# Radioimmunoassay for the Quantitation of Lisinopril and Enalaprilat

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Received September 20, 1985, from the *University of Melbourne, Clinical Pharmacology Unit, Department of Medicine, Austin Hospital, Heidelberg, Victoria, 3084, Australia.* Accepted for publication February 10, 1986.

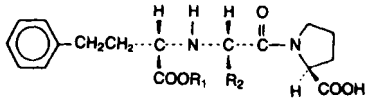
**Abstract** □ A sensitive radioimmunoassay (RIA) capable of measuring either lisinopril (1-[$N^2$-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl]-L-proline) or enalaprilat (1-[*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl]-L-proline), the active metabolite of enalapril has been developed. A suitable antiserum was raised against an immunogen prepared from conjugation of lisinopril, the lysyl analogue of enalapril, with succinoylated keyhole limpet hemocyanin. A novel radiotracer was also prepared for use in the assay by acylation of the epsilon amine group on the lysyl side chain of lisinopril with *N*-succinimidyl [2,3-$^3$H]propionate. The antiserum was used at a final dilution of 1:44,500 and the sensitivity of the assay for enalaprilat was estimated at 2 pmol/mL plasma sample and 0.4 pmol/mL for lisinopril. Enalapril, the ethyl ester of enalaprilat, exhibited little cross-reactivity (0.005%), and several other compounds (captopril, proline, lysine, tyrosine, hippuric acid, and tryptophan) were found not to cross-react. In rabbits given a 2.03 μmol/kg iv dose of enalapril, plasma concentrations of enalaprilat were determined by the RIA technique and compared with an estimation of the enalaprilat concentrations derived from the extent of inhibition of plasma angiotensin converting enzyme (ACE). The plasma levels estimated by ACE inhibition were less than those obtained by the RIA in the first 45 min but were always greater in the samples taken after this time. Both assay methods showed that the conversion of enalapril to enalaprilat was rapid, and also indicated that there was initial rapid clearance of enalaprilat from the plasma.

Enalapril, a prodrug that requires de-esterification to provide an active metabolite (enalaprilat, 1-[*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl]-L-proline),[1] is a relatively new angiotensin converting enzyme (ACE) inhibitor[2] that is currently undergoing extensive clinical and experimental investigation as an antihypertensive drug.[3] Lisinopril, 1-[$N^2$-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl]-L-proline, the lysyl analogue of enalaprilat, does not require any form of metabolic activation and has more recently been utilized in clinical trials to assess its effectiveness as a antihypertensive agent.[4]

Enalapril has been found to be a relatively weak inhibitor of ACE in vitro, with a typical $IC_{50}$ value of $1.2 \times 10^{-6}$ M. In comparison, the active metabolite, enalaprilat, has an in vitro $IC_{50}$ value of $1.2 \times 10^{-9}$ M.[2] However, for the in vivo inhibition of a pressor response to injected angiotensin I, enalapril has proved to be effective within a dose range similar to that of enalaprilat in rats, but not dogs.[1] This difference has been attributed to the more rapid de-esterification of enalapril by the esterase enzymes in the hepatic system and plasma of rats. In dogs, de-esterification of enalapril has not been observed in plasma.[5]

Reports of studies in which isotopes of enalapril or the extent of ACE inhibition were used to determine the disposition of enalapril and its pharmacologically active metabolite, enalaprilat, in rats[6-8] and dogs[5] have recently been published. Tocco et al.[5] utilized the extent of inhibition of a partially purified porcine plasma ACE preparation and found a good correlation between the determined enalaprilat concentrations in biological samples and those determined isotopically in the same samples. Other methods relying on the extent of endogenous ACE inhibition as both a marker of pharmacological activity and as an index of drug levels[6,9] are not effective for directly determining the conversion of enalapril in metabolically active tissues that do not contain ACE, and do not provide the quantitative data required for pharmacokinetic studies.

There have been a number of clinical studies, [10-13] for example, that have utilized the RIA described by Hichens et al.[14] However, as antisera with the appropriate reagents are not commercially available, we have developed our own RIA for these compounds using a novel radiotracer that can be simply prepared. We used this RIA to study the time-course of enalaprilat in the plasma of rabbits after an intravenous bolus dose of enalapril. Since it has been suggested that the extent of endogenous ACE inhibition may be used as an index of enalaprilat concentrations in plasma,[15] we compared concentrations determined from the extent of endogenous ACE inhibition with those measured by the newly developed RIA.



