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# High-performance liquid chromatographic determination of enalapril in human plasma by enzyme kinetic analytical method

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**Abstract**

A high-performance liquid chromatographic method for indirect determination of enalapril in human plasma, was developed and validated. An exogenous angiotensin converting enzyme after drug inhibition was determined by reacting with hippuryl–histidyl–leucine to produce hippuric acid (HA) which was inversely proportional to the amount of enalaprilat in plasma. The HPLC was carried out on a Lichrosphere® 60RP–select B, C18, 5 μm (125 mm × 4.0 mm i.d.) column at flow rate of 1.0 ml/min. The analysis time per injection was within 6.5 min. The lowest concentration of enalaprilat to be quantitated was 3.0 ng/ml with the acceptable accuracy and precision. This successfully developed method was practically and accurately used for pharmacokinetics and bioequivalent study of enalapril.


*Keywords:* Enzyme kinetic analytical method; High-performance liquid chromatography; Enalapril in plasma; Enalaprilat in plasma; Angiotensin converting enzyme inhibitor

## 1. Introduction

Enalapril is a prodrug which is hydrolyzed after absorption forming the active angiotensin converting enzyme (ACE) inhibitor, enalaprilat. Enalaprilat inhibits ACE on the rennin–angiotensin system affecting angiotensin II formation. The action of angiotensin II in the body, not only as the vasoconstrictor, but also increases the re-absorption of sodium and has non-hemodynamically and hemodynamically mediated effects to cardiovascular structure. Therefore, enalapril has been used as the potential drug for the treatment of hypertension and congestive heart failure [1–5].

According to chemical structures of enalapril and enalaprilat (Fig. 1), the properties of ultraviolet absorption could only be characterized from an unconjugated phenyl moiety resulting the very low absorptivity values of both compounds [6]. Even though the determination of enalapril or enalaprilat in pharmaceutical products at the microgram concentration level, applying the derivative ultraviolet spectrometry or high-performance liquid chromatography (HPLC) with ultraviolet (UV) detector have been reported [7–9], the determination of enalapril and/or enalaprilat in plasma samples at the nanogram level utilizing HPLC with UV detection is hardly achieved [10]. For pharmacokinetic and bioequivalent studies of enalapril, the reliable and convenient analytical technique is needed. However, only indirect analytical methods have been reported including pre-column derivatization for HPLC, radiometric, enzyme inhibition and radioimmuno-methods [10–12]. These indirect methods involve tedious sample preparations that are inconvenient and time consuming. In addition, special equipments are required for those assays with radioactive compounds. Recently, the liquid chromatography/tandem–mass spectrometry for direct determination of enalapril and enalaprilat in plasma has been reported [13]. However the instrument is too expensive to be practically used in most laboratory. Leverage these

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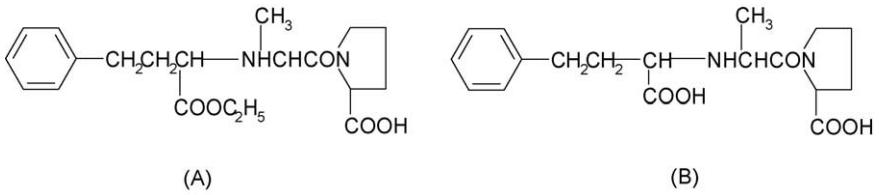


Fig. 1. Chemical structures of enalapril (A) and enalaprilat (B).

constraints, the enzyme kinetic analytical method for ACE inhibitor drug determination is proposed in this study.

Enzyme kinetic analytical method is defined as the method of analysis based on enzyme kinetic principle. To express any enzyme kinetic, the activity of enzyme that catalyzing enzyme–substrate reaction have to be determined either in term of rate of product formation or substrate utilization [14,15]. Therefore, by measuring ACE activity related to ACE inhibitor drug, enalaprilat, the analysis of enalaprilat could be performed. The method is simple and does not require any sample extraction. The method was modified from ACE determination [16,17], with the development of sample preparation and chromatographic analysis.

