3. Bioenhancement of Lisinopril

Lisinopril, have been shown to improve survival and reduce morbidity in patients with heart failure or myocardial infarction. Lisinopril is a hydrophilic molecule belonging to BCS class III.

Lisinopril inhibits Angiotensin Converting Enzyme *in vitro*, as well as after parenteral and oral administration to humans; Despite the fact that lisinopril has a second free carboxylic moiety on the C-22 position which is responsible for its carrier-mediated transepithelial transport but it has comparatively less affinity for peptide transporters than other ACE inhibitors (1).

Lisinopril transport in rats has been shown to be a combination of both passive and active processes, involving carrier mediated peptide transport system (2). Lisinopril is slowly and incompletely absorbed following oral administration. About 25 % of a given dose is absorbed on average, but absorption varies considerably between individuals, ranging from about 6 to 60 % (3). Metabolism does not occur as it is already an active diacid (a lysine derivative of enalaprilat). Peak concentrations in plasma are reported to occur after about 7 hr. Lisinopril is reported not to be significantly bound to plasma proteins. It is excreted unchanged in the urine. The effective half-life for is 12 hr in patients with normal renal function (4).

In the present chapter piperine and glycyrrhizic acid ammonium salt were incorporated with lisinopril. Effect of both bioenhancers on permeability of lisinopril was determined by *ex vivo* permeation studies and *in vivo* pharmacokinetic studies in rats.

3.1 LISINOPRIL-PIPERINE BINARY SYSTEMS

In the present section piperine (PI) was incorporated with lisinopril. Effect of PI on lisinopril permeability was determined. PI was incorporated with lisinopril (LI), using three different concentrations of PI, by means of two methods such as physical mixture and solvent evaporation method. Binary systems with each weight ratio, of both methods were evaluated by following parameters,

- FTIR – to determine any physical interaction of PI with LI
- DSC – to determine compatibility of PI with LI
- *Ex vivo* permeation study – to determine the effect of PI on permeation of LI

3.1.1 Materials

The Model drug Lisinopril (LI) was procured as a gift sample from Wockhardt Ltd., Mumbai, India. Piperine (PI) was purchased from Sigma Aldrich Ltd., Mumbai, India. Potassium bromide, sodium hydroxide, potassium dihydrogen orthophosphate and methanol were purchased from Qualigence fine chemicals, Mumbai, India. All the other chemicals and solvents were of analytical grade and were used without any further purification. Deionized double distilled water was used through out the study.

3.1.2 Methods

LI – PI binary systems were prepared at three different weight ratios (LI: PI – 2:1, 2:2, and 2:3 w/w). Two methods such as physical mixture method and solvent evaporation method were used to prepare LI – PI binary systems with three ratios. Both methods are described in detail below.

Physical Mixtures (**PM**):

The required and accurately weighed amounts of LI and PI were prepared by simply mixing the powders in a polythene bag.

Solvent Evaporation Method (**SE**):

The required amounts of LI and PI were accurately weighed and dissolved in methanol. Both solutions were mixed uniformly followed by allowing the solvent evaporated to get a dried powder.

3.1.3 Evaluation Parameters

3.1.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR studies were performed to get an idea about possible physical interaction of LI and PI. FTIR transmission spectra of a pure LI, PI and LI – PI binary systems of PM method and SE method were obtained using a Fourier Transform infrared spectrophotometer (Avatar™ 360 E.S.P™ FTIR spectrometer, Thermo Nicolet Corp., Madison, WI, USA). A total of 2 % (w/w) of sample (with respect to the potassium bromide-KBr) was mixed with dry KBr. The mixture was ground into a fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned 16 times at 4 mm s^{-1} at a resolution of 2 cm^{-1} over a wave number region of 500–4000 cm^{-1}. The characteristic peaks were recorded.

3.1.3.2 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric analysis was used to characterize thermal behaviour of LI, PI and LI-PI binary systems and to check compatibility of PI with LI. DSC thermograms were obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan). Temperature calibration was performed using indium as a standard. Samples (2.5 to 5 mg) were crimped in a standard aluminium pan and heated from 30–250 °C at a heating rate of 10 °C min^{-1} under constant purging of dry nitrogen at 40 ml min^{-1}. An empty pan, crimped in same manner as sample, was used as a reference. The characteristic endothermic peaks and specific heat of the melting endotherm were recorded.

3.1.3.3 Spectrophotometric analysis and standard curves

Shimadzu, UV-1700 Double beam UV, Visible spectrophotometer, Kyoto (Japan) was used for spectrophotometric analyses of LI in presence of PI in phosphate buffer (pH 6.0). An analysis was done at 258.4 nm where LI has maximum absorbance. Standard curves were constructed by serially diluting a stock solution (in methanol) of LI to obtain concentrations in the range of 10 to 900 µg/ml using phosphate buffer (pH 6.0) as diluent. Each concentration was analyzed in triplicate against reference blank containing PI.

3.1.3.4 Ex Vivo permeation studies

All binary systems containing LI and PI were assessed for *ex vivo* permeation studies. The permeation study was carried out same as described in section 2.1.3.4 using goat intestinal membrane. LI concentration in donor compartment was taken to maintain sink condition (1 mg/ml). Samples were withdrawn from receptor compartment at predetermined time interval 5, 15, 30, 60, 90, 120, 180, and 240 min and replaced with same volume of fresh medium to maintain sink condition. The withdrawn samples were filtered through 0.45 µm whatman filter paper and diluted suitably and analyzed spectrophotometrically with first derivative method at 258.4 nm for LI. The absorbance values were transformed to concentration by reference to a standard calibration curve obtained experimentally. The amount of drug permeated was determined and plotted as a function of time. The permeability coefficients (P_{eff}) and permeation enhancement ratio were calculated from the linear part of the curves as described in section 2.1.3.4. These studies were performed in triplicate for each of control and binary systems and average values were considered for data analysis.

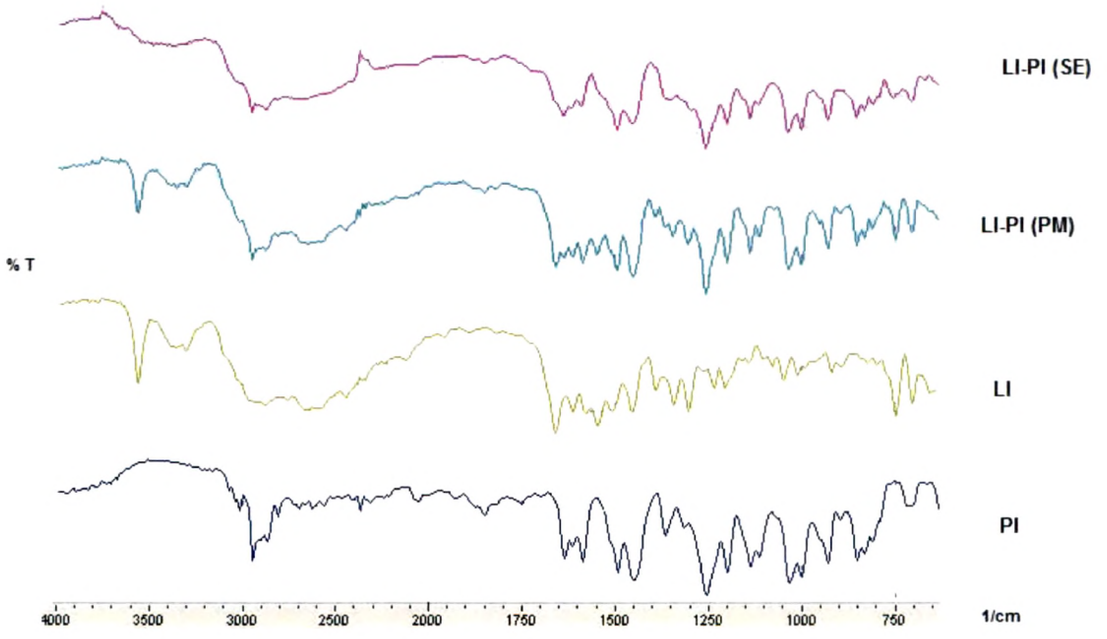
The results of experiments performed (n = 3) are presented as mean $\pm$ SD. Significance between the mean values was calculated using ANOVA using SPSS version 15.0. Tukey HSD post-hoc multiple comparison test was done to detect significant differences ($P<0.05$) between the permeability of LI in presence and absence of PI.

3.1.4 Results and Discussion

3.1.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of LI, PI, LI – PI binary systems of PM method and SE method are shown in Figure 3.1.1. FTIR spectrum of pure LI shows characteristic peak of carbonyl band of 1655 – 1570 cm^{-1} (5,6). Peak at 3554.56 cm^{-1} and coupled doublet at 3348 and 3294 cm^{-1} is due to N-H stretching of primary amine. Broad peak between 3000 to 2500 cm^{-1} ascribed to O-H stretching. C-H stretching ascribed peaks between 3000-2800 cm^{-1}. Peak at 1544 cm^{-1} is of N-H bending and peaks in between 1340-1266 cm^{-1} is of C-N stretching. All these peaks of LI remain unaffected in the IR spectrum of binary systems of both methods. There is slight change in intensity but that is insignificant from pure LI. Thus FTIR spectra remains unchanged, explained that there was no interaction between LI and PI in binary system of both methods.