1, $R_1$ = $CH_2CH_3$; $R_2$ = $CH_3$

2, $R_1$ = H; $R_2$ = $CH_3$

3, $R_1$ = H; $R_2$ = $(CH_2)_4NH_2$

## Experimental Section

**Materials**—Enalapril maleate, enalaprilat (1-[*N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-alanyl]-L-proline), and lisinopril (1-[$N^2$-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl]-L-proline) (Merck, Sharp and Dohme, Rahway, NJ), and captopril (Squibb Inc., Princeton, NJ) were provided by the manufacturers. Keyhole limpet hemocyanin suspension (from *Megathura crenulata*) supplied as a slurry in 65% ammonium sulfate (Calbiochem-Behring Corp., La Jolla, CA), succinic anhydride (Fluka, Switzerland), bovine serum albumin, hippuric acid, histidine, lysine, phenylalanine, proline, tryptophan, tyrosine, and ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Sigma Chemical Co. St. Louis, MO), *N*-succinimidyl [2,3-$^3$H]propionate, specific activity 59 Ci/mmol (Amersham, Australia), incomplete and complete Freund's adjuvant and *B. pertussis* cell suspension (CSL Australia), $F_{254}$ HP-TLC plates (Merck, Darmstadt), 2,5-diphenyloxazole and 1,4-bis-[2-(5-phenyloxazolyl)]benzene (Packard Instrument Co., Downers Grove, IL), propanidid (Bayer, Germany), and medical grade silastic tubing (0.76 mm i.d. × 1.65 mm o.d.) (Dow Corning, MI) were purchased.

**Preparation of Immunogen**—Keyhole limpet hemocyanin (1 mL of a 35-mg/mL protein of the hemocyanin suspension) was centrifuged at 40,000 × *g* for 10 min and the supernatant discarded. The pellet was resuspended in 9 mL of distilled water, and 1 mL of a concentrated phosphate buffered saline (PBS, 1.5 M NaCl:100 mM $NaH_2PO_4$-$Na_2HPO_4$, pH 7.4) was added. This mixture was dialyzed



*0022-3549/86/0500-0512$01.00/0*

overnight against 1 L of PBS (150 mM NaCl:10 mM $NaH_2PO_4$-$Na_2HPO_4$, pH 7.4). Succinic anhydride (100 mg) was added successively in 20-mg aliquots to the dialyzed hemocyanin solution with continuous stirring and the pH was maintained between 7 and 8 with addition of 0.6 M NaOH in 0.2 M $Na_2CO_3$ in a dropwise manner. Following the addition of the last aliquot of succinic anhydride, the mixture was stirred an additional 30 min. The mixture was then dialyzed three times against PBS (3 × 1 L) over 5 h.

**Conjugation of Lisinopril with Succinoylated Hemocyanin**—Lisinopril (10 mg in 1 mL of distilled water) was added with 0.5 mL of ethyl-3-(3-dimethyl-aminopropyl)carbodiimide HCl (10 mg/mL solution prepared immediately prior to use) to 2.5 mL of the dialyzed succinoylated hemocyanin. The pH of the mixture was maintained between 5 and 6 and the solution was continuously stirred while protected from light for 24 h at 25°C. The reactants were then dialyzed against a 150 mM NaCl solution (1 L) which was changed at ~7 h intervals over 36 h. Aliquots of the immunogen were then stored frozen until used for immunization.

**Immunization**—Young male New Zealand crossbred rabbits (1.5–2 kg) were immunized at multiple sites on their backs with a water–oil emulsion of the respective dialyzed immunogen using Freund's adjuvant as the oil base. The complete adjuvant was used on the first immunization and the incomplete on the second and subsequent immunizations at monthly intervals. At each immunization, an injection of *B. pertussis* cell suspension (0.5 mL) was injected intramuscularly to further stimulate the immune system. Blood was obtained from the ear vein ~10 d following the second and subsequent immunizations.

