Determination of enalapril and enalaprilat by enzyme linked immunosorbent assays: application to pharmacokinetic and pharmacodynamic analysis

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Keywords
ELISA, enalapril, enalaprilat, pharmacodynamics, pharmacokinetics

Received 26 November 2001; revised 28 January 2002; accepted 1 March 2002

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ABSTRACT

We have developed two enzyme linked immunosorbent assay (ELISA) methods for determining enalapril and enalaprilat in plasma. In this study, 48 healthy subjects received an oral dose of either 10 or 20 mg of enalapril and plasma concentrations of enalapril and enalaprilat were determined by their specific ELISA methods. These plasma concentrations and blood pressure measurements were applied to evaluate the pharmacokinetic (PK) and pharmacodynamic (PD) parameters of both enalapril and enalaprilat. The enalapril values for the area under the curve (AUC0–∞) were 480 ± 216 and 832 ± 325 ng·h/mL, maximum plasma concentrations (Cmax) were 310 ± 187 and 481 ± 185 ng/mL, and times required to reach the maximum concentration (tmax) were 1.13 ± 0.22 and 1.09 ± 0.33 h for 10 and 20 mg doses, respectively. The enalaprilat values for AUC0–∞ were 256 ± 122 and 383 ± 158 ng·h/mL, Cmax values were 57 ± 29 and 72.9 ± 33.6 ng/mL, and tmax values were 4.28 ± 1.45 and 4.05 ± 0.12 h for 10 and 20 mg doses, respectively. The Cmax values of enalaprilat were ~10 times higher than those in the literature, which were determined by angiotensin converting enzyme (ACE) inhibition assays following alkaline hydrolysis, but similar to those of enalaprilat. The PD profiles revealed a significant correlation between enalaprilat concentrations in plasma and the decrease in systolic and diastolic blood pressures (r = -0.95 with P < 0.001 and r = -0.95 with P < 0.001), respectively, following a single oral dose of enalapril. These ELISA methods have the advantage of being simple, accurate, sensitive, and do not depend on enalaprilat binding to ACE. Such methods can be used for analysis and kinetic testing of enalapril and enalaprilat in biological fluids.

INTRODUCTION

Enalapril maleate, N-(N-S-1-ethoxycarbonyl-3-phenyl-propyl)-L-alanyl-L-proline hydrogen maleate, is a prodrug that requires de-esterification to an active diacid metabolite enalaprilat [1]. Enalaprilat is the active angiotensin converting enzyme (ACE) inhibitor that has been shown to be effective in the treatment of hypertension and congestive heart failure by dilating peripheral vascular resistance without causing significant change in heart rate or cardiac output [2–4]. Following oral administration of enalapril in healthy subjects, absorption is rapid [2–5]. Maximum plasma concentrations of unchanged enalapril are reached in about 1 h and it disappears from circulation after about 4 h. However, maximum plasma concentrations of the active metabolite, enalaprilat, are reached in about 3–4 h after enalapril administration [3–5].

The available methods for measuring enalapril in plasma depends on the conversion of enalapril to enalaprilat by alkaline hydrolysis and then assaying the latter form by a fluorometric [1] or radio-enzymatic
MATERIALS AND METHODS

Materials
All the following chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO, USA): bovine serum albumin, ovalbumin, avidin, glutaraldehyde (25%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), N-hydroxysuccinimide ester, goat antirabbit alkaline phosphatase conjugate, p-nitrophenyl phosphate, tween-20, sodium azide and diethanolamine. Tris buffer and sodium chloride were purchased from Acros Organics (New Jersey, NY, USA). Biotin-X-hydrazide was purchased from Calbiochem Co., UK. Microtiter 96-well flat bottom plates were purchased from Greiner, Labor-technik, Germany. ELISA WellScan reader and Wellwash were obtained from Denley Co. (Billinghurst, UK).

Subjects, protocol and blood sampling
The blood sampling was conducted at the Jordan Red Crescent and at Al-Mowasah Hospitals in Amman-Jordan and was approved by the Ethical Committee at both hospitals. The study was performed in accordance with the relevant articles of the Declaration of Helsinki (1964) as revised in Tokyo (1975), Venice (1983), Hong Kong (1989), and Somerset West, RSA (1996). Each volunteer has agreed to participate in the study and signed an informed consent prior to the initiation of the study.