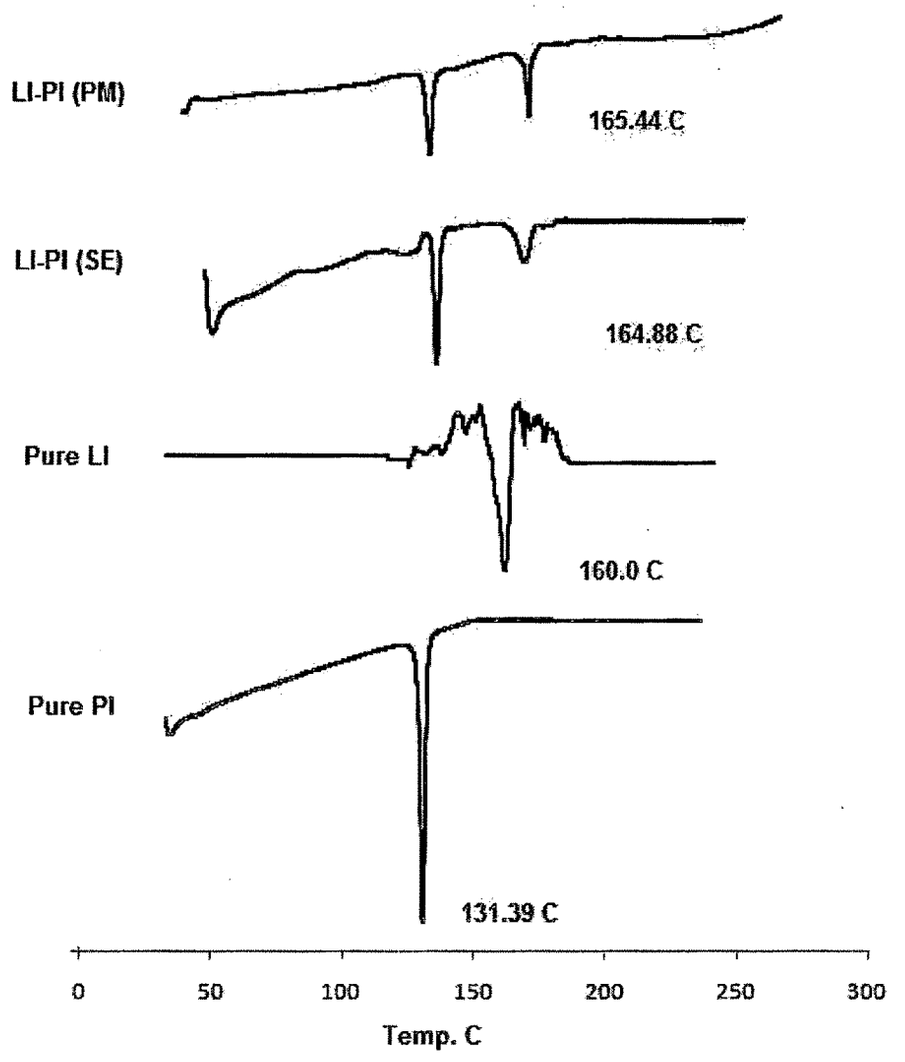
Figure 3.1.1 The FTIR spectra of LI, PI, LI-PI binary systems of each method.



3.1.4.2 *Differential Scanning Calorimetry (DSC)*

The thermogram of pure LI, PI, LI – PI binary systems of PM method and SE method are shown in Figure 3.1.2. Thermogram of pure LI showed a sharp endothermic peak at 160.0 °C (7), which is due to a melting point as it consumes energy. Thermogram of PI indicates a sharp endothermic peak at 131.39 °C. In the binary system of PM method endothermic peak of LI found at 155.44 °C, shift in peak to higher temperature which may be due to some interaction between LI and PI. Binary system of SE method has endothermic peak found at 164.88 °C. The intensity is almost same as pure LI. Thus all thermograms prove that there is very less to almost negligible interaction between LI and PI in all binary systems of both methods.

Figure 3.1.2 The thermograms of pure LI, PI and LI-PI binary systems of each method.



3.1.4.3 *Spectrophotometric analysis and standard curves*

UV spectrophotometric method was successfully applied for determining permeated LI content in *ex-vivo* permeation study without interference of PI. A UV spectrum of LI is shown in Figure 3.1.3. All the validation parameters for determination of LI are shown in Table 3.1.1. The method is simple and sensitive enough to measure the LI content in *ex-vivo* permeation study.

Figure 3.1.3 The representative zero order spectra of LI.

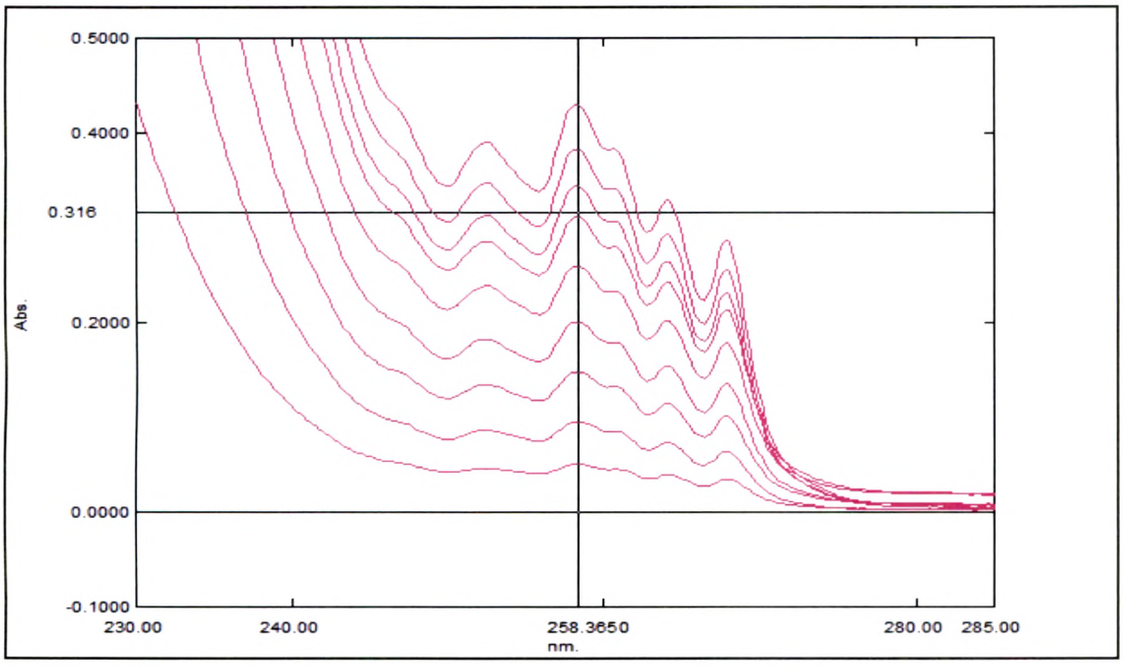


Table 3.1.1 The spectral and statistical data for determination of LI.

Parameters	LI in presence of PI
Wavelength	258.4 nm
Range	10-900 µg/ml
Linearity	0.9935
Intercept	0.0005
Slope	0.0215
LOD	2.1 µg/ml
LOQ	6.3 µg/ml
Intra day precision	% RSD < 2
Inter day precision	% RSD < 2

3.1.4.4 *Ex Vivo permeation studies*

Permeation coefficient (P_{eff}) was calculated for LI and LI-PI binary systems of both methods. Table 3.1.2 summarised the mean permeation coefficient ± SD of LI in absence (control) and presence of PI in binary systems of both methods. Release profile of LI in each binary system with ratio of LI: PI- 2:1, 2:2 and 2:3 is represented respectively in Figure 3.1.4, Figure 3.1.5 and Figure 3.1.6.

Table 3.1.2 Mean permeation coefficient ± SD of LI in absence (control) and presence of PI for binary systems of both methods.

Ratio of LI:PI	**Mean Permeation coefficient ± SD of LI, P_{eff} x 10^{-5} (cm/sec)(n=3)**		**Enhancement Ratio (ER)**	
LI (control)	0.316 ± 0.002*		1.00	
	PM Method*	**SE Method***	**PM Method**	**SE Method**
LI:PI 2:1	0.579 ± 0.01	0.763 ± 0.15	1.83	2.41
LI:PI 2:2	**1.158 ± 0.03**	0.500 ± 0.12	**3.67**	1.58
LI:PI 2:3	0.421 ± 0.06	0.342 ± 0.01	1.33	1.08

*Note: p value < 0.05; significant difference from control (Tukey's multiple comparison test)

Binary systems of both methods have higher permeation than control LI. i.e. PI significantly ($p<0.05$) increasing the permeation coefficient (P_{eff} cm/sec) of LI. Both methods supports increase in permeation, but PM method with LI: PI 2:2 ratio shows 3.67 fold increase in permeation coefficient of LI than control (LI without PI).

As found in bioenhancement of AT, PI causing increase in permeation of LI in *ex-vivo* permeation study. The probable mechanism could be, PI modulates membrane dynamics and due to its brush broader mechanism (8). PI causes more permeation enhancement of LI (3.67 fold) than it does of AT (2 fold increment). The probable mechanism may be due to; it can interact with the cells of small intestine and increasing their ability to absorb more amino acids. These may be causing more permeation of LI through the intestinal membrane.

Figure 3.1.4 The release profile of LI with ratio LI: PI- 2:1 for both methods.