**Radiotracer Preparation**—Immediately prior to use, the toluene solvent containing the *N*-succinimidyl [2,3-$^3$H]propionate (2.5 mCi, 42 nmol) was evaporated to dryness under a stream of nitrogen and then reconstituted with borate buffer (200 μL, 50 mM, pH 7.5 or 9.0). An equal number of moles of lisinopril and *N*-succinimidyl [2,3-$^3$H]propionate were then mixed in 50 mM borate buffer at either pH 7.5 or 9.0. The mixture (final volume 400 μL) was stirred continuously using a magnetic stirrer during the addition of reactants and then left protected from light at room temperature for 3 h. A small quantity of *N*-succinimidyl [2,3-$^3$H]propionate was left in the borate buffer (pH 7.5) as a control to monitor the hydrolysis products. The products from the reaction were then separated by TLC with a butanol:water:glacial acetic acid (4:1:1) solvent on silica gel $F_{254}$ TLC plates. After the plates were developed, they were sectioned into 1-cm strips, and the radioactivity was eluted with three 0.5 mL-aliquots of 0.01 M HCl.

**Radioimmunoassay Procedures**—All procedures were done at 4°C. For incubation, the antiserum and radiotracer (10,000 dpm per assay tube) were diluted appropriately in PBS (pH 7.5) and added separately to the glass mini-vials (7 mL) containing the assay sample (up to 100-μL sample volume). All samples were assayed in duplicate. The final volume of the incubation tube was brought to 0.7 mL with PBS. The incubate contained 6% normal sheep sera, 0.1% sodium azide, and 25 mM EDTA. Samples were incubated for 20–24 h at 4°C and the separation of bound from free ligand was obtained by precipitation of the bound complex using an equal volume of saturated ammonium sulfate solution. After centrifugation (1,600 × *g* for 30 min), the supernatant was aspirated and the pellet was washed with 0.5 mL of half-saturated ammonium sulfate solution, recentrifuged, and the supernatant discarded. The pellet was then dissolved in 0.5 mL of distilled water and, after the addition of 5.5 mL of a liquid scintillation cocktail (3 g 2,5-diphenyloxazole, 0.2 g 1,4-bis-[2-(5-phenyloxazolyl)]benzene in 0.75 L xylene and 0.25 L Triton X-100), the radioactivity was determined by liquid scintillation counting. Counting time was limited to 10,000 counts or 10 min and quenching was corrected by the channels ratio method.

**Angiotensin Converting Enzyme Assay**—The ACE activity was determined using the fluorometric method of Freidland and Silverstein.[16] The extent of ACE inhibition was expressed as a percentage of the ACE activity from a predose plasma sample. Standard curves for the inhibition of plasma ACE were obtained by adding known amounts of enalaprilat to drug-free plasma from the rabbit in which the drug level was to be determined. In this way, the system could be regarded as a competitive protein binding assay where the percent of predose ACE activity remaining after enalaprilat had been administered was treated as the bound fraction from an RIA in the data analysis. Standards and samples were assayed under the same conditions.

**Preparation of the Rabbits for Kinetic Studies**—Male New Zealand crossbred rabbits (n = 3) were anesthetized by continuous infusion of propanidid solution (25 mg/mL at 0.5 mL/min) and, under sterile conditions, a silastic cannula (0.76 mm i.d. × 1.65 mm o.d.) was inserted into the facial vein and passed down to the level of the right atria. The cannula was then exteriorized at the back of the neck for convenient access, and the rabbits were allowed at least 7 d to recover prior to the experiment. On the day of the experiment, enalapril maleate (as an 8.12 μmol/mL solution in sterile saline, 2.03 μmol/kg dose) was given as an intravenous bolus, followed by 2 mL of saline to flush the cannula, and blood was sampled at intervals thereafter. Preliminary tests determined that samples obtained from the venous line were not contaminated by prior drug administration via that line. Rabbits, which are convenient laboratory animals to maintain, were selected for this study because repeated blood samples can be taken without significant depletion of blood volume.

