Ultra Science Vol. 15(2), 153-160 (2003).

Determination of Lisinopril in Tablets by Competitive Inhibition of Angiotensin-Converting Enzyme

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(Acceptance Date 3rd March, 2003)

Abstract

A rapid and accurate method for lisinopril, **I**, determination in tablets by competitive inhibition of angiotensin-converting enzyme (ACE) was described. First, a solution of **I** was mixed in a 1 to 10 ratio with a serum containing high ACE activity. Enzymatic activity (inhibition) was determined by spectrophotometric kinetic method using 0.8 mmol/L FAPGG (N-[3-(Furyl)-Acryloyl]-L-Phenylalanyl-GlycylGlycyne) as the substrate. The concentration of lisinopril was determined from a standard curve of inhibitor concentration versus percent of ACE inhibition. The close correlation between the drug concentration and the degree of ACE inhibition was demonstrated and was found to correlate well with values obtained by HPLC method (r = 0.92). The determination of lisinopril concentration via competitive inhibition also reflects the physiological activity *i.e.* the efficiency of the preparation.

Keywords : Lisinopril, ACE, Competitive Inhibition, Tablets

Introduction

In the past two decades, much attention has been focused on the renin-angiotensin system in relation to mechanisms controlling blood pressure and renal function. The angiotensin-converting enzyme (ACE, dipeptidyl carboxypeptidase EC 3.4.15.1) generates a powerful vasoconstrictor substance[1] angiotensin-II , and inactivates the vasodilating substance bradykinin. Angiotesin-converting enzyme inhibitors have been of particular clinical interest. Lisinopril, **I**, (Fig. 1) a lysine analogue of elanaprilat, is a potent long acting oral nonsulfhydryl inhibitor of ACE. It was discovered and developed by the Merck Sharp and Dohme Research Laboratories[2,3]. It is an important advance in the treatment of patients with hypertension and congestive heart failure[5], exclusively eliminated by renal excretion[4]. Its therapeutic effects are the decrease in angiotensin-II, and aldosteron and increase

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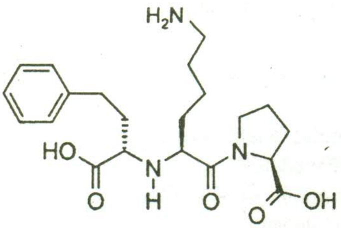
in bradykinin concentrations.



I

Fig l. Structure of lisinopril (N-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline)

It is of great importance to have a reliable and yet simple method for a quantitative determination of lisinopril. There are several methods available for determination of lisinopril in tablets: spectrophotometric[6], fluorometric[7], UV derivative spectrophotometric and HPLC method[8]. We would like to describe a simple, rapid, sensitive and accurate spectophotometric method for determination of lisinopril in tablets by competitive inhibition of ACE. Enzymatic activity (inhibition) was determined by Holmquist's kinetic method[9], modified by Ronca-Testoni[10], with 0.8 mmol/L FAPGG as the substrate in 50 mmol/L Tris buffer at pH 8.2 at 37 °C. The enzymatic activity was determined by monitoring the decrease in absorbance at 340 nm. as a result of hydrolysis of the substrate. (The substrate is a tripeptide blocked at the N-terminal by a furanacryloyl group which acts as a chromophoric monitor of substrate hydrolysis).

Experiments

Apparatus: All absorbance measurements were made using Milton Roy Spectronic 401 spectrophotometer, at 340 nm and 37 °C. The HPLC analysis was performed on Varian Star HPLC with LiChrosfer 100, RP -18; 250mm X 4mm column, at 25 °C, phosphate buffer pH = 4.0 and methanol (4:1) as the mobile phase, 1.2 ml/min, injection volume 20µL and UV detection at 261nm.

Reagents: The assay buffer consisted of 50 mmol/L Tris buffer (2-amino-hydroxymethyl-1,3-propandiol) adjusted to pH 8.2 (37 °C) with 0.1 mol/L HCl and addition of 300 mmol/L NaCl. The assay buffer components and lisinopril were obtained from Merck Darmstadt FRG. The enzyme substrate FAPGG and ACE elevated control serum were purchased from Sigma Chemical Co St. Louis MO., USA. All reagents were of analytical grade.