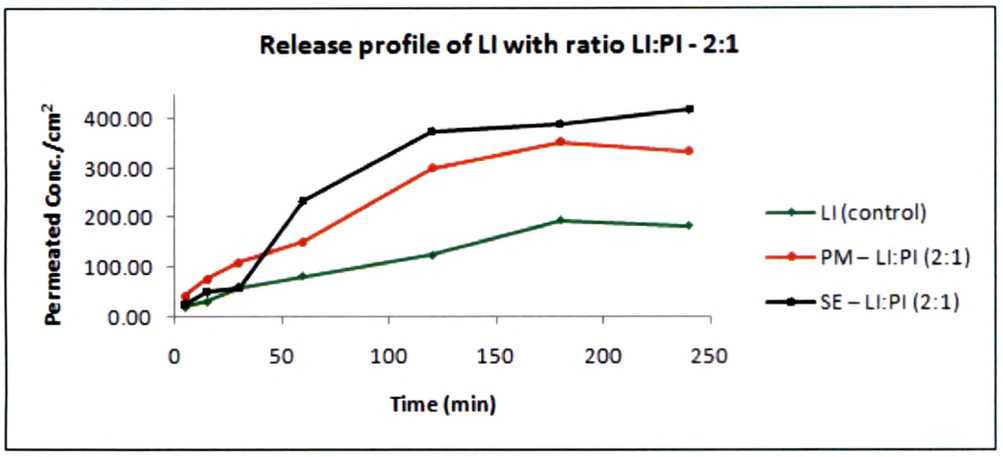


Figure 3.1.5 The release profile of LI with ratio LI: PI- 2:2 for both methods.

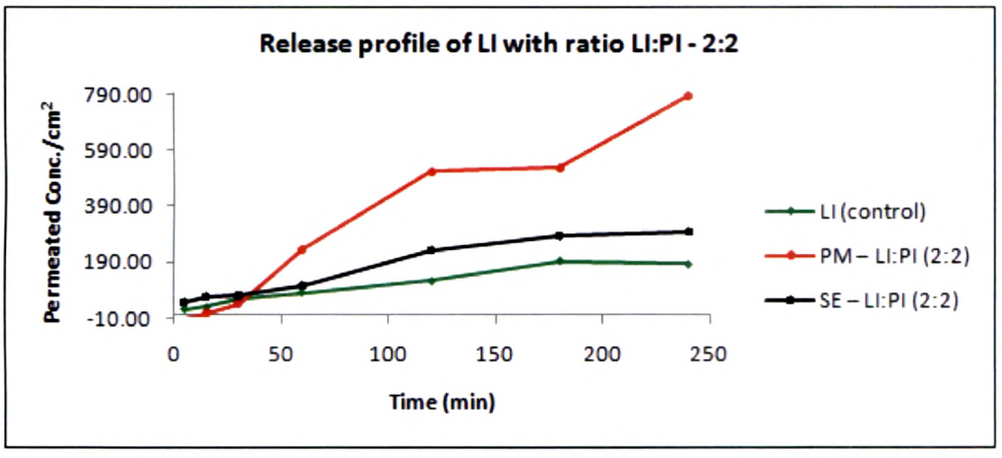
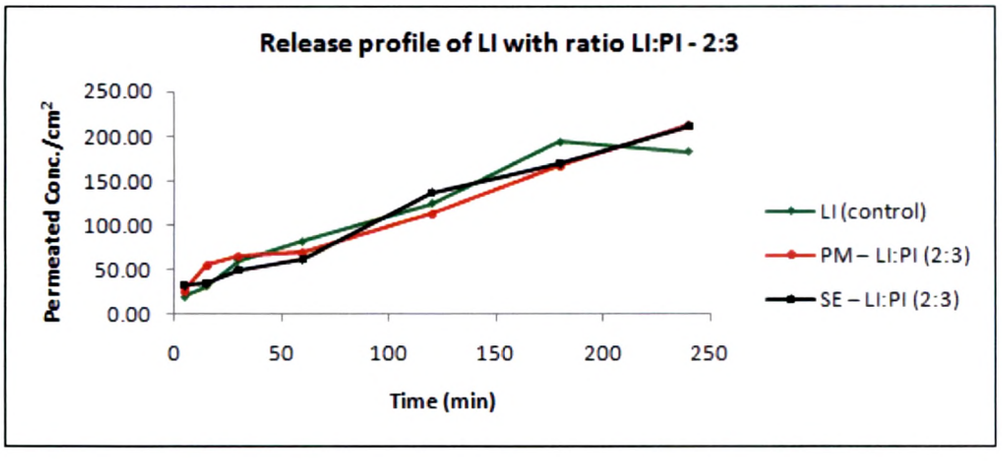


Figure 3.1.6 The release profile of LI with ratio LI: PI- 2:3 for both methods.



3.1.5 Conclusion

Results of DSC and IR suggest that no physical interaction between LI and PI. There was very weak to almost negligible interaction between LI and PI found in both FTIR and DSC studies. Results of *ex-vivo* permeation, ratio of LI: PI 2:2 showed 3.67 fold increment in permeation coefficient of LI in presence of PI than pure drug LI.

PM method has more enhancement of absorption of LI than SE method. Therefore it is concluded from all these studies, only PM method will be used for further studies.

3.1.6 References

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3.2 LISINOPRIL-GLYCYRRHIZIC ACID AMMONIUM SALT BINARY SYSTEMS

In the present section glycyrrhizic acid ammonium salt (GA) was incorporated with lisinopril. Effect of GA on lisinopril permeability was determined. GA was incorporated with lisinopril (LI), using three different concentrations of GA, by means of two methods such as physical mixture and solvent evaporation method. Binary systems with each weight ratio, of two methods were evaluated by following parameters,

- FTIR – to determine any physical interaction of GA with LI
- DSC – to determine compatibility of GA with LI
- *Ex vivo* permeation study – to determine the effect of GA on permeation of LI

3.2.1 Materials

The Model drug Lisinopril (LI) was procured as a gift sample from Wockhardt Ltd., Mumbai, India. Glycyrrhizic acid ammonium salt (GA) was purchased from Sigma Aldrich Ltd., Mumbai, India. Potassium bromide, sodium hydroxide, potassium dihydrogen orthophosphate and methanol were purchased from Qualigence fine chemicals, Mumbai, India. Sodium acetate, glacial acetic acid, acetyl acetone and formaldehyde were purchased from S. D. Fine Chem. Ltd., Mumbai, India. All the other chemicals and solvents were of analytical grade and were used without any further purification. Deionized double distilled water was used through out the study.

3.2.2 Methods

LI – GA binary systems were prepared at three different weight ratios, where GA was taken as 1 %, 5 % and 10 % of w/w of LI dose (10 mg). Two methods such as physical mixture method and solvent evaporation method were used to prepare the LI – GA binary systems with three ratios. Both methods are described in detail below,

Physical Mixtures (**PM**):

The required and accurately weighed amounts of LI and GA were prepared by simply mixing the powders in a polythene bag.

 # Solvent Evaporation Method (SE):

The required amounts of LI and GA were accurately weighed and dissolved in methanol. Both solutions were mixed uniformly followed by allowing the solvent evaporated to get a dried powder.

3.2.3 Evaluation Parameters

3.2.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR studies were performed to get an idea about possible physical interaction of LI and GA. FTIR transmission spectra of a pure LI, GA and LI – GA binary systems of PM method and SE method were obtained using a FTIR spectrophotometer (Avatar™ 360 E.S.P™ FTIR spectrometer, Thermo Nicolet Corp., Madison, WI, USA). A total of 2 % (w/w) of sample (with respect to the potassium bromide-KBr) was mixed with dry KBr. The mixture was ground into a fine powder using an agate mortar before compressing into KBr disc under a hydraulic press at 10,000 psi. Each KBr disc was scanned 16 times at 4 mm s^{-1} at a resolution of 2 cm^{-1} over a wave number region of 500–4000 cm^{-1}. The characteristic peaks were recorded.

3.2.3.2 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetric analysis was used to characterize thermal behaviour of LI, GA and LI-GA binary systems and to check compatibility of GA with LI. DSC thermograms were obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan). Temperature calibration was performed using indium as a standard. Samples (2.5 to 5 mg) were crimped in a standard aluminium pan and heated from 30–250 °C at a heating rate of 10 °C min^{-1} under constant purging of dry nitrogen at 40 ml min^{-1}. An empty pan, crimped in same manner as sample, was used as a reference. The characteristic endothermic peaks and specific heat of melting endotherm were recorded.

3.2.3.3 *Spectrophotometric analysis and standard curves*

Shimadzu, UV-1700 Double beam UV, Visible spectrophotometer, Kyoto (Japan) was used for spectrophotometric analyses of LI in presence of GA in phosphate buffer (pH 6.0). An analysis was done by reported colorimetry method (1) at 347.0 nm. The reagent used for a colour formation is prepared by mixing 10 ml of walpole acetate buffer solution pH 3.6 (2) with 2.1 ml of acetylacetone and 5 ml of formaldehyde and the mixture was diluted to 25 ml with distilled water. Standard curves were constructed by taking aliquots of standard LI solution, within concentration range (5 – 50 µg/ml) which were transferred into 10-ml volumetric flasks. To each flask, 1 ml of reagent was added, mixed well and heated in a boiling water bath for 10 min. The volume was made up with water and absorbance of solution was measured at 347.0 nm against reagent blank. Each concentration was analyzed in triplicate.

3.2.3.4 *Ex Vivo permeation studies*

Pure LI, LI – GA binary systems of PM method and SE method were assessed for *ex vivo* permeation studies. The permeation study was carried out same as described in section 2.1.3.4 using goat intestinal membrane. LI concentration in donor compartment was taken to maintain sink condition (1 mg/ml). Samples were withdrawn from receptor compartment at predetermined time interval 5, 15, 30, 60, 90, 120, 180, and 240 min and replaced with same volume of fresh medium to maintain sink condition. The withdrawn samples were filtered through 0.45 µm whatman filter paper and subsequently the reagent was added and procedure described in section 3.2.3.3 was applied and analyzed spectrophotometrically at 347.0 nm for LI coloured complex. The absorbance values were transformed to concentration by reference to a standard calibration curve obtained experimentally. The amount of drug permeated was determined and plotted as a function of time. The permeability coefficients (P_{eff}) and permeation enhancement ratio were calculated from the linear part of the curves as described in section 2.1.3.4. These studies were performed in triplicate for each of control and binary systems and average values were considered for data analysis.