**Data Analysis**—The plasma concentrations of enalaprilat estimated by RIA and ACE inhibition were calculated using:

$$y = A/(C + x^E) \quad (1)$$

where $x$ is the concentration of unknown substance, $y$ is the measured response, and $A$, $C$, and $E$ are constants for the particular assay, using a FORTRAN program (modified by the authors) from Burger, et al.[17] The linear regression for two variables where both are determined with some associated error was also done using a FORTRAN program written by the authors utilizing the approach outlined by Cornbleet and Gochman[18] and Snedecor and Cochran.[19] The area under the curve (AUC) was calculated using the trapezoidal rule and the mean residence time (MRT) was calculated as the ratio of the area under the moment curve (AUMC) and AUC (AUMC/AUC) as described by Gibaldi and Perrier.[20]

## Results and Discussion

**Lisinopril Tritiation**—The elution profiles using TLC from the tritiation reactions are shown in Fig. 1. A single

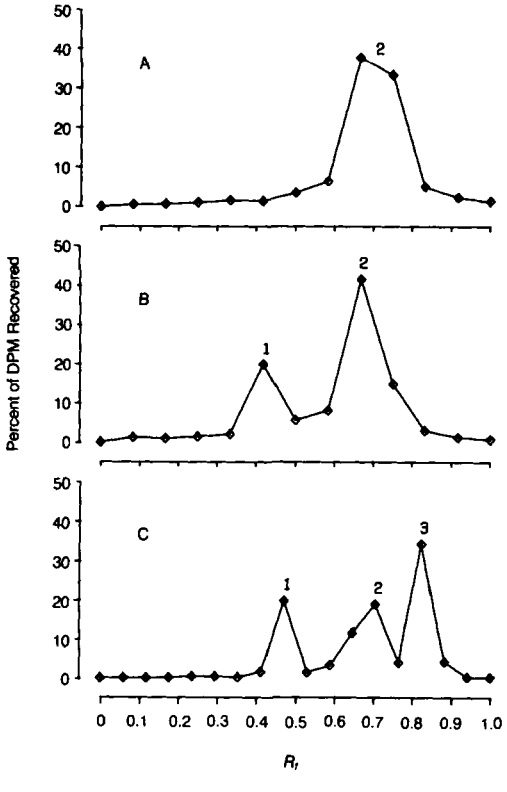



**Figure 1**—*Elution profiles of radioactivity from the conjugation of N-succinimidyl [2,3-$^3$H]propionate with lisinopril. Panel A: N-succinimidyl [2,3-$^3$H]propionate alone in borate buffer, pH 7.5. Panel B: lisinopril and N-succinimidyl [2,3-$^3$H]propionate at pH 7.5. Panel C: lisinopril and N-succinimidyl [2,3-$^3$H]propionate at pH 9.0.*

peak was observed from the control reaction where only *N*-succinimidyl [2,3-$^3$H]propionate was present in pH 7.5 borate buffer (Fig. 1, panel A). When lisinopril was added to the reaction at pH 7.5, two peaks of radioactivity occurred (Fig. 1, panel B), and when the reaction was carried out at pH 9.0 in borate buffer, three peaks were observed (Fig. 1, panel C). The binding of radioactivity from all of the peaks was assessed using the antiserum raised against the lisinopril–succinoylated hemocyanin immunogen at a final dilution of 1:700. Peak 1 (Fig. 1, panels B and C) was the only one to bind with the antiserum (91 and 92.5%, respectively) and radioactivity from the other peaks bound <1%. The estimated overall recovery of the radiolabel added to the reaction at pH 9.0 and 7.5 from the TLC was 54% and 63%, respectively, and the radioactivity from peak 1 was 19.7 and 19.9%, respectively, of this amount. In Fig. 1 (panel C), peaks 2 and 3 represent 64.5% of the recovered radiolabel; in panel B, peak 2 represents 64.2% of the recovered radiolabel. From the reaction containing only *N*-succinimidyl [2,3-$^3$H]propionate at pH 7.5 (Fig. 1, panel A), peak 2 represents 82.7% of radioactivity recovered. The prepared radiotracer obtained from peak 1 (Fig. 1, panels B and C) has proved to be stable over long periods of time (12 months) when stored frozen (−20°C) in portions suitably sized to avoid repeated thawing and refreezing. It is apparent that there is no advantage to running the reaction at pH 9.0, where additional side products are formed, when adequate tritium incorporation, into the lisinopril occurs under the milder conditions at pH 7.5.