## 2. Experimental

### 2.1. Reagents and chemicals

Enalapril and enalaprilat as reference compounds were kindly supplied by Merck Sharp and Dome Research Laboratory (West Point, Pennsylvania, USA). Analytical grade hippuric acid (HA), hippuryl–histidyl–leucine (HHL) and 2-methyl hippuric acid were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was obtained from Lab-Scan (Thailand). Analytical grade sodium chloride, sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate anhydrous and sodium dodecyl hydrogen sulfate (SDS) were all from Merck (Darmstadt, Germany).

### 2.2. Human plasma

Human plasma for method development was generously supplied by the Plasma Division, Thai Red Cross Society, Thailand.

### 2.3. Preparation of standards

Individual stock solutions of enalapril and enalaprilat was prepared in methanol to give the concentration of 1 mg/ml. For standard samples, the appropriate volumes of enalaprilat stock solution were pipetted into volumetric tubes, the solvent was dried off before adding blank plasma to give enalaprilat concentration range of 10–120 ng/ml plasma. For 2-methyl hippuric acid as an internal standard (IS), the stock solution of 90 μg/ml in acetronitrile was prepared. These stock solutions were kept frozen and used within 1 month. The stock solution of HHL 2.5 mM have to be freshly prepared in phosphate saline solution (pH 8.3).

### 2.4. Preparation of solutions

A phosphate saline solution was consisted of 0.1 M di-sodium hydrogen phosphate and 0.3 M sodium chloride, the solution was adjusted to pH 8.3 with 8% phosphoric acid. The mobile phase buffer was the mixture of 40 mM sodium dihydrogen phosphate and 2 mM SDS, adjusted to pH 2.3 with 1 M phosphoric acid.

### 2.5. Chromatographic conditions

A spectral system TSP consisted of P4000 gradient pump, AS3000 autosampler and UV2000 detector were used. Data acquisition was performed on a PC1000 system software integrator.

Separations were accomplished on a reversed phase Lichrosphere® 60RP-select B, C18, 5 μm (125 mm × 4.0 mm i.d.) column from Merck (Darmstadt, Germany) and operated at ambient temperature (27 ± 1 °C). The mobile phase consisted of acetonitrile and mobile phase buffer, was set to gradient condition from the volume ratio of 13:87 at time 0–7.4 min to 75:25 from time 7.4 to 30 min with the equilibration time of 10 min before next injection. The mobile phase was delivered at constant flow rate of 1.0 ml/min. The effluent was monitored at 228 nm. The mobile phase was freshly prepared and degassed by ultrasonication before used.

### 2.6. Sample preparation

A series of calibration standard samples were analyzed along with plasma samples. A 50 μl of either standard or plasma sample was mixed with 1.0 ml of phosphate saline solution. The mixture was heated at 75 °C for 5 min to destroy ACE in the sample. The mixture was then cooled down to room temperature before transferring only 100 μl into centrifuge glass tube containing 100 μl of human plasma as standard exogenous ACE. This treated mixture was incubated in the shaker bath with regulated temperature of 37 ± 1 °C for 10 min before adding 50 μl of 2.5 mM HHL. The ACE–HHL reaction (Fig. 2) was proceeded for 60 min. A solution of methanol containing 8% phosphoric acid (2:1, v/v) in the volume of 200 μl was added into the reaction mixtures to stop the ACE–HHL reaction. A 1.0 ml of IS (3.6 μg/ml in acetonitrile) was then added. The tube was centrifuged at