The results of experiments performed (n = 3) are presented as mean ± SD. Significance between the mean values was calculated using ANOVA using SPSS version 15.0. Tukey HSD

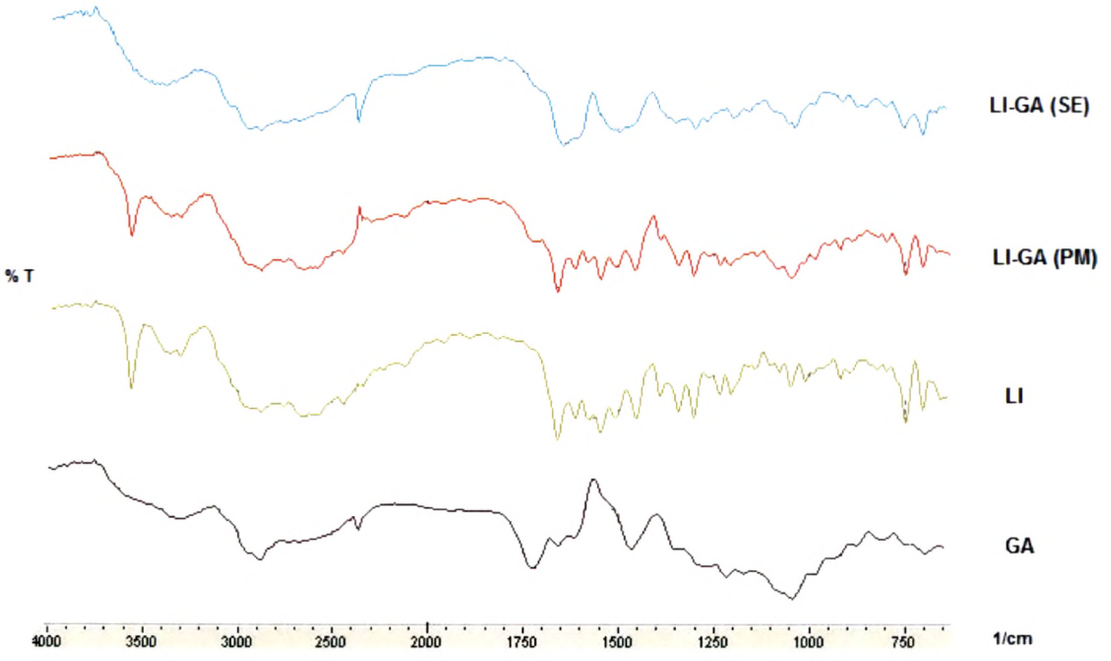
post-hoc multiple comparison test was done to detect significant differences ($P<0.05$) between the permeability of LI in presence and absence of GA.

3.2.4 Results and Discussion

3.2.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of LI, GA, LI – GA binary systems of PM method and SE method are shown in Figure 3.2.1. FTIR spectrum of pure LI shows characteristic peak of carbonyl band of 1655 – 1570 cm^{-1} (3,4). Peak at 3554.56 cm^{-1} and coupled doublet at 3348 and 3294 cm^{-1} is due to N-H stretching of primary amine. Broad peak between 3000 to 2500 cm^{-1} ascribed to O-H stretching. C-H stretching ascribed peaks between 3000-2800 cm^{-1}. Peak at 1544 cm^{-1} is of N-H bending and peaks in between 1340-1266 cm^{-1} is of C-N stretching. All these peaks of LI remain unaffected in the IR spectrum of binary systems of both methods. Some change in the intensity is there but it is insignificant from pure LI. Thus FTIR spectra remains unchanged, explained that no interaction between LI and GA in binary system of both methods.

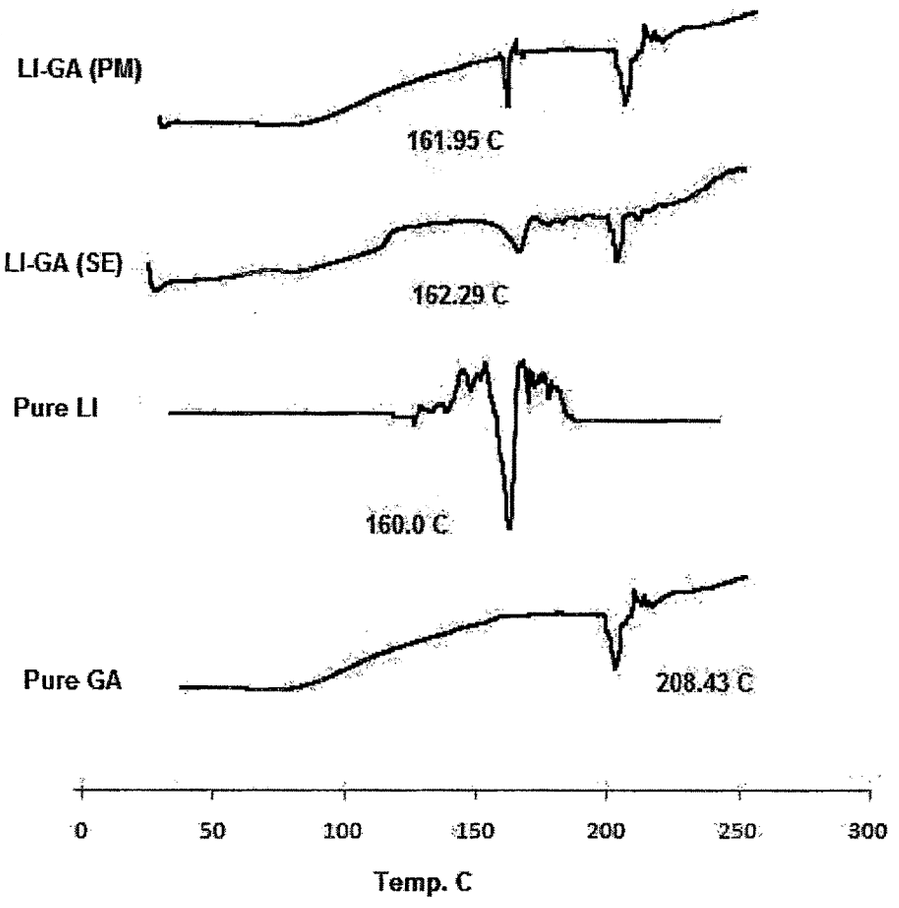
Figure 3.2.1 The FTIR spectra of LI, GA, LI-GA binary systems of each method.



3.2.4.2 *Differential Scanning Calorimetry (DSC)*

The thermogram of pure LI, GA, LI – GA binary systems of PM method and SE method are shown in Figure 3.2.2. The thermogram of pure LI showed a sharp endothermic peak at 160.0 °C (5), which is due to a melting point as it consumes the energy. The thermogram of GA indicates an endothermic peak at 208.43 °C. In the binary system of PM method endothermic peak of LI found at 161.95 °C, thus there may be possibility of some weak interaction between LI and GA. Binary systems of SE method has endothermic peak at 162.29 °C, the peak intensity is less than pure LI. Thus all the thermograms prove that there is very less interaction between LI and GA in binary systems of both methods.

Figure 3.2.2 The thermograms of pure LI, GA and LI-GA binary systems of each method.



3.2.4.3 *Spectrophotometric analysis and standard curves*

The method was successfully applied for determining permeated LI content in *ex-vivo* permeation study without interference of GA. A UV-Visible spectrum of LI is shown in Figure 3.2.3. All the validation parameters for determination of LI are shown in Table 3.2.1. The method is simple and sensitive enough to measure the LI content in *ex-vivo* permeation study.

Figure 3.2.3 The representative zero order spectra of LI.

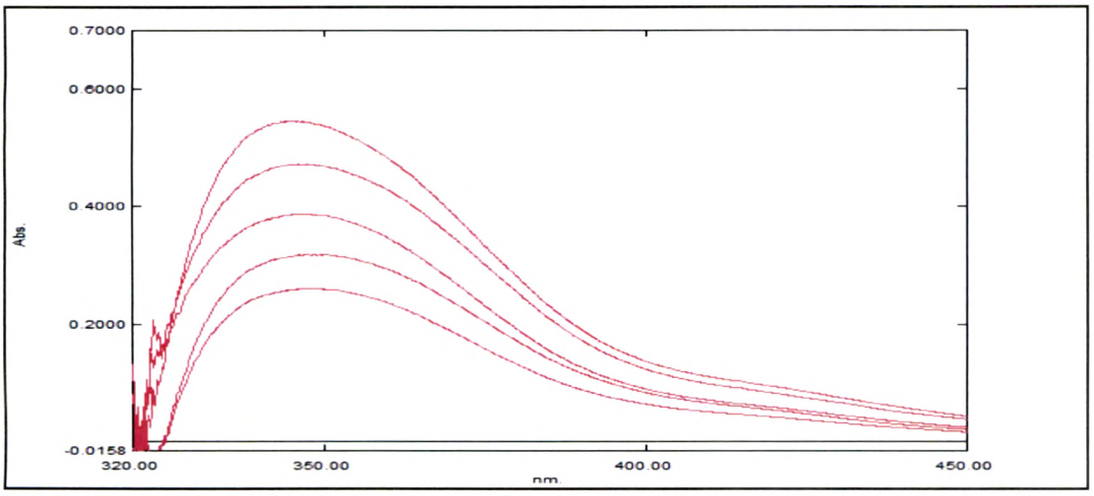


Table 3.2.1 The spectral and statistical data for determination of LI.