Preparation of solutions: Substrate solution: 0.8 mmol/L. (FAPGG (32 mg) was dissolved in 100 mL of 50 mmol/L Tris buffer pH 8.2). Stock solution of lisinopril: 20 mg /100 mL (Lisinopril dihydrate (21.8 mg) was dissolved in 100 mL of deionized water). Working solution: 2000 ng /mL was obtained by 1:100 dilution of the stock solution with deionized water. Final concentrations for the calibration curve: 0, 2, 5,10, 20 and 30 ng/mL were made by dilution of the working solution with deionized water.

Preparation of pool serum: The serum with high ACE activity was obtained from patients without antihypertensive therapy with normal biochemical parameters, normal bilirubin, nonlipidemic and without hemolysis. The prepared pool serum was stored at -18 °C. The determination was performed also with a commercial control serum with elevated enzyme activity from Sigma (porcine ACE in a buffered human serum base with stabilizer and preservative). Calibration curves were prepared for both the commercial control serum and the pool serum.

Enzyme activity (inhibition) determination: Measurements were performed in 50 mmol/L Tris buffer with 0.8 mmol/L substrate FAPGG and presence of 300 mmol/L NaCl. ACE hydrolyses the substrate by the following reaction:

$$\text{FAPGG} \xrightarrow{\text{ACE}} \text{FAP} + \text{G-G}$$

The reagent solution (1 mL) was brought to reaction temperature, 0.1 mL of was added and thoroughly mixed The cuvet was placed in the constant temperature cuvet compartment and allowed to equilibrate for 5 minutes. The initial absorbance value of the test sample was recorded at 340 nm. The final absorbance was recorded exactly 5 minutes later. ACE activity (U/L) was calculated using equation 1:

$$\text{ACE}\,(\text{U/L}) = \Delta A \frac{V_t}{V_s} \cdot \frac{1}{t} \cdot \frac{1}{\Delta\varepsilon} .1000 \qquad (1)$$

ΔA - Absorbance after 5 min.

Vt - total assay volume

Vs - sample volume

t - time (min.)

$\Delta\varepsilon$ - difference absorptivity FAPGG / FAP.

Maximal $\Delta\varepsilon$ for total hydrolysis of 0.8 mmol/L substrate at 340 nm is $\Delta\varepsilon = 0.59$

Competitive inhibition method for determination of lisinopril: Preparation of tablets for determination of lisinopril: From 10 pulverized tablets, was measured an amount of average tablet weight (224 mg) corresponding (equivalent) to 10 mg of lisinopril. This was dissolved in 50 mL of deionized water, mixed for 20 minutes, diluted to 100 mL and filtered. 1 mL of the filtrate was diluted to 100 ml with deionized water. 1ml of this solution was diluted to 100 ml with deionized water to a final concentration of 10 ng/mL. This was mixed in a 1 to 10 ratio with serum containing high ACE activity. ACE activity was then determined (after 30 min incubation at 25 °C with inhibitor) via spectrophotometric kinetic method with 0.8 mmol/L FAPGG substrate. Percent of enzyme inhibition is then calculated using equation 2·

$$\%\ \text{Inhibition} = \frac{(\text{Uninhibited Activity-Inhibited Activity})}{\text{Uninhibited Activity}} .100 \qquad (2)$$

The values of lisinopril concentration were determined from a standard curve of concentration of added inhibitor versus percent of inhibition of ACE activity. One-day storage in a refrigerator at + 4 °C did not influence the stability of the enzyme-inhibitor complex. Freezing and thawing caused dissociation of enzyme-inhibitor complex and decreased the percent of inhibition by 15 %. The correlation between the concentration of the drug and the degree of inhibition of ACE has been demonstrated.