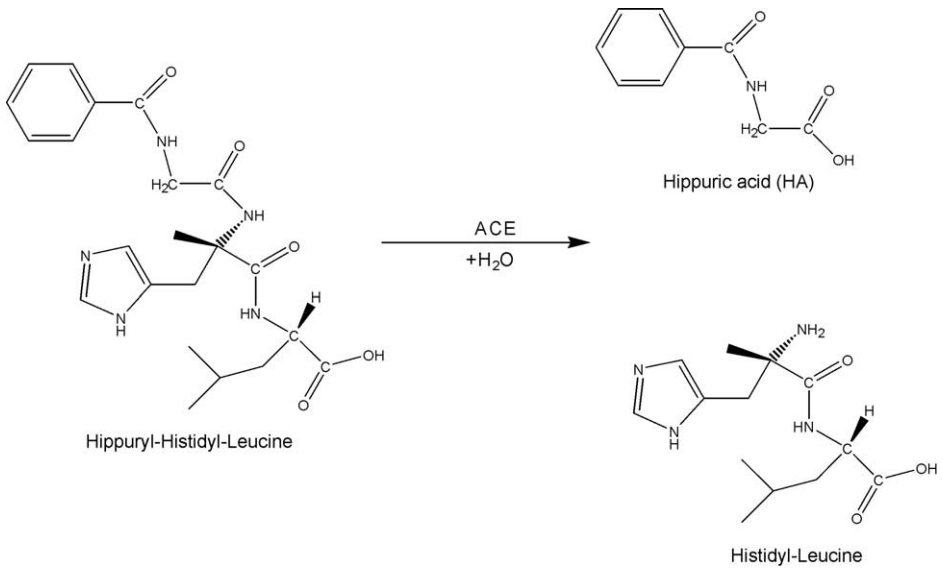


Fig. 2. The reaction between ACE and HHL as substrate to yield hippuric acid.

3200 × *g* for 10 min and only supernated solution was carried to HPLC analysis with the injection volume of 10 μl. This developed analytical method was validated according to the bioanalytical method validation.

### 2.7. Validation of the method

The linearity of the HPLC method for determination of enalaprilat was evaluated by analyzing spiked plasma standards containing nine different concentrations of enalaprilat. They were 5, 10, 15, 20, 40, 60, 80, 100 and 120 ng/ml plasma, respectively. The analysis was processed according to the aforementioned procedure. The peak area ratio of HA and IS was plotted against enalaprilat concentrations to determine the pattern of the calibration curve before doing the regression analysis. The reproducibility of linearity was confirmed by analyzing the other three series of spiked plasma standards of enalaprilat.

Spiked plasma standards of enalaprilat at 10, 40, 80 and 100 ng/ml were assayed in the set of six replicates within 1 day to evaluate the intra-day accuracy and precision. For the inter-day assay, the same analysis was used but performed on separated different days.

The lowest limit of quantitation (LOQ) for enalaprilat in plasma was determined as enalaprilat plasma concentration that could reduce the detectable HA to at least 10% comparing to drug free concentration.

Three replicates of 10, 40 and 80 ng/ml of spiked plasma enalaprilat were used for stability studies. The stability of enalaprilat in plasma at laboratory room temperature was determined in term of the difference of mean peak area responses of HA at 5 and 10 h comparing to the initial time. The stability of IS was also determined. For the stability at storage temperature (−48 °C), the mean concentration of enalaprilat in plasma at time 15, 30 and 45 d were determined comparing to the initial control concentration. The stability of processed analyte in autosampler in term of mean peak area responses of HA and also IS were determined up to 45 h comparing between 0 and 4 °C. For freezed–thaw cycle stability, enalaprilat concentrations in plasma were determined up to three cycles.

### 2.8. Application for pharmacokinetic study

Twelve adult healthy males with the age range of 25–40 were included in the study. They have to pass the physical examination criteria and have not taken any medication and alcohol for at least 1 week prior to the study. Each fasted subject was administered a single dose of 5 mg Renitec® with a 200 ml of water. Blood samples were withdrawn from the forearm vein following administration and at consecutive sampling time till 14 h post dose. Plasma was obtained through centrifugation at 3000 × *g* for 10 min and stored at −48 °C for subsequent analysis within 2 weeks. Plasma enalaprilat concentration–time profile was generated for each individual such that peak plasma concentration ($C_{max}$) and the time to peak concentration ($t_{max}$) could be visually determined. Linear trapezoidal method was utilized for area under the plasma concentration time curve (AUC) calculation.