Parameters	**LI in presence of GA**
Wavelength	347 nm
Range	5-50 µg/ml
Linearity	0.9980
Intercept	-0.035
Slope	0.028
Sandell's sensitivity ($\mu g/cm^2$ per 0.001 absorbance unit)	0.042
LOD	0.52 µg/ml
LOQ	1.57 µg/ml
Intra day precision	% RSD < 2
Inter day precision	% RSD < 2

3.2.4.4 *Ex Vivo permeation studies*

Permeation coefficient (P_{eff}) was calculated for LI and LI-GA binary systems of both methods as per equation 2.1.1. Table 3.2.2 summarised the mean permeation coefficient ± SD of LI in absence (control) and presence of GA in binary systems. Release profile of LI in each binary system with ratio of LI: GA- 1:0.01, 1:0.05 and 1:0.1 is represented respectively in Figure 3.2.4, Figure 3.2.5 and Figure 3.2.6.

Table 3.2.2 Mean permeation coefficient ± SD of LI in absence (control) and presence of GA for binary systems of both methods.

Ratio of LI:GA	**Mean Permeation coefficient ± SD of LI, P_{eff} x 10^{-5} (cm/sec)(n=3)**		**Enhancement Ratio (ER)**	
LI (control)	0.316 ± 0.002*		1.00	
	PM Method*	**SE Method***	**PM Method**	**SE Method**
LI:GA 1:0.01	2.974 ± 0.9	1.553 ± 0.19	9.42	4.92
LI:GA 1:0.05	**4.185 ± 1.2**	2.342 ± 0.24	**13.25**	7.42
LI:GA 1:0.1	1.026 ± 0.14	1.448 ± 0.45	3.25	4.58

*Note: p value < 0.05; significant difference from control (Tukey's multiple comparison test)

Binary systems of both methods have higher permeation than control LI. i.e. GA significantly ($p<0.05$) increasing the permeation coefficient (P_{eff} cm/sec) of LI. Both methods confirm increase in permeation, but PM method with LI: GA 1:0.05 shows incredible (13.25 fold) enhancement in permeation of LI than control (LI without GA). It is observed from the results that GA causing maximum enhancement of LI comparative to AT, even it enhances permeation more than PI does of both AT and PI.

Figure 3.2.4 The release profile of LI with ratio LI: GA - 1:0.01 for both methods.

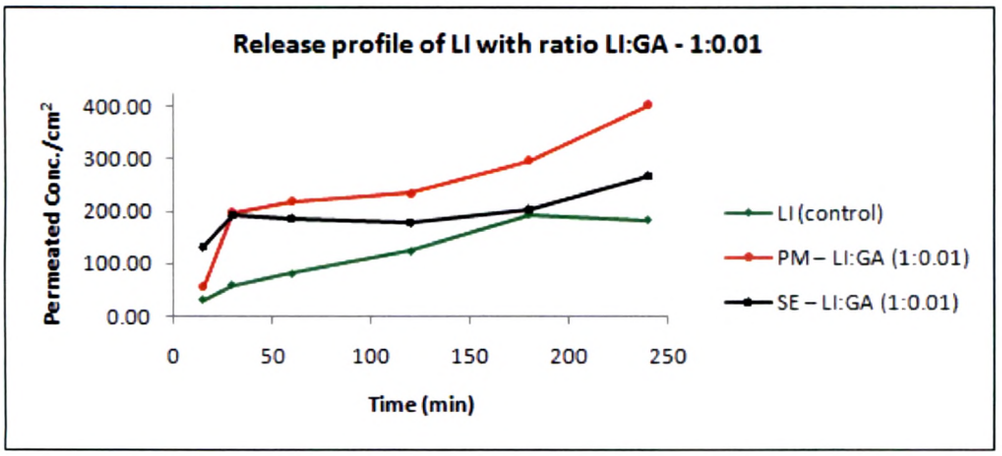


Figure 3.2.5 The release profile of LI with ratio LI: GA - 1:0.05 for both methods.

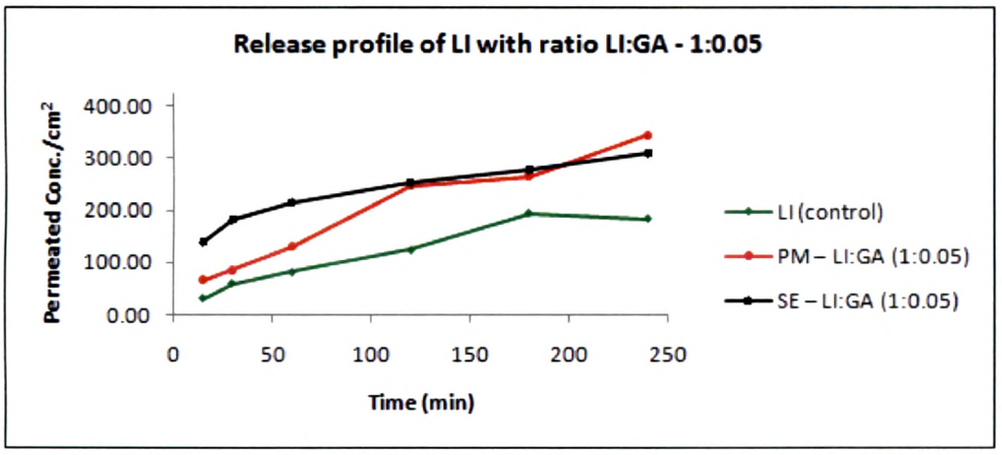
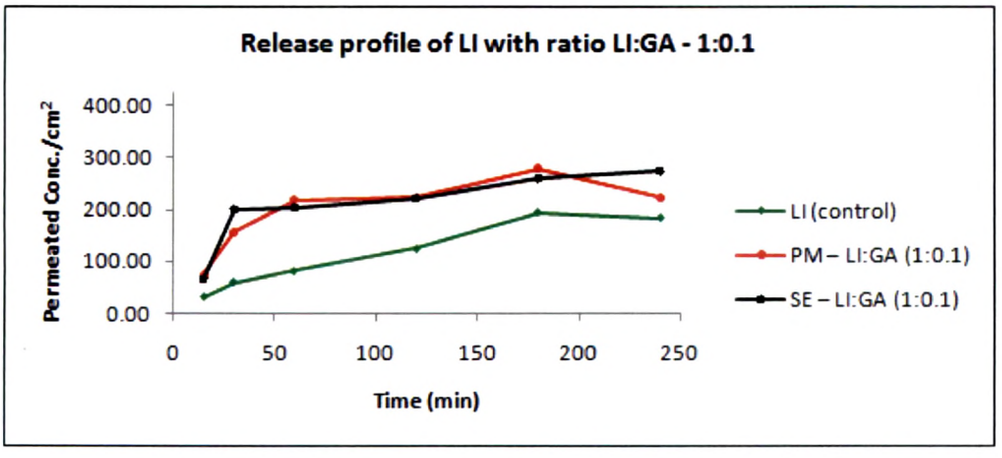


Figure 3.2.6 The release profile of LI with ratio LI: GA - 1:0.1 for both methods.



3.2.5 Conclusion

DSC and IR graphs suggest that there is no interaction between LI and GA in each of binary systems of PM and SE method. Both methods cause incredible enhancement in permeation in *ex vivo* permeation studies. It has been found that GA cause maximum enhancement (13.25 fold) of LI comparative to it does for AT as well more than the enhancement found with PI for both drugs. PM method has more enhancement of absorption of LI than SE method. Therefore it is concluded that, only PM method will be used for *in vivo* bioavailability determination.

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5 J. O. Maryadele, S. Ann, E. H. Patricia, The Merck Index, Edn 13, Merck and Co Inc., New Jersey, USA, **2001**, 1225.

3.3 Pharmacokinetics Studies of Lisinopril

In the present section, effect of bioenhancers (PI and GA) on lisinopril bioavailability was determined. Bioavailability determination of lisinopril in presence of natural bioenhancers was done by performing *in vivo* studies in rats. The prime objectives were,

- To develop simple and cost effective bio-analytical method for lisinopril in presence of PI and GA
- To perform *in vivo* studies of lisinopril and lisinopril with both bioenhancers (PI and GA) using rat as an animal model
- To determine all pharmacokinetic parameters using the data obtained from *in vivo* studies
- To draw significant conclusions by statistical treatment to data obtained

As described in section 2.4 pharmacokinetic variable is used to describe the time course of drug concentrations in blood in mathematical terms so that, performance of pharmaceutical dosage forms can be evaluated in terms of the rate and amount of drug they deliver to blood. Thus when poor absorption improved by incorporating absorption enhancers, *in vivo* absorption experiments give most comprehensive results.

To understand achievement of bioavailability enhancement of Lisinopril, *in vivo* studies were performed in albino rats. In present study, all pharmacokinetics parameters were calculated in MS Excel (1) using Wagner nelson method (2,3). All the calculations of pharmacokinetic parameters were done in same manner as for atenolol.

The experiments were conducted as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals, Reg. No. 404/01/a/CPCSEA) guidelines. The protocol for the study was approved by the Institutional ethical committee at The M. S. University of Baroda, India.

3.3.1 Materials

The Model drug lisinopril (LI) was procured as a gift sample from Wockhardt Ltd., Mumbai, India. Piperine (PI) and Glycyrrhizic acid ammonium salt (GA) were purchased from Sigma Aldrich Ltd., Mumbai, India. Potassium dihydrogen orthophosphate, orthophosphoric acid, hydrochloric acid were purchased from S. D. fine chemicals Ltd., Mumbai, India. Acetonitrile, methanol and water were of HPLC grade (Qualigence fine chemicals, Mumbai, India) and used without further purifications. Sep-Pak® C_{18} solid phase extraction (SPE) cartridges were from Waters Corporation, MA, USA. All the other chemicals and solvents were of analytical grade and were used without any further purification. Deionized double distilled water was used through out the study wherever needed. Animal feed and husk was obtained Amrut Laboratory Animal Feed.