Determination with HPLC method: Preparation of tablets for determination of lisinopril: 10 Tablets were pulverized mixed and aliquot of tablet weight equivalent to 10 mg lisinopril was dissolved in 7 mL of mobile phase. This was mixed and diluted to 10 ml with mobile phase (buffer pH = 4.0 and methanol 4:1). The mobile phase consisting of phosphate buffer pH = 4.0 and methanol (4:1) was previously filtered and degassed. The buffer (pH = 4.0) was prepared from 0.02 mol/L KH_2PO_4 (2.722 g/L adjusted with phosphoric acid). Preparation of standard solution: 27.23 mg of lisinopril dihydrate equivalent to 25 mg lisinopril was dissolved in 15 mL mobile phase, then mixed and diluted to 25 mL with mobile phase. The sample and the standard solution prior to injection were filtered through a 0.45 μm cellulose acetate filter. The quantity (mg) of lisinopril per tablet was calculated according to equation 3:

$$mg/tbl = \frac{F(P_1).m(P_2).0.91864.x}{F(P_2).m(P_1).2.5} \qquad (3)$$

F (P_1) - area under peak for sample solution

F (P_2) - area under peak for standard solution

m (P_2) - lisinopril dihydrate in standard solution

m (P_1) - tablet weight in mg in sample solution

0.91846 - ratio of relative molecular weights of lisinopril and lisinopril dihydrate

x - average tablet weight in mg

Results and Discussion

Optimal condition for determination :

We have examined optimal condition for the determination of lisinopril in tablets by the competitive inhibition of ACE: concentration of substrate, ratio of serum to inhibitor, stable percent of inhibition. We have also examined the influence of substrate concentration on the percent of inhibition of enzymatic reaction with pool serum and range of concentration (0.2- 3.2 mmol/L). The optimal concentration of the substrate is 0.8 mmol/L for the optimal range of inhibition and measurement of differences in enzymatic activity. A relation between substrate concentration and percent of inhibition with 10 ng/ml lisinopril (1:10 with pool serum after 30 min. incubation) was established. The ratio between the serum and inhibitor was determined with inhibitor concentrations of 2 ng/mL and 20 ng/mL, with control serum and pool serum. The optimal ratio is 1:10 for range of inhibitor concentration of 2-30 ng/mL (Table 1).

Table 1. Percent of inhibition depending on ratio inhibitor / serum

ratio inhibitor/serum	Percent of inhibition			
	2 ng/mL Lisinopril		20 ng/mL Lisinopril	
	Control serum	Pool serum	Control serum	Pool serum
1: 5	24	22	79	64
1:10	12.5	11	64	47
1:15	10	7.5	31	23
1:20	8.2	5.0	26	18

The rate of formation of enzyme-inhibitor complex was determined by the percent of inhibition of ACE in the control serum and the pool serum with 2ng/mL and 20 ng/mL in 5 minute interval. Stable complex and percent of inhibition was achieved after 30 minutes.

The calibration curve was prepared with the control serum and the pool serum in previous defined conditions (0.8 mmol/L substrate in Tris buffer pH 8.2, 300 mmol/L NaCl, 340 nm, 37 °C). The range of concentration is 2-30 ng/mL and determination was done with the control serum and the pool serum (Table 2). Results were presented as a logarithm of lisinopril concentration versus percent of inhibition (Fig. 2).

Table 2. Correlation between lisinopril concentration and percent of inhibition

Lisinopril concentration ng / mL	Percent of inhibition	
	Control serum	Pool serum
0	-	-
2	12.1	11.0
5	28.3	17.7
10	42	32
20	61.8	46.8
30	69.1	54.8

The optimal concentration of the substrate for determination of enzymatic activity and inhibition is 0.8 mmol/L[10]. Higher concentrations of the substrate were not needed and they are not rational. The effect of substrate concentration on the hydrolysis of FAPGG by serum ACE is presented in Fig. 3. Michaelis' constant (Km) for uninhibited enzymatic reaction is 0.376 mmol/L. The percent of inhibition is optimal with concentration of substrate 0.8 mmol/ L for range of concentration of inhibitor 2-30 ng/mL (Table 1). The substrate FAPGG is specific for the determination of ACE. activity and inhibition. The presence of chloride ions in the reaction mixture was necessary for a change in conformation of the catalytic center and stabilization of enzyme-substrate or enzyme inhibitor complex. The substrate is stable in solution for 30 days if stored in a cold and dark place whereas the frozen pool serum is stable for 60 days.