Prior to the study the participants were asked not to take any medication for two weeks before the study or during the course of the investigation. A comprehensive check-up for each volunteer was performed, including physical examination, clinical chemistry, haematology evaluation and urine analysis, which revealed no evidence of disease in any of the volunteers.

Forty-eight healthy male volunteers aged between 18 and 43 years (31 ± 7.4) and weighing 60–90 kg (74.1 ± 8.6), received either a 10 or 20 mg oral dose of enalapril. An oral dose containing either 10 or 20 mg of enalapril was given in the morning with 240 mL of water for each of the 48 volunteers. Blood samples (3–4 mL) were collected in heparinized tubes at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2.0, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 12, and 24 h post dosing. Plasma was separated and stored at −20 °C until the date of analysis. Blood pressure was measured at 0, 1, 2, 4, 6, 8, 12 and 24 h post dosing. A light breakfast was given to all participants after 2 h of the oral dose. A standard lunch was given to all participants after 8 h of drug intake.

Reagents for ELISA of enalapril
The reagents for ELISA was prepared as described in details elsewhere [10–12]. Briefly, antienalapril antibodies were raised in rabbits (White New Zealand) against BSA-enalapril conjugate, which was made first by separating enalapril from maleic acid by HPLC. Secondly, the purified enalapril was linked to BSA via carbodiimide and then purified by gel chromatography. Following immunization, the cross-reactivity of the antisera was tested with enalapril, enalaprilat, proline, phenylalanine, alanine-proline, and captoril. All, except enalapril, showed no binding with the antisera.

Enalapril was linked to biotin via its carbohydrate group using long chain biotin (biotin-X-hydrazide). The biotin was conjugated to enalapril using carbodiimide and N-hydroxysuccinimide ester. The complex was purified by HPLC and tested for binding with the antienalapril antisera (Ibarani, pers. comm.).

The standards of enalapril assay was prepared by dissolving 1 mg of enalapril in 1 mL of dlH2O and then diluted in pooled heparinized plasma to prepare a stock solution of 200 μg/mL. From that stock solution, 1000, 500, 200, 100, 50, 10, 1 and 0 ng/mL of enalapril standards were prepared.

ELISA for enalapril
An ELISA was used for the determination of enalapril in plasma samples [10–12]. Briefly, flat bottom 96-well microtiter plates were coated with 0.1 mL of 5 μg/mL of avidin in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6, overnight at room temperature. Wells
were washed three times with 25 mM Tris-HCl buffer, pH 7.3, containing 0.05% Tween-20, 0.15 M NaCl, and 0.02% sodium azide, blocked with the same washing buffer for 30 min, and washed twice. Biotin-enalapril conjugate (1:8000) was added (0.1 mL/well), incubated for 30 min, and the wells then were washed twice. In duplicates, plasma standards or samples were added (10 μL/well) followed by the addition of antienalapril antisera (0.1 mL) diluted (1:6000) in assay buffer 150 mM Tris-HCl buffer, containing 1% BSA, 0.05% Tween-20, 0.15 M NaCl, 20 mM EDTA and 0.02% sodium azide). The wells were left incubating at 22 °C for 1 h with orbital shaking. The wells were then washed three times, and antirabbit antibody alkaline phosphatase conjugate, diluted in assay buffer without EDTA, was added as 0.1 mL/well and left incubating for 1 h at 22 °C with orbital shaking. The wells were washed four times, and 0.1 mL/well of 1 mg/mL of p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, containing 1 mM MgCl₂ and 0.02% sodium azide was added. After 30 min, colour development was stopped by the addition of 2 M NaOH (0.1 mL/well), and absorbance was read at 405 nm and 600 nm differential filters. Absorbance values were transformed to logit binding to construct a straight line on a semilog graph.

Reagents for ELISA of enalaprilat

The reagents for ELISA was prepared as described in details elsewhere [10–12]. The method is based on developing antibodies to the drug lisinopril because lisinopril is a lysyl analogue of enalaprilat. Lisinopril was conjugated to BSA or OVA via glutaraldehyde [10–12]. Briefly, antilisinopril antisera were raised in rabbits (White New Zealand) against BSA-lisinopril conjugate. Following immunization, the cross-reactivity of the antisera was tested with enalapril, enalaprilat, proline, phenylalanine, alanine-proline, and captopril. All, except enalaprilat, showed no binding with the antisera.