3.3.2 Estimation of Drug in Plasma

3.3.2.1 HPLC method for Lisinopril

Lisinopril (LI) was determined according to previously reported method (4), which was modified. The method was applied for LI determination in presence of PI and GA from plasma samples. Various parameters for HPLC analysis of LI from plasma samples of in vivo studies are explained in Table 3.3.1.

3.3.2.2 Plasma extraction procedure

Standard or sample of LI was extracted from plasma by modifying previously reported method (5,6). Extraction from plasma was accomplished by solid phase extraction (Sep-Pak® C_{18} cartridges SPE). Plasma sample (400 µl) was acidified by adding 0.1 M HCl (600 µl) mixed and allow to stand for 5 min at room temperature. The mixture was dispensed slowly into SPE cartridge (which was previously conditioned with 2 ml methanol and twice with 2 ml water). SPE columns were washed five times with 2 ml of 0.1 M hydrochloric acid. An extracts were eluted into clean test tubes with 1 ml methanol and evaporated to dryness at 40 °C. The residue was reconstituted with 500 µl of methanol and 20 µl was injected into HPLC column, where lisinopril was separated from endogenous plasma substances.

Table 3.3.1 Various HPLC parameters of LI from plasma samples of in vivo studies.

Parameter	Details
System	Shimadzu, Kyoto, Japan
Pump	LC-20AT Prominence solvent delivery module, Double piston operated with 10 ml stainless steel pump head
Injector	Rheodyne manually driven injector with a 20 µl fixed loop with 60° rotation.
Detector	UV detector – SPD-20A Prominence Range of measurement – 0-2 AU Integrator output - ± 1.0 V Auto-zero – Full scale
Software	Spinchrom Chromatographic Station® CFR Version 2.4.0.195 (Spinchrom Pvt. Ltd., Chennai, India)
Stationary Phase	Analytical Column – Phenomenex (Torrance, USA) C_{18} column, Particle size 5 µm; 250 mm X 4.6 mm i.d. Guard Column – ODS column, Particle size 10 µm; 10 mm X 5 mm i.d.)
Mobile Phase Composition	Potassium dihydrogen phosphate 20 mM (pH set to 3 with orthophosphoric acid) : Acetonitrile (80:20 v/v)
Temperature	Ambient
Flow Rate	0.6 ml/min
UV detection wavelength (λ)	215 nm
Retention time	5.2 min, with Asymmetry – 1.2 and Resolution – 2.01
Internal Standard	Enalapril

3.3.2.3 *Extraction efficiency*

The extraction efficiency was calculated by adding known amount of LI (100, 250 and 500 ng/ml; n = 6 per concentration) or internal standard (enalapril 25 µl, 100 µg/ml) to 400 µl of blank rat plasma. LI was extracted as described in section 3.3.2.2. The residue was reconstituted in mobile phase. The known amount of sample was injected into the chromatographic system.

The peak area of sample was compared to those obtained from equivalent volumes of standard solution of drug in mobile phase directly injected into the HPLC system. The determination of un-extracted samples was performed in triplicate for each concentration.

The developed methods were validated for following parameters.

3.3.2.4 *Linearity and range*

The linear detector response for the assay was tested as follows. These determination (n=6) from minimum of five concentration levels (50, 100, 250, 500, and 1000 ng/ml) of the analyte were made. Detector response was correlated against analyte concentration by least squares regression.

3.3.2.5 *Accuracy and precision*

For the determination of intra day and inter day accuracy and precision of the assay, various quantities of LI was added to aliquots of 400 µl rat plasma to yield 50, 100, 250, and 500 ng/ml. Accuracy was expressed as Mean percent,

Equation 3.1

$$Accuracy = \left(\frac{Mean\ measured\ Concentration}{Expected\ Concentration} \right) \times 100$$

Precision was calculated as inter and intra day coefficient of variation

Equation 3.2

$$\%\ CV = \left(\frac{SD}{Mean} \right) \times 100$$

3.3.3 Pharmacokinetic study of LI

3.3.3.1 Animal treatment

Female Wistar albino rats (weighing about 250-300 gm) were used in all the experiments. Rats were provided by Sun Pharma Advanced Research Company Ltd., Vadodara, India. Aanimals were housed over two weeks in a temperature (20 – 25 °C) and relative humidity (between 50 and 60 %) controlled room and were given standard rat chow and water which were freely available. The protocol for studies was approved by the Institutional ethical committee at The M S University of Baroda, India. The experiments were conducted as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals, Reg. No. 404/01/a/CPCSEA) guidelines. All animals were fasted overnight with free access to tap water before experiments.

3.3.3.2 Calculation of dose of the drug in rats

A dose calculation was done same as described in section 2.4.4.2. Maximum dose of LI that can be given to human in single day is 50 mg. In this study, the dose given to rats was 1 mg/kg which was below LD_{50} dose.

3.3.3.3 Route of administration and withdrawal of blood samples

Different groups of rats were taken for pharmacokinetic studies. Each group has three rats. Table 3.3.2 represents all groups and dose of LI and bioenhancers administered to rats. All rats were fasted for at least 12 hr prior to experiment, with free access to water. LI (control), LI along with PI, and LI along with GA were administered orally with gavage needle. LI and GA was dissolved, while PI was suspended in 1% carboxymethyl cellulose (sodium salt – solution was made in hot distilled water).

Table 3.3.2 The Details of groups and dose given to the rats.

Group No.	Group Details (3 rats/group)	Dose (mg/kg)
1	LI (Control)	LI 1
2	LI + PI 1	LI 1 + PI 0.09
3	LI + PI 2	LI 1 + PI 0.45
4	LI + PI 3	LI 1 + PI 0.9
5	LI + GA 1	LI 1 + GA 0.0225
6	LI + GA 2	LI 1 + GA 0.1125
7	LI + GA 3	LI 1 + GA 0.225

After oral administration 1 ml of blood samples were collected from the retro orbital plexus of rat at 0, 1, 2, 3, 4, 6, 8, 12 and 24 hr time points into heparinized collection tubes. The blood samples were immediately centrifuged (3000 rpm) for 10 min at an ambient temperature. Supernatant plasma was separated and stored at –20 °C until analyzed. Plasma samples collected from rats were analyzed using modified HPLC method (as described in Section 3.3.2) and drug plasma concentration values were determined from calibration curve.

3.3.3.4 *Statistical analysis*

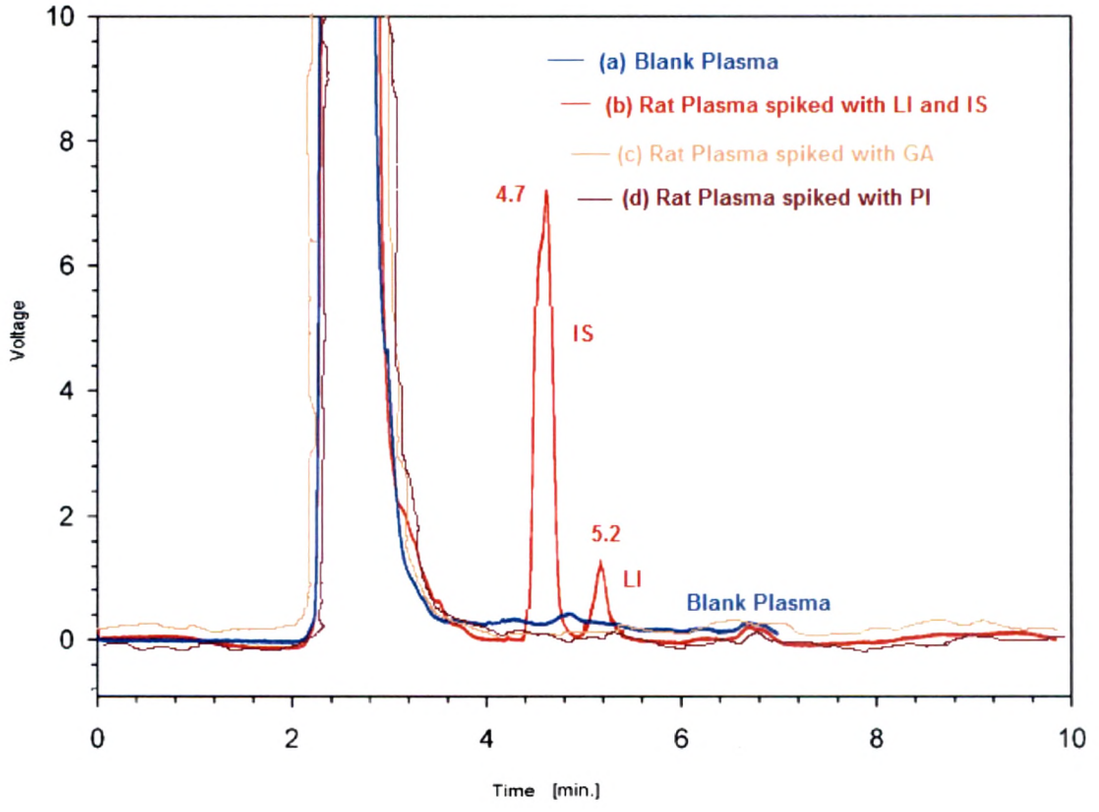
Primary pharmacokinetic parameters were determined using none compartmental analysis. Maximum plasma concentration C_{max}, and time to reach maximum concentration (T_{max}) were estimated directly from plasma concentration time curve. All other pharmacokinetic parameters were calculated as described section 2.4.1. All data were expressed as mean $\pm$ SD. Plasma drug concentrations as well as pharmacokinetic parameters were compared by student's t-test at α= 0.05.

3.3.4 Results and discussion

3.3.4.1 HPLC method for Lisinopril

The chromatograph of rat blank plasma and plasma spiked with LI and internal standard is given in Figure 3.3.1. It also represents plasma spiked with GA and PI. It is clearly observed from the chromatogram that no interference from blank plasma peaks to peaks of drug and internal standard; as well bioenhancers are not showing any peak at analytical wavelength of LI. The peaks of LI and the internal standard were sufficiently separated with typical retention times of 5.2 min for LI and 4.7 min for internal standard. Usual running time for the sample was 10 min.