The Lineweaver-Burk plot of hydrolysis of the substrate in absence and presence of lisinopril (Fig. 3) is typical for competitive inhibition. Michaelis' constant (Km) is 0.376 mmol/L for an uninhibited reaction, and 0.523 mmol/L for an inhibited reaction. The inhibition of lisinopril was competative and K_i value of 2.25 nmol/L was obtained from Dixon plots (Fig. 4). Two substrate concentrations (0.4 mmol/L and 0.8 mol/L) in the incubation mixtures were used.

The calibration curve is prepared with the control serum and the pool serum (Table 2). In Fig. 2 a standard curve for lisinopril determination in range 2-30 ng/ml is presented. A difference exists in percent of inhibition between the control serum and the pool serum. The concentration of inhibitor of 13.5 ng/mL inhibits 50% of ACE activity in the control serum and for the pool serum 25 ng/ml is needed. Lisinopril does not bind significantly to the serum proteins, however the serum is more complex system in comparison to the purified ACE. The use of different pool serums does not have an effect on the obtained values for the percent of inhibition.

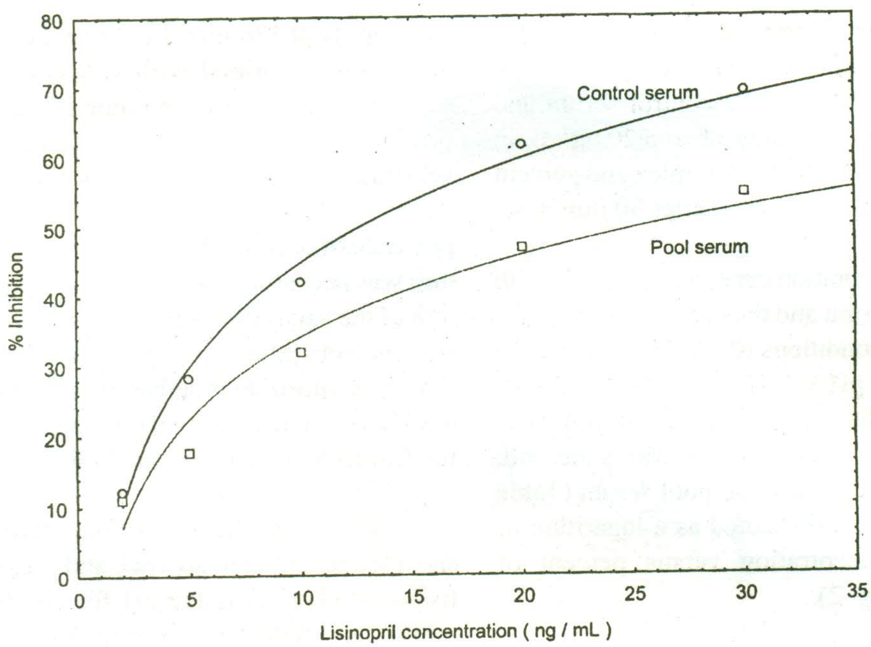


Fig. 2. Correlation between lisinopril concentration and percent of inhibition

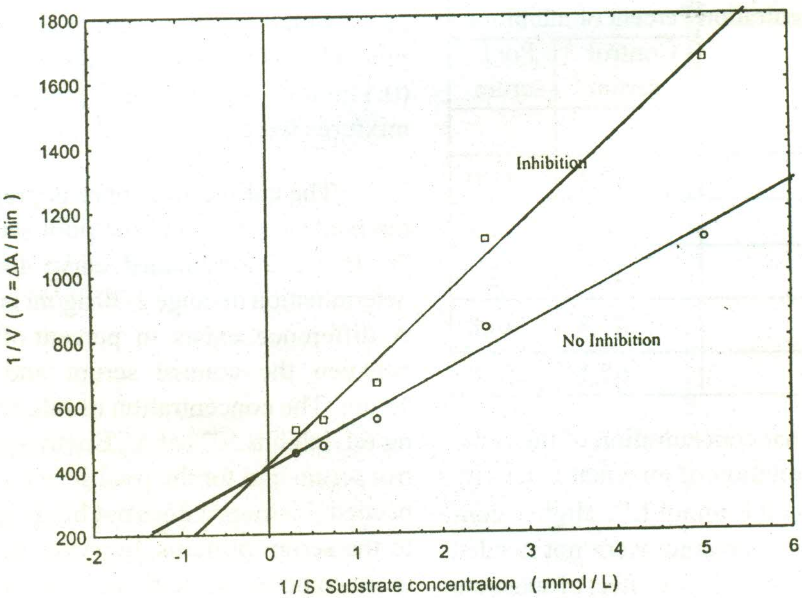


Fig. 3. Effect of substrate concentration on FAPGG hydrolysis by serum ACE (Lineweaver-Burk plot) in presence and absence of lisinopril

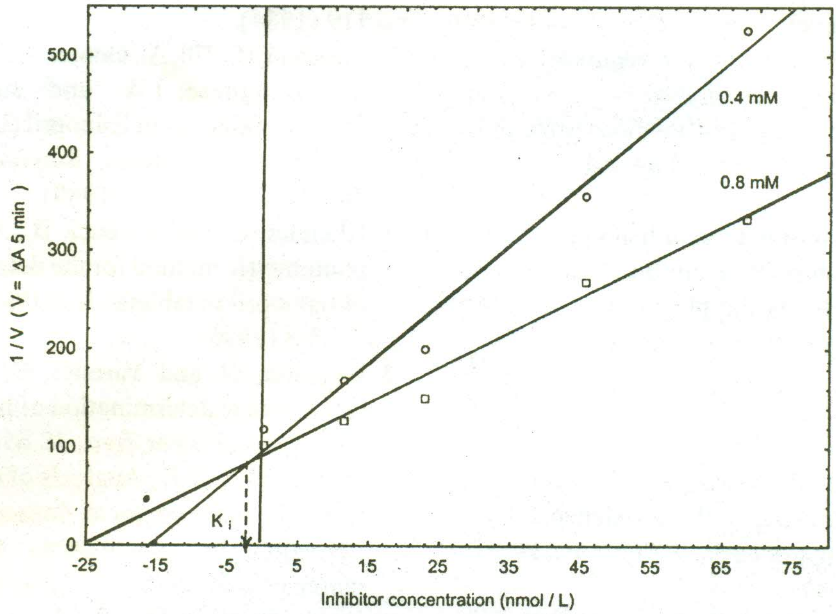


Fig. 4. Dixon plots of the relationship between the reciprocal of reaction velocity and various amounts of lisinopril.