## 3. Results and discussion

### 3.1. Enzyme kinetic analytical method for enalapril and enalaprilat determinations

As an ACE inhibitor, enalaprilat concentration in plasma sample should be related to ACE activity. ACE activity could be determined by either reacting with natural or synthetic substrate [18]. This study selected HHL, synthetic substrate which have been proven for its high specificity to ACE [16]. The appropriate reaction time between ACE and HHL at 37 °C was determined by measuring the peak area of HA

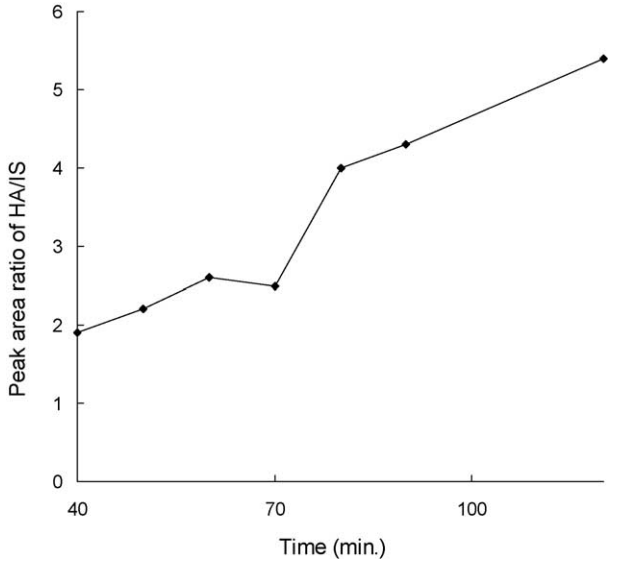


Fig. 3. ACE–HHL reaction (37 °C) at different incubation time.

formation and IS at different reaction times of 40, 50, 60, 70, 80, 90 and 120 min. The formation of HA showed the least variable at the reaction time of 60 min (Fig. 3). Therefore, this 60 min time period was used for further analysis.

As already reported, the level of endogenous ACE could be affected by various factors including disease state [16,19]. This causes the variation of endogenous ACE among individual. To eliminate this uncontrollable endogenous ACE in plasma sample, endogenous ACE in plasma was destroyed by heat and substituted the source of ACE externally. Both rabbit serum and human plasma were compared for the suitability as standard ACE according to the chromatographic condition proposed for HA. Rabbit serum showed the endogenous interference at HA peak but not for human plasma (chromatogram not shown). Therefore, human plasma was selected as the standard exogenous ACE for enalapril and enalaprilat determinations.

The activity of endogenous ACE in plasma samples were completely destroyed at 75 °C within 5 min (Table 1). Enalaprilat in plasma sample was proven to be unaffected at this high temperature. By comparing the remaining ACE activity from the heated plasma spiked with enalaprilat in the concentration of 20, 40 and 60 ng/ml to the unheated aqueous enalaprilat at the same concentration as the control solution, enalaprilat in heated plasma samples could still inhibit ACE activity as that does in control solution. This confirms the stability of enalaprilat at high temperature (Table 2).