Figure 3.3.1 The Chromatograph of rat blank plasma, plasma spiked with LI and IS, plasma spiked with GA and PI.



Calibration curve (peak area ratio of LI to IS versus LI concentration) in plasma was constructed by spiking five different concentrations of LI and fixed concentration of IS. Chromatographic responses were found to be linear over an analytical range of 50–1000 ng/ml and found to be quite satisfactory and reproducible with time. Linear regression equation was calculated by least squares method using Microsoft Excel® program and summarized in Table 3.3.3. Correlation coefficient equals 0.9995, indicating a strong linear relationship between the variables. Extraction efficiency was greater than 85 % which is represented Table 3.3.4.

Table 3.3.3 The spectral data for determination of LI by proposed HPLC method.

Parameters	**Values**
Linearity Range (ng/ml)	50 – 1000
Coefficient of determination (r^2)	0.9990
Correlation coefficient (r)	0.9995
*Regression equation (Y=m·x + c)	
Slope (m)	0.019
Intercept (c)	2.818
Limit of detection (ng/ml) [a]	14.79 ± 1.9
Limit of quantitation (ng/ml)[a]	44.84 ± 3.5

* Y=m·x + c, where x is the concentration (ng/ml). [a] Data represents Mean ± SD (n = 6).

Table 3.3.4 Mean Extraction Recovery of LI and internal standard from the spiked rat plasma.

	Internal Standard	**LI**		
Concentrations (ng/ml)	2500	100	250	500
Mean Extraction Recovery (%)*	93.14 ± 3.98	90.32 ± 2.8	90.82 ± 5.9	89.71 ± 5.6
Average Extraction Recovery (%)	93.14 ± 3.98		90.28 ± 4.77	

*Data represents Mean ± SD (n = 6).

Accuracy data in the present study ranged from 99.22 to 101.24 % (Table 3.3.5) indicates that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of LI, gave an R.S.D. very low (7), revealed that the proposed method is highly precise. Accuracy of method was evaluated by using *t*-test at four concentration levels. The *t*-values obtained for 50, 100, 250 and 500 ng/ml were 1.59, 1.51, 0.87 and 2.18 for inter-day whereas, 1.89, 0.95, 0.47 and 0.14 for intra-day, respectively. The *t*-value required for significance at 5 % level at 5 degrees of freedom is 2.57, and obtained values were well below this value. Thus no significant difference was observed between amounts of drug added and recovered. Overall, data summarized in Table 3.3.5, enables that an excellent accuracy and high precision was obtained.

Table 3.3.5 Inter-day and intra-day precision and accuracy of the method for LI estimation in rat plasma.

Concentrations (ng/ml)						
Nominal	**Found mean** [a]	**SD**	**RSD %**	**Mean Accuracy*** **(%)**	**t_{cal}**#	**CI**
Intra-day precision and accuracy of LI determination in rat plasma (n=6)						
50	49.65	0.42	0.85	99.30	1.89	50 ± 0.34
100	99.22	1.28	1.29	99.22	0.95	100 ± 1.03
250	250.68	1.30	0.52	100.27	0.47	250 ± 1.04
500	499.79	1.59	0.32	99.95	0.14	500 ± 1.27
Inter-day precision and accuracy of LI determination in rat plasma (n=6)						
50	49.70	0.32	0.64	99.41	1.59	50 ± 0.26
100	101.24	1.44	1.42	101.24	1.51	100 ± 1.15
250	248.75	2.49	0.99	99.50	0.87	250 ± 1.99
500	503.33	2.66	0.53	100.67	2.18	500 ± 2.13

[a] Average of six determinations at four concentration levels for inter-day and intra-day respectively.

*All the mean accuracy was calculated against their nominal concentrations.

$t_{cal} = |100 - R|\sqrt{n} / R.S.D$ Where t_{cal} is the calculated t value, n is the number of replicated, and R is mean accuracy. Tabulated t-value for 95 % two sided confidence interval for 5 degree of freedom was (t_{tab} =) 2.57.

3.3.4.2 *Pharmacokinetic Study of LI with PI*

Plasma samples collected from the rats were analyzed using proposed reverse phase HPLC method and LI plasma concentration values were determined from calibration curve. Mean plasma concentrations of LI in each of rats i.e. control rat and rats treated with each of three concentrations of PI is shown in Table 3.3.6. The average plasma LI concentrations versus time profiles in presence of each concentrations of PI are represented in Figure 3.3.2, Figure 3.3.3 and Figure 3.3.4. It is clearly observed that plasma drug concentrations with each concentrations of PI are highly significant ($p < 0.001$) and increased than control LI.

Table 3.3.6 The mean plasma concentrations of LI after administrations of LI, LI with three concentrations of PI.

Time (hr)	Observed Mean ± SD Plasma Concentrations of LI (ng/ml) (n = 3)			
	LI (Control)	LI + PI 0.09 mg/kg	LI + PI 0.45 mg/kg	LI + PI 0.9 mg/kg
0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.00	53.14 ± 3.12	65.33 ± 4.39	50.67 ± 2.50	52.50 ± 7.97
2.00	72.92 ± 3.10	91.16 ± 5.12	70.93 ± 4.18	73.84 ± 10.90
3.00	92.86 ± 7.04	114.57 ± 5.98	107.24 ± 4.46	112.40 ± 7.16
4.00	112.83 ± 8.50	147.60 ± 6.40	165.96 ± 5.04	145.34 ± 3.42
6.00	125.95 ± 6.89	219.92 ± 4.64	199.39 ± 2.69	182.86 ± 5.85
8.00	103.04 ± 4.15	175.65 ± 5.46	162.98 ± 6.64	151.89 ± 1.34
12.00	89.36 ± 4.98	133.32 ± 8.15	127.42 ± 2.66	119.24 ± 1.08
24.00	47.68 ± 3.61	82.41 ± 2.28	73.62 ± 3.70	67.02 ± 2.38

Figure 3.3.5, Figure 3.3.6, Figure 3.3.7, and Figure 3.3.8 represents the natural log of mean plasma concentrations of LI with time plot for absorption and elimination rate constant. Each Figure indicates there is no much change in elimination rate constant. Table 3.3.7 compares various pharmacokinetic parameters of LI with and without PI administration. It is found that C_{max}, $AUC_{0\text{-}24\ hr}$, $T_{1/2a}$, and K_a were significantly increased, whereas no difference observed in T_{max}, K_{el}, and $T_{1/2el}$. Relative bioavailability of LI with PI is almost 1.5 fold more than the pure drug LI.

Figure 3.3.2 The Mean plasma concentrations versus time profile following a single oral administration of LI, and LI in presence of PI 0.09 mg/kg. Each value is Mean ± SD of three determinations.

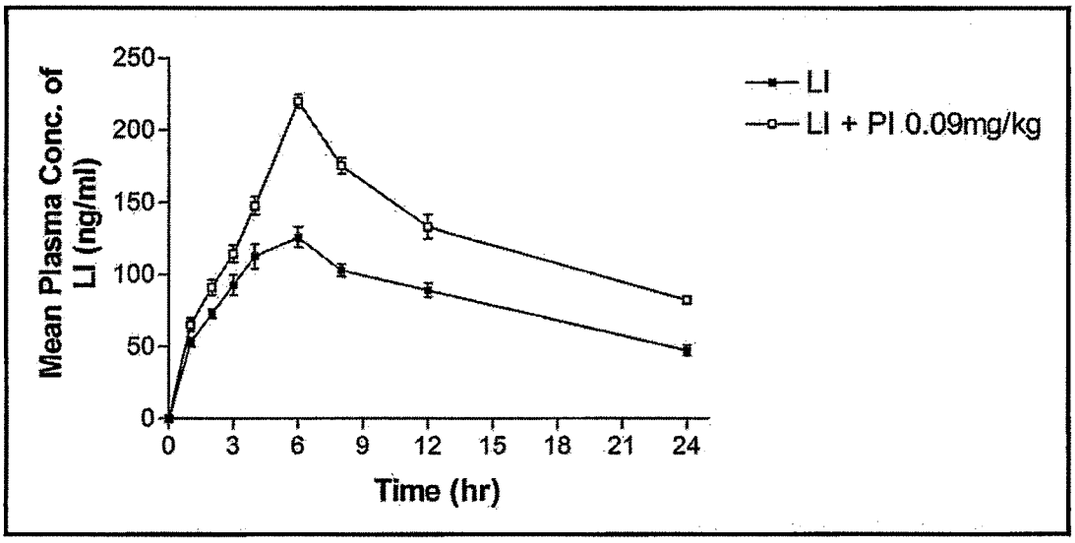


Figure 3.3.3 The Mean plasma concentrations versus time profile following a single oral administration of LI, and LI in presence of PI 0.45 mg/kg. Each value is Mean ± SD of three determinations.