Table 3. Comparison between HPLC and Competitive Inhibition methods

Run N°	Lisinopril concentration mg / tbl	
	Competitive Inhibition	HPLC
1	10.5	10.27
2	9.5	9.48
3	10.0	9.95
4	9.8	9.97
5	10.3	10.4
6	9.9	9.94
7	10.0	9.9
8	9.4	9.65
9	9.7	9.74
Mean	9.9	9.92
SD	± 0.35	± 0.29
CV	3.5 %	2.9 %
Corr. Coeff.	0.92	

The accuracy of the competitive inhibition method for concentration determination of lisinopril in tablets was found to be satisfactory. Permitted variation is 90-110% of the declared content of lisinopril in tablet. The method described may be adapted for other competitive inhibitors of ACE, and may be an introduction for determination of lisinopril in biological fluids. The method is fast, simple, reliable and with satisfactory precision and accuracy.

The competitive inhibition method for determination of lisinopril is compared with the HPLC method. The same sample of pulverized tablets was analyzed for the amount of lisinopril. From the calculated results (Table 3) it can be seen that the variance p-value is less than 0.01. There is a statistically significant relationship between competitive inhibition method and HPLC method at the 99%

confidence level. The correlation coefficient equals 0.9198, indicating a relatively strong relationship between the variables. Accuracy for competitive inhibition methods is 98-105% and the detection limit is 2 ng /mL.

The determination of lisinopril concentration via competitive inhibition at the same time, also reflects the physiological activity *i.e.* efficiency of the preparation.

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