Table 1
Effect of temperature on the ACE activity in blank human plasma

| Temperature (°C) | Relative mean ACE activity (%) |
|---|---|
| 35 | 100.0 |
| 50 | 49.90 |
| 75 | 0 |

Table 2
The relative mean ACE activity in control standard enalaprilat solution and heated standard plasma samples (70 °C, 5 min)

| Enalaprilat concentration (ng/ml) | Relative mean ACE activity (%) ($n = 3$) | |
|---|---|---|
| | Control solution | Heated standard plasma |
| 0 | 100.00 | 100.00 |
| 20 | 88.94 | 90.22 |
| 40 | 58.03 | 57.11 |
| 60 | 52.08 | 53.91 |

The appropriateness of relating the formation of HA to only enalaprilat in plasma sample was clarified. As illustrated in Fig. 4, enalaprilat could inhibit ACE activity more significantly than enalapril. It is then confirmed again that enalaprilat is the active metabolite of enalapril. By adding enalapril in the constant concentration into each series concentration of enalaprilat, the performance of inhibiting ACE varied with concentration of enalaprilat rather than enalapril such that the same pattern of inhibition profiles for different concentration of enalapril was observed (Fig. 5). In addition, the $IC_{50}$ value (drug concentration that could inhibit 50% of ACE activity in human plasma) for enalapril has been reported to be $4.5 \times 10^{-9}$ M of enalaprilat [16]. Therefore, it was rationale to determine only enalaprilat concentration in plasma for explaining the performance of total plasma drug (enalapril and enalaprilat) concentration.

### 3.2. Hippuric acid analysis

For the pretreatment of plasma sample prior to HPLC analysis of hippuric acid, the plasma deproteinization with acetonitrile was compared with the liquid extraction using ethyl acetate as reported by Chiknas [17]. It was found that the use

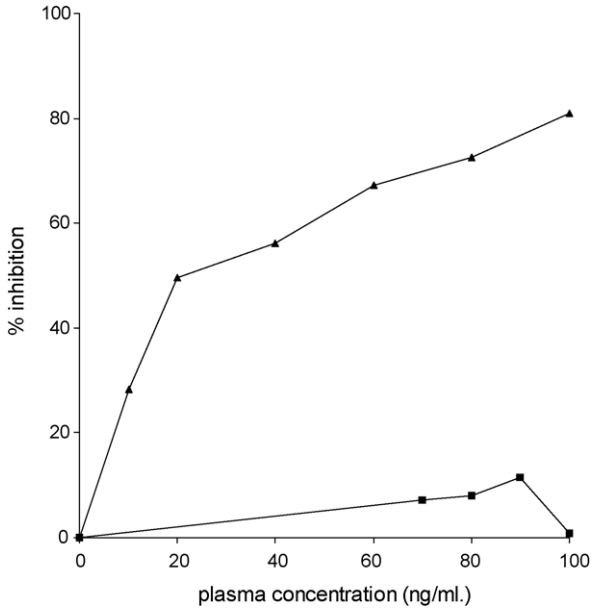


Fig. 4. The inhibition of ACE activity by (■) enalapril or (▲) enalaprilat. % Inhibition = $100 \times [(PAR_{drug\ free} - PAR_{drug\ concentration}) / PAR_{drug\ free}]$.

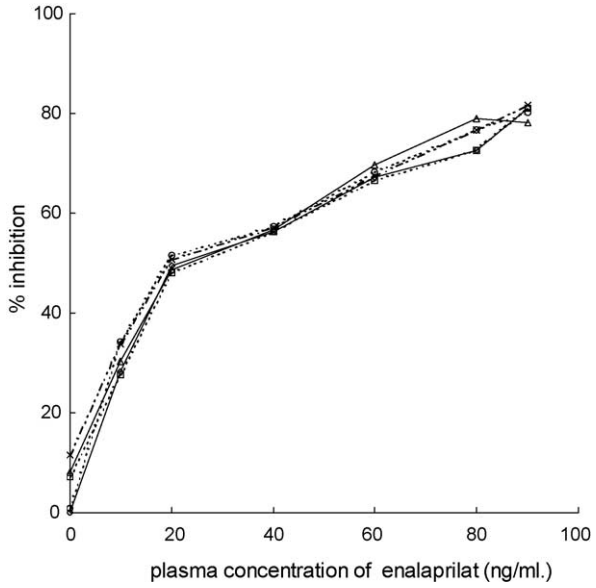


Fig. 5. The inhibition of ACE activity by the mixture of enalapril and enalaprilat. Five series of standard plasma samples containing the mixture of enalaprilat 10, 20, 40, 60, 80, 100 ng/ml and constant concentration of enalapril for each series at the concentration of 0 (◊), 70 (□), 80 (△), 90 (×), 100 (◯) ng/ml were prepared and analyzed followed the developed method.