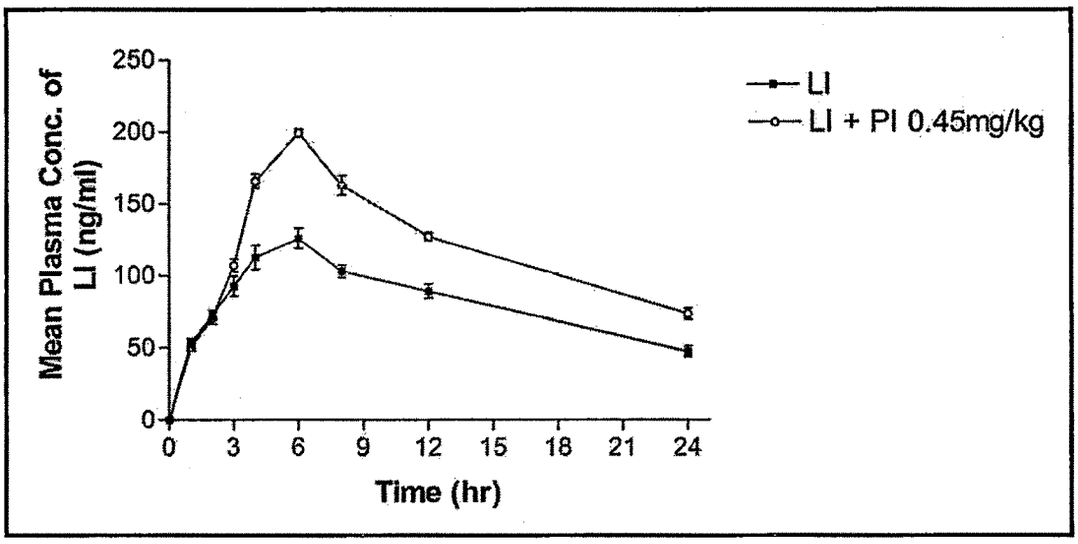


Figure 3.3.4 The Mean plasma concentrations versus time profile following a single oral administration of LI, and LI in presence of PI 0.9 mg/kg. Each value is Mean ± SD of three determinations.

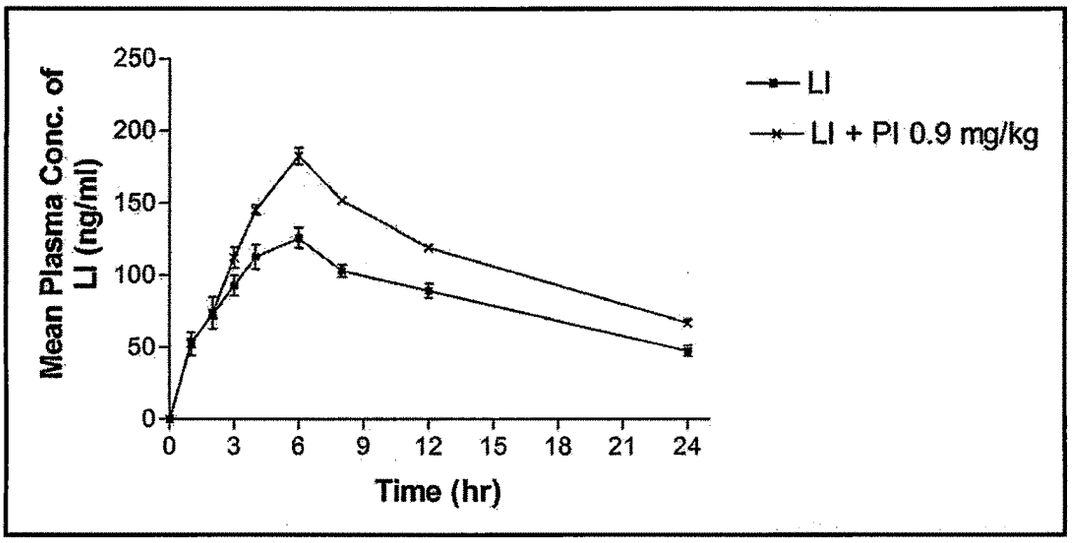


Figure 3.3.5 The Natural log of mean plasma concentrations of LI versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.

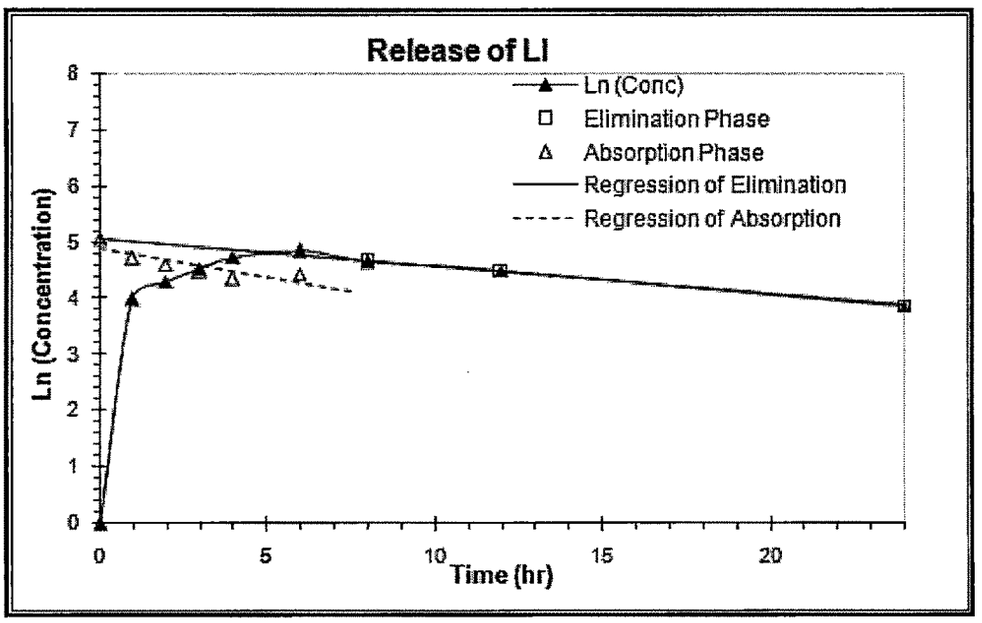


Figure 3.3.6 The Natural log of mean plasma concentrations of LI with PI 0.09 mg/kg versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.

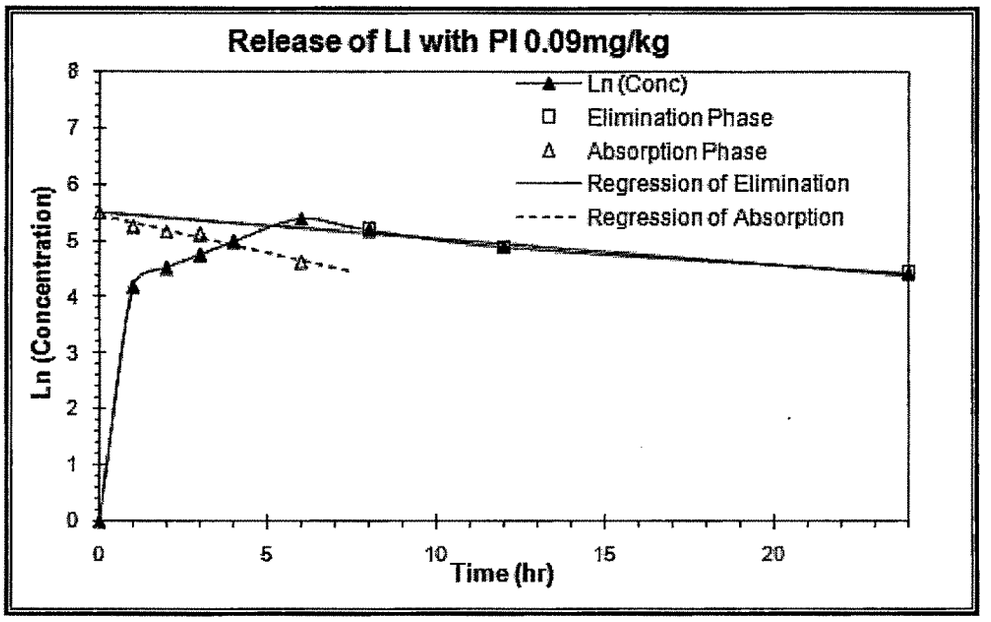


Figure 3.3.7 The Natural log of mean plasma concentrations of LI with PI 0.45 mg/kg versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.

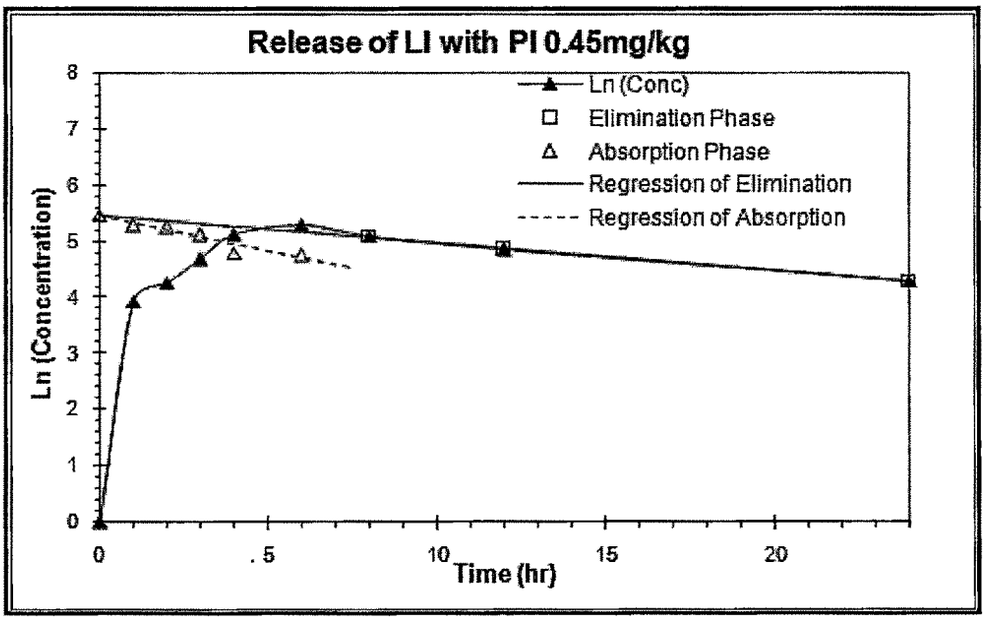