The standards for enalaprilat assay was prepared by dissolving 1 mg of enalaprilat, prepared from enalapril by base hydrolysis using sodium hydroxide, and was confirmed by HPLC and mass spectra, in 1 mL of dH₂O (femtol. perm. comm.). The enalaprilat was then diluted in pooled heparinized plasma to prepare a stock solution of 200 μg/mL. From that stock solution, 500, 200, 100, 50, 10, 1 and 0 μg/mL of enalaprilat standards were prepared.

ELISA for enalaprilat

An ELISA was used for the determination of enalaprilat in plasma samples [10–12]. Briefly, flat bottom 96-well microtiter plates were coated with 0.1 mL of 12.6 μg/mL of OVA-lisinopril in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6, overnight at room temperature. Wells were washed three times with 25 mM Tris-HCl buffer, pH 7.3, containing 0.05% Tween-20, 0.15 M NaCl, and 0.02% sodium azide, blocked with the same washing buffer for 30 min, and washed twice. In duplicates, plasma standards or samples were added (50 μL/well), followed by the addition of antilisinopril antisera (1:1000, 50 μL/well) diluted in assay buffer 50 mM Tris-HCl buffer, containing 1% BSA, 0.05% Tween-20, 0.15 M NaCl, 20 mM EDTA and 0.02% sodium azide). The wells were left incubating at 22 °C for 1 h with orbital shaking. The wells were then washed three times, and antirabbit antibody alkaline phosphatase conjugate, diluted in assay buffer without EDTA, was added in 0.1 mL/well and left incubating for 1 h at 22 °C with orbital shaking. The wells were washed four times, and 0.1 mL/well of 1 mg/mL of p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, containing 1 mM MgCl₂ and 0.02% sodium azide was added. After 30 min, colour development was stopped by the addition of 2 M NaOH (0.1 mL/well), and absorbance was read at 405 nm and 600 nm differential filters. Absorbance values were transformed to logit binding to construct a straight line on a semilog graph.

Data analysis

Non-compartmental PK calculations were used for the evaluation of the area under the plasma concentration–time profile from time zero to time t (AUCₜ), zero to infinity (AUC₀₋∞) elimination half-life (t¹/₂) and minimum residual time (MRT) [13]. Briefly, AUCₜ was calculated using the linear trapezoidal rule, where t is the last measurable time point, which begins at time 0 and finishes at the last quantifiable point. The area under the plasma concentration–time profile from time zero to infinity (AUC₀₋∞) was calculated by adding AUCₜ to the value of dividing the last measurable concentration at time t over elimination rate constant. The elimination t₁/₂ was calculated from the slope of the semilogarithmic of the last plasma concentration points vs. time. The MRT was calculated by dividing the area under a moment curve from time 0 to infinity (AUMCₜ₋∞) over AUC₀₋∞. The maximum plasma drug concentration (Cmax) and time to reach the maximum drug concentration (tmax) were calculated by averaging the highest plasma concentration and its corresponding time of the drug or metabolite in each individual.
Statistical analysis
A two-sided t-test was used to compare the PK parameters obtained at the 10 and 20 mg doses and the differences in blood pressure at each time point. In addition, a Pearson correlation test with ANOVA two-sided significance was used to evaluate if there was an association between the change in blood pressure and enalaprilat or enalapril plasma concentrations.

RESULTS
ELISA for enalapril
The standard curves constructed exhibited good linearity ($r^2 = 0.989–0.995$) following transforming the absorbance values to logit binding and plotted vs. concentration of enalapril on a semilog paper (Figure 1a). The intra- ($n = 10$) and inter-assay ($n = 8$) assay accuracy (% recovery) ranged from 90.2 to 110.5% and 98.9–102%, respectively, over 1–1000 ng/mL range of enalapril in human plasma. The intra- and inter-assay precision (% CV) ranged from 1.06 to 9.98% and 5.06–7.09%, respectively, over 1–1000 ng/mL range of enalapril in human plasma.

ELISA for enalaprilat
The standard curves constructed exhibited good linearity ($r^2 = 0.988–0.996$) after transforming the absorbance values to logit binding and plotted vs. concentration of enalaprilat on a semilog paper (Figure 1b). The intra- ($n = 9$) and inter-assay ($n = 9$) assay accuracy (% recovery) ranged from 80.7 to 117.3% and 87.6–111.0%, respectively, over 1–500 ng/mL range of enalaprilat in human plasma. The intra- and inter-assay precision (% CV) ranged from 1.87 to 9.87% and 1.89–7.47%, respectively, over 1–500 ng/mL range of enalaprilat in human plasma.