of acetonitrile as plasma deproteinizing agent required less time consuming for sample preparation. Meanwhile, chromatogram of HA was not interfered from any endogenous substances. These results were superior to ethyl acetate extraction method (chromatogram not shown). Therefore, the deproteinization method was used in this study.

HA and 2-methyl hippuric acid (IS) could be analyzed under isocratic mode of HPLC at UV detector wavelength of 228 nm with the resolution of 1.3 when introducing SDS into the composition of mobile phase. The chromatographic peak shape of HA was optimized under the varying concentration of SDS and phosphate buffer. The presence of SDS 2.0 mM could improve the tailing of HA peak when combining with 40 mM of the buffer. However, this isocratic mode was replaced by the gradient elution mode when applying for enalaprilat analysis in plasma. In the presence of enalaprilat, HA could only be occurred by the reaction of ACE post drug inhibition with excess HHL that showed as huge chromatogram at the very late time of 37 min. This could be resolved by utilizing the gradient mode.

### 3.3. Linearity

The linear relationship between peak area ratio of HA/IS and enalaprilat concentration could be expressed as two calibration curves in the exponential pattern. The low-end concentrations of enalaprilat covered the concentration range of 3.0–20.0 ng/ml and the high end was in the range of 20.0–120.0 ng/ml. The linear regression was determined and the reproducibility of the calibration curves were proven as tabulated in Table 3.

Table 3
Reproducibility of the calibration equations for the proposed HPLC method for enalaprilat determination in plasma

| Calibration parameters | Calibration range (ng/ml) | |
|---|---|---|
| | 0–20 | 20–120 |
| Regression equation PAR = $a\,e^{-bC}$ | | |
| Slope ($b$) | 0.033 | 0.012 |
| Standard deviation of the slope ($S.D._b$) | 0.001 | 0.001 |
| Relative standard deviation of the slope (%) | 4.02 | 5.54 |
| Intercept ($a$) | 0.101 | 0.348 |
| Standard deviation of intercept ($S.D._a$) | 0.011 | 0.018 |
| Coefficient of determination ($R^2$) | 0.998 | 0.999 |
| Relative standard deviation of $R^2$ (%) | 0.14 | 0.02 |

### 3.4. Accuracy and precision

The intra-day and inter-day accuracy for enalaprilat determination in term of % bias were within ±10% in all concentrations investigated while % relative standard deviation (R.S.D.) for both intra-day and inter-day precision were less than 10% at any concentration studied (Table 4).

### 3.5. Limit of quantitation

The lowest limit of quantitation for enalaprilat was determined to be 3.0 ng/ml. With this lowest detectable concentration, the accuracy as % bias was within ±10% and % R.S.D. of six replicate determinations was in the range of 8.69–10.90% ($n = 6$). Therefore, this concentration was included as the initial concentration in the calibration curve.

### 3.6. Specificity of the method

The specificity of the analytical method was determined to ensure that the method can be used to quantitate enalaprilat validly. The specificity was studied by analyzing five different blank plasma samples individually. The retention times of HA and IS were 4.8 and 6.0 min, respectively. No any detectable endogenous interference was observed at these time points