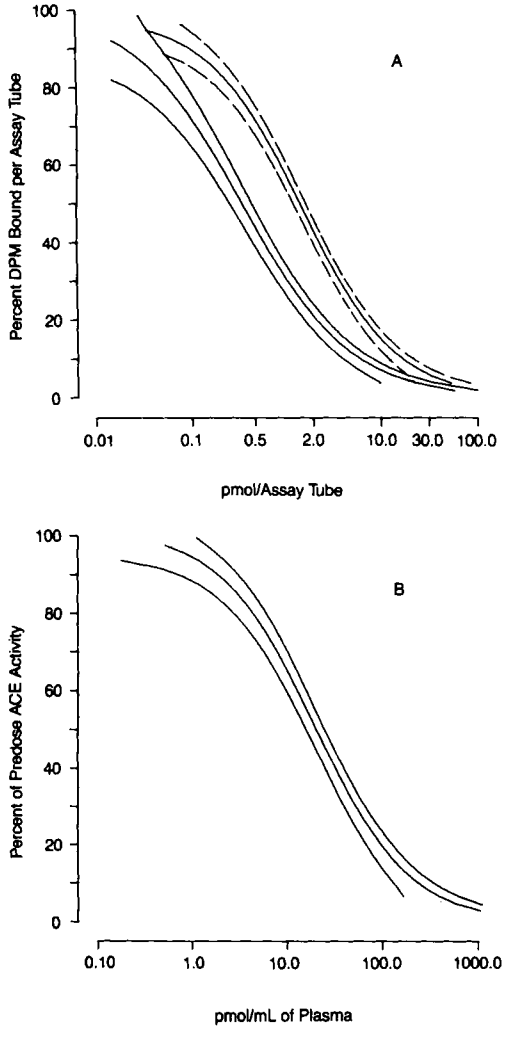



**Figure 2**—*Computer derived standard curves with 95% confidence limits. Panel* A: *RIA standard curves for lisinopril (solid line, n = 22) and enalaprilat (dashed line, n = 22). Panel* B: *ACE standard curve (n = 16).*

**Titer of Antiserum and Standard Curves**—The antiserum raised against the prepared immunogen was tested for its ability to bind the prepared radiotracer. A final dilution of 1:44,500 of the antiserum proved to be appropriate for 30% zero-binding. Standard curves for lisinopril and enalaprilat could then be constructed. Typical standard curves and computed 95% confidence limits are shown in Fig. 2A. Under incubation conditions, the effective range of the assay for lisinopril was 0.4–100 pmol/mL of plasma and for enalaprilat was 2–200 pmol/mL of plasma. Appropriate dilution of the samples allowed extension of the assay to concentrations >200 pmol/mL plasma. As a recent report has indicated, the effectiveness of lisinopril as an antihypertensive agent,[4] this antiserum will be useful in pharmacokinetic studies with lisinopril. At these detection limits, this assay would be capable of adequately determining enalaprilat or lisinopril plasma concentrations to low levels published from clinical trials.[4,12] Parallelism for the assay was observed when samples were diluted from 1/2 to 1/240 and no interfering effects of the plasma were found. Nonspecific binding was typically 1.5–2.0% of the total counts added.

Coefficients of variation for interassay variability for enalaprilat were 9.92% (n = 9) and 9.9% (n = 9) for known concentrations of 6.5 and 1.5 pmol/tube, respectively. The intra-assay CV for enalaprilat was determined to be 7.9% (n = 16) and 7.5% (n = 16) for concentrations of 6.5 pmol/tube and 1.5 pmol/tube respectively.

**Selectivity of the Antiserum**—Enalapril cross-reacted to 0.005% compared with enalaprilat. Other compounds, including captopril, hippuric acid, histidine, lysine, phenylalanine, proline, salicylic acid, tryptophan, and tyrosine, were unable to cross-react when tested at concentrations to 0.43 mM or where solubility became limiting, indicating the high selectivity of the antiserum. Heparin at 100 units per tube did not interfere with the assay.

The importance of the second carboxyl group on the enalaprilat molecule is shown by the much lower affinity of the antiserum for enalapril. Obviously, this carboxyl group is a significant immunological determinant since esterification with an ethyl group (as in enalapril) results in a compound with low cross-reactivity for the antibody. It has also been noted that the section of the molecule between the hapten and carrier protein (the bridge) may also be involved in the antibody formation process.[21,22] The greater affinity of the antiserum for lisinopril is probably another example of this, where the lysyl side chain is involved in the specific binding to the antibody.

