11–12 h postdose was greater than time-matched placebo values. By this time postdose, the inhibitor may have been sufficiently cleared to marginally reveal an increased enzyme concentration. This increase may represent induction of endopeptidase-24.11 synthesis by the inhibitor over the previous 3 days of dosing. Such an induction of enzyme synthesis by its inhibitor is well established for angiotensin-converting enzyme, which is induced by angiotensin-converting enzyme inhibitors, resulting in increased concentrations of angiotensin-converting enzyme in plasma (27) or tissue (28, 29).

In conclusion, the development of this assay and its application to the action of endopeptidase-24.11 inhibitors in vivo established the time course of in vivo inhibition of endopeptidase-24.11 by an oral prodrug inhibitor and showed that hypertensive subjects may have higher circulating concentrations of endopeptidase-24.11 than do normotensive subjects. The assay allows correlation of in vivo enzyme inhibition with biological responses (e.g., changes in clearance of atrial natriuretic factor). Further studies are obviously required to confirm the difference in endopeptidase activity between normotensive and hypertensive subjects.

We gratefully acknowledge support for this work from the Health Research Council of New Zealand, and the National Heart Foundation. A. M. R. holds a senior fellowship from the National Heart Foundation of New Zealand.

References