Kinetic analysis for enalapril and enalaprilat
The plot of the plasma concentration–time curve and the calculated PK parameters for enalapril are presented in Figure 2(a,c) and Table I. The AUC$\text{[0→∞]}$ values for enalapril were 480 ± 216 and 832 ± 325 ng/h/mL for 10 and 20 mg doses, respectively. The $C_{\text{max}}$ values for enalapril were 310 ± 187 and 481 ± 185 ng/mL for 10 and 20 mg doses, respectively (Table I). In addition, $T_{\text{max}}$ values were 1.13 ± 0.22 and 1.09 ± 0.33 hours for 10 and 20 mg doses of enalapril, respectively. Furthermore, the elimination $t_{1/2}$ values were 1.31 ± 0.99 and 0.93 ± 0.37 h for the 10 and 20 mg doses of enalapril, respectively. In comparison between the two doses, AUC$\text{[0→∞]}$ and $C_{\text{max}}$ were the only values that showed statistically significant differences (Table I).

The plot of the plasma concentration–time curve and the calculated PK parameters for enalaprilat are presented in Figure 2(b,c) and Table I. The values of AUC$\text{[0→∞]}$ for enalaprilat were 256 ± 122 and 383 ± 158 ng/h/mL for 10 and 20 mg doses, respectively. The $C_{\text{max}}$ values for enalaprilat were 57 ± 29 and 72.9 ± 33.6 ng/mL for 10 and 20 mg doses, respectively (Table I). In addition, $T_{\text{max}}$ values were 4.28 ± 1.45 and 4.05 ± 1.22 hours for 10 and 20 mg doses of enalaprilat, respectively. Furthermore, the elimination $t_{1/2}$ values were 3.47 ± 2.47 and 3.95 ± 0.248 h for the 10 and 20 mg doses of enalaprilat, respectively. In comparison between the two doses, AUC$\text{[0→∞]}$ and $C_{\text{max}}$ were the only values that showed statistically significant differences (Table I).

Blood pressure and its correlation with enalapril and enalaprilat concentrations
Both doses of enalapril resulted in a significant decrease ($P < 0.001$) in the blood pressure (systolic and diastolic)
PK and PD of enalapril and enalaprilat determined by ELISA

Figure 2 (a) Plasma enalapril concentrations profile after oral administration of 10 (▲) and 20 mg (■) enalapril. Error bars represent ± SE. (b) Plasma enalaprilat concentrations profile after oral administration of 10 (▲) and 20 mg (■) enalapril. Error bars represent ± SE. (c) Plasma enalapril (dashed lines) and enalaprilat (solid lines) profiles on semilog after 10 (▲) and 20 mg (■) oral administration of enalapril. Error bars represent ± SE.

(Figure 3a,b). The maximum range of decrease in the systolic (6-7.6% and 11-12%) and diastolic (11.3-12.5% and 17.2-18.5%) blood pressures were at 4-6 h post dosing of 10 and 20 mg doses of enalapril, respectively. In addition, a significant correlation between enalaprilat concentrations in plasma and the
Table 1 Pharmacokinetic parameters of enalapril and enalaprilat following 10 and 20 mg oral administration of enalapril maleate.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>10 mg dose</th>
<th>20 mg dose</th>
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<tbody>
<tr>
<td></td>
<td>Enalapril</td>
<td>Enalaprilat</td>
</tr>
<tr>
<td>AUC$_{0-24}$ (ng/min/ml)</td>
<td>480 ± 216</td>
<td>256 ± 122</td>
</tr>
<tr>
<td>C$_{max}$ (ng/ml)</td>
<td>310 ± 187</td>
<td>57 ± 29</td>
</tr>
<tr>
<td>T$_{max}$ (h)</td>
<td>1.13 ± 0.52</td>
<td>4.28 ± 1.45</td>
</tr>
<tr>
<td>T$_{1/2}$ (h)</td>
<td>1.31 ± 0.09</td>
<td>3.47 ± 0.47</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.4 ± 1.0</td>
<td>7.4 ± 2.8</td>
</tr>
</tbody>
</table>

$^1P < 0.0001$ when compared to 10 mg dose, $^2P = 0.0032$ when compared to 10 mg dose, $^3P = 0.0026$ when compared to 10 mg dose, $^4P = 0.023$ when compared to 10 mg dose.