**Angiotensin Converting Enzyme Standard Curve**—A standard curve with 95% confidence limits constructed using rabbit plasma ACE is shown in Fig. 2B. The effective range for enalaprilat concentrations to inhibit the ACE activity is similar to that for binding in the RIA. The inter- and intra-assay CV for the ACE assay were 10.4% (n = 15) and 8.6% (n = 5), respectively, at a concentration of 50 pmol/mL of enalaprilat.

**Plasma Concentrations**—Plasma concentrations of enalaprilat after administration of a 2.03 μmol/kg dose of enalapril to rabbits are shown plotted against time in Fig. 3. Up to 0.75 h following drug administration, enalaprilat concentrations were lower when estimated by the ACE inhibition method, but in the latter phases (0.75–6.0 h), were greater than the levels determined by the RIA. The MRT and $AUC_{0-6}$ measurements for both methods of drug assay are shown in Table I. The MRT values calculated for both assay methods were significantly different.

The linear-regression analysis of the enalaprilat concen-

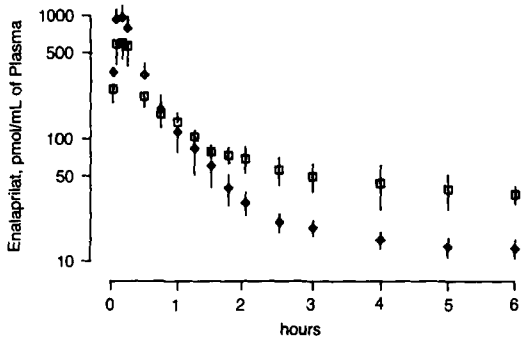



**Figure 3**—*Plasma concentrations of enalaprilat after intravenous administration of enalapril (2.03 μmol · kg⁻¹) to rabbits (n = 3). Key: (◇) RIA-determined drug levels; (□) ACE-determined drug levels. Results shown as mean ± SEM.*

**Table I—Calculated AUC and MRT for enalaprilat Following Intravenous Administration of Enalapril[a]**

| Parameters | Assay Method | |
|---|---|---|
| | RIA | ACE |
| $AUC_{0-6}$, pmol · h/mL | 534.9 (30.9%) | 562.9 (30.7%) |
| MRT, h | 0.827[b] (18.2%) | 1.521[b] (26.6%) |

[a] Values in parentheses are the percent SD. [b] Significantly different, $p < 0.05$.

trations estimated by both the ACE and RIA methods was computed. The data and fitted line are shown in Fig. 4. The equation from the regression analysis is: $y = 0.58x + 0.05$ when the enalaprilat concentrations determined by RIA are taken as the x variable (95% C.I. for the slope ±0.08 and y intercept ±0.02). A high correlation of the data was found ($r = 0.9$, $p < 0.01$), although a slope different from unity was obtained.

In this report, the extent of ACE inhibition is represented by the data showing transformation of percent inhibition of ACE into enalaprilat concentrations. It was anticipated that the regression analysis (Fig. 4) would directly correlate with a slope of unity, but this was not the case. Where the enalaprilat levels are lower when estimated by ACE inhibition (0–0.75 h), it is probable that enalaprilat concentrations are approaching those required to maximally inhibit the enzyme, which is also a region of imprecision within the assay. It should be noted that in the regression analysis (Fig. 4), those values from the early time points (Fig. 3) show a large variation around the regression line.