Table 4
Precision and accuracy of proposed HPLC method for enalaprilat determination

| Nominal concentration added (ng/ml) | Concentration analyzed (ng/ml) mean ± S.D. | R.S.D. % | Accuracy % bias |
|---|---|---|---|
| Intra-assay ($n = 6$) | | | |
| 10.0 | 9.00 ± 0.20 | 2.22 | −10.02 |
| 40.0 | 43.86 ± 0.40 | 0.91 | 9.64 |
| 80.0 | 80.88 ± 1.24 | 1.53 | 1.10 |
| 100.0 | 97.46 ± 3.68 | 3.78 | −2.54 |
| Inter-assay ($n = 24$) | | | |
| 10.0 | 9.49 ± 0.43 | 4.56 | −5.14 |
| 40.0 | 39.48 ± 3.46 | 8.76 | −1.31 |
| 80.0 | 80.22 ± 3.19 | 3.98 | 0.28 |
| 100.0 | 98.03 ± 1.84 | 1.88 | −1.97 |

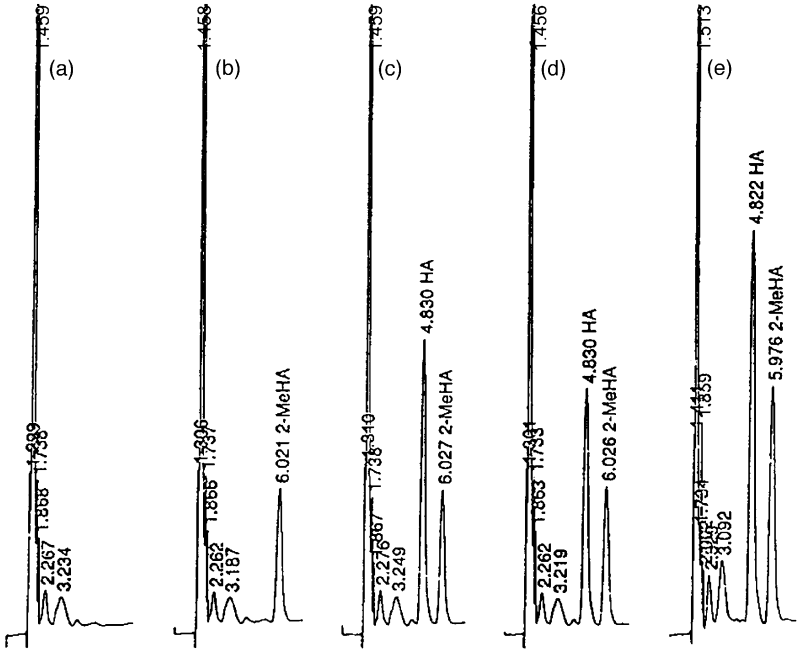


Fig. 6. Chromatograms of: (a) blank human plasma; (b) blank human plasma spiked with 3.6 μg/ml of IS; (c) blank human plasma spiked with 100 ng/ml of HA and 3.6 μg/ml of IS; (d) blank human plasma spiked with 100 ng/ml of enalaprilat and 3.6 μg/ml of IS; (e) plasma sample from volunteer following administration of 5 mg enalpril tablet.

for every standard plasma sample studied and plasma samples from volunteer (Fig. 6).

### 3.7. Stability

The stability of standard plasma samples and in-processed analyte of enalaprilat were summarized in Table 5. The processed analytes in the autosampler which was controlled at 0 °C demonstrated the less difference and less fluctuate in the mean response of both HA and IS than those observed at 4 °C (Table 6). Therefore, the autosampler was controlled at 0 °C during analysis for the whole study. At this controlled temperature, the processed analytes could be stable up to 40 h with the difference in percentage of peak area response from the initial time less than 1%. The standard plasma samples could be kept frozen and thaw within only two cycles with no deterioration of the drug. The stability of standard plasma containing enalaprilat was concentration independent when restored at −48 °C within 1 month.