Figure 3 (a) Systolic blood pressure after 10 (▲) and 20 mg (●) oral administration of enalapril. Error bars represent ± SE. (b) Diastolic blood pressure after 10 (▲) and 20-mg (●) oral administration of enalapril. Error bars represent ± SE.

Figure 4 (a) Correlation between the percent decrease in systolic blood pressure and the concentration of enalapril in plasma ($r = 0.95$). (b) Correlation between the percent decrease in diastolic blood pressure and the concentration of enalapril in plasma ($r = 0.95$). Each point represents the mean of blood pressure to all individuals at time $t$ with its corresponding enalaprilat concentration.

between the decrease in blood pressure and enalapril concentrations.

Adverse effects
No serious adverse effects occurred in the course of the study. Only mild adverse effects were noticed in 40% of the subjects, such as headache and dizziness.

DISCUSSION
ELISA methods were developed to measure enalapril and enalaprilat in plasma. The PK parameters for enalapril for
such as AUC\textsubscript{0-\infty}, T\textsubscript{max}, elimination t\textsubscript{1/2} and MRT were all similar to those published in the literature [2-5]. In addition, the PK parameters for enalaprilat as AUC\textsubscript{0-\infty}, C\textsubscript{max}, T\textsubscript{max}, elimination t\textsubscript{1/2} and MRT were similar to those published in the literature [3,14,15]. However, C\textsubscript{max} values for enalapril obtained using ELISA following 10 and 20 mg doses of enalapril were ~10 times higher than values obtained by ACE inhibition assay.

The high enalapril plasma samples were tested for enalapril binding to proteins by running plasma samples at 0.5 h and 3 h post dosing on gel filtration column (Sephadex G 25, 32 ml gel). The eluted enalapril from the plasma sample were at the same position as the standard free enalapril whereas in the 3 h sample ~20% of it only eluted with protein peak (data not shown). In addition, plasma-containing enalapril or standards of enalapril solution was incubated with 10 Units/ml of carboxyesterase enzyme for 24 h at room temperature samples and then followed by ELISA testing. Carboxyesterase is expected to convert enalapril to enalaprilat. Samples treated with the enzyme did not bind with the antienalapril antibodies, which means that the antienalapril antibodies only bind to enalapril and do not cross react with enalaprilat (data not shown).

Several reports analyzed enalapril using fluorometric ACE inhibition assay following alkaline hydrolysis. In the former method, the assay depends on the kinetics and binding of the drug to the enzyme. Using radioimmunoassay, Worland et al. [8] have shown that enalaprilat concentrations were ~two-times higher than those concentrations obtained by ACE inhibition assay especially those between 0 h and 0.75 h post intravenous administration of enalapril to rabbits. It was postulated that enalaprilat concentrations were approaching those required to maximally inhibit the enzyme and therefore, the full extent of ACE inhibition is relatively slow in onset [8]. This, in part, explains why in our assay the C\textsubscript{max} enalapril results were much higher than those in the literature. On the other hand, enalaprilat concentrations following 0.75 hours were found to be higher using ACE inhibition assay than radioimmunoassay [8]. In our study, enalaprilat concentrations at 12 and 24 h were lower than in the literature that were determined by ACE inhibition assay [1].

In this study, the blood pressure returned to predose level within 12–24 h following a single 10 and 20 mg oral dose of enalapril. This, however, is not as described in the literature that blood pressure reduction lasts for 24–36 h. The plasma concentration values of enalaprilat at 12 and 24 h were lower than those in the literature but showed significant correlation with the lower reduction in the blood pressure (Figure 4a,b). It has to be mentioned that the age of the healthy subjects in this study may in part explain the lower hypotensive effect at 12 and 24 h post dosing. It has been shown that the apparent clearance of enalapril after oral administration and the clearance of enalaprilat after intravenous administration were about 30% lower in elderly than young individuals [3].

CONCLUSION

Two ELISA methods were developed to measure enalapril and enalaprilat in plasma. These ELISA methods revealed that C\textsubscript{max} for enalapril were different than those in the literature, and therefore, re-evaluation of the PK of enalapril in humans should be addressed. In addition, ELISA adds the advantage of being simple, accurate, and sensitive. Such a method can be used in kinetic studies and therapeutic drug monitoring of enalapril and enalaprilat in biological fluids, following long-term use of enalapril, especially with its known accumulation property in patients with congestive heart failure and chronic renal insufficiency [15,16].

REFERENCES