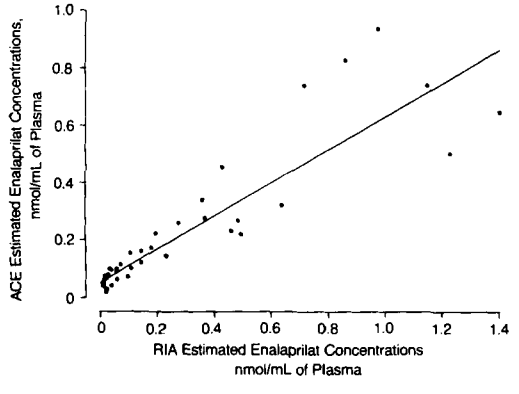



**Figure 4**—*Linear regression of estimated plasma enalaprilat levels by RIA (abscissa) and ACE inhibition (ordinate).*

In the latter stages, where the concentration of enalaprilat estimated by ACE activity is greater than those estimated by RIA, a mechanism based on the kinetics of enalaprilat binding to the enzyme and the conditions of standard preparation for the ACE assay may be the cause of the difference. Enalaprilat is a tight-binding, but slow-binding, inhibitor of plasma ACE,[23,24] which means a high degree of enzyme inhibition is achieved with comparable concentrations of inhibitor to enzyme, but at these concentrations the full extent of inhibition is relatively slow in onset.[24] However, with greater concentrations of inhibitor, the time for maximal inhibition is greatly reduced. In the preparation of the standard curves for the estimation of enalaprilat concentration by ACE activity, inhibitor was added to drug-free plasma and allowed to stand 30–60 min prior to assay for ACE activity, assuming full inhibition would be achieved. Using literature values for turnover number, $K_m$ for hippuryl-histidyl-leucine[25,26] as a substrate for ACE, and the kinetic on-and-off rate constants for the binding of enalaprilat to ACE,[23,27] the percent inhibition of ACE was simulated using the SAAM/CONSAM programs[28,29] over a range of inhibitor concentrations to assess the effect of non-equilibrium on the standard curves. Values simulated at 45 and 405 min were not parallel, with increasing differences apparent at lower concentrations of inhibitor. This indicates that, under conditions where equilibrium has not been attained in the preparation of the standards, greater concentrations of inhibitor will be estimated in the plasma sample than are actually present. Unless appropriate precautions are taken, the use of endogenous ACE activity as a means of measuring enalaprilat concentrations could result in serious error.

In another study, workers used a partially purified preparation of porcine plasma ACE to estimate the concentration of enalapril and enalaprilat in biological samples and compared this with isotopically determined concentrations. A high correlation was obtained with a slope of 1.18 which suggests that using an exogenous source of ACE might be more suitable than utilizing the extent of endogenous ACE inhibition.

In order to avoid problems of endogenous plasma ACE competing with the antiserum for both radiotracer and enalaprilat, EDTA (25 mM) was included in the incubation medium for chelating the zinc atom of the ACE. The removal of zinc from the ACE has been shown to decrease the affinity of ACE for enalaprilat by a factor of $2.5 \times 10^4$.[27] Therefore, under these conditions, it would be unlikely to observe any interference in the RIA from endogenous ACE.

Using gas chromatographic-mass spectrometric techniques, Tocco et al.[5] did not discover metabolites of enalapril, other than enalaprilat, in dogs. Pang et al.[7] also found no significant evidence of other metabolites produced from isolated perfused rat liver. It is possible that the rabbit, also, does not produce metabolites of enalapril other than enalaprilat which may interact with the antibody used in the RIA. It would be unlikely for enalapril to interfere with the assay as the cross-reactivity is negligible.

Enalapril concentrations can also be determined indirectly using the RIA by hydrolysis of the ethyl ester group to transform enalapril to enalaprilat using either base hydrolysis or a rat liver homogenate with esterase activity. Enalapril concentrations can then be calculated from the difference between this and a nonhydrolyzed sample. This approach would not be readily applicable for our described endogenous ACE method of determining enalapril concentrations.

The results of the $AUC_{0-6}$ calculations for both assay methods were not different, but the calculation of MRT yielded a significantly greater value for the data from ACE (see Table I). Although inhibition of plasma ACE from a single dose has been shown to persist for at least 24 h in

humans[10] it does not mean that the larger MRT value is the real value in view of the above discussion and the fact that enalaprilat is a remarkably potent inhibitor of ACE.[27]

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## Acknowledgments


This work was supported by a National Health and Medical Research Council of Australia Program Grant. PJW was the recipient of an NH & MRC Biomedical Postgraduate Research Scholarship. The authors would also like to thank Merck Sharp & Dohme for the provision of enalapril, enalaprilat, and lisinopril, and Dr. F. A. O. Mendolsohn for the assays of ACE activity in the plasma samples.