Table 6
The range of % difference of HA and IS in the autosampler 40 h at 0 and 4 °C

| Autosampler temperature (°C) | Enalaprilat concentration (ng/ml) | % Difference from the initial response | |
|---|---|---|---|
| | | HA | IS |
| 0 | 10 | 0.22–0.97 | 0.06–0.97 |
| | 40 | 0.07–0.48 | 0.14–0.52 |
| | 80 | 0.09–2.17 | 0.01–2.18 |
| 4 | 10 | −1.03–3.31 | −2.05–6.87 |
| | 40 | −2.87–4.82 | 0.08–8.68 |
| | 80 | −2.82–14.06 | 0.09–7.58 |

### 3.8. Pharmacokinetics

The mean plasma concentration–time profile of enalaprilat after administration of 5 mg enalapril tablet to healthy volunteers is illustrated in Fig. 7. Only half an hour after enalapril administration, enalaprilat concentration could be detected.

Table 5
Stability of enalaprilat in standard plasma samples and processed analytes

| Stability studies | Storage condition | | % Difference from the initial response/concentration | | | |
|---|---|---|---|---|---|---|
| | Time | Temperature (°C) | Enalaprilat concentration (ng/ml) | | | IS |
| | | | 10 | 40 | 80 | |
| Laboratory temperature[a] | 10 h | 27 ± 1 | −0.33 | −0.52 | −0.76 | 0.11 |
| Storage temperature[b] | 30 d | −48 | −2.0 | 1.50 | 1.25 | – |
| Autosampler[a] | 40 h | 0 | 0.36 | 0.37 | 0.81 | 0.52 |
| Freeze–thaw cycle[b] | 2 cycles | | 0.0 | 2.0 | 1.25 | – |

[a] % Difference: [(peak area at initial time − peak area at time *t*) / peak area at initial time] × 100.
[b] % Difference: [(concentration at initial time − concentration at time *t*) / concentration at initial time] × 100.

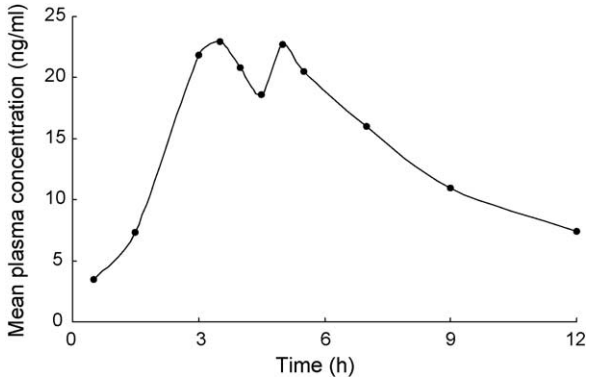


Fig. 7. The mean plasma concentration–time profile following the administration of 5 mg enalapril tablet to 12 volunteer subjects.

Table 7
Pharmacokinetic parameters of enalaprilat following a single oral administration of 5 mg Retinec® tablet to 12 healthy volunteers

| Pharmacokinetic parameters | Mean values ± S.D. ($n = 12$) |
|---|---|
| $C_{max}$ (ng/ml) | 28.24 ± 6.76 |
| $t_{max}$ (h) | 4.3 ± 1.0 |
| $AUC_{(0–t)}$ (ng/ml h) | 160.84 ± 12.74 |

This implies the rapid biotransformation of enalapril to enalaprilat in the body. The maximum concentration of enalaprilat were observed within the time range of 3.0–5.5 h ($t_{max}$) at the concentration ($C_{max}$) of 20.93–39.98 ng/ml. For $AUC_{(0–t)}$, an average of 160.84 ng/ml h was determined with the range of 81.09–243.90 ng/ml h (Table 7). These pharmacokinetic parameters correspond to the other reports [20,21]. Therefore, this should confirm the reliability of this developed method.

## 4. Conclusion

The suitability of enzyme kinetic to quantify enalapril in human plasma has been successfully demonstrated. The method is simple, sensitive, specific and reproducible. The method was proven for its appropriateness in pharmacokinetic study. This enzyme kinetic application was also applied for other ACE inhibitor drug analysis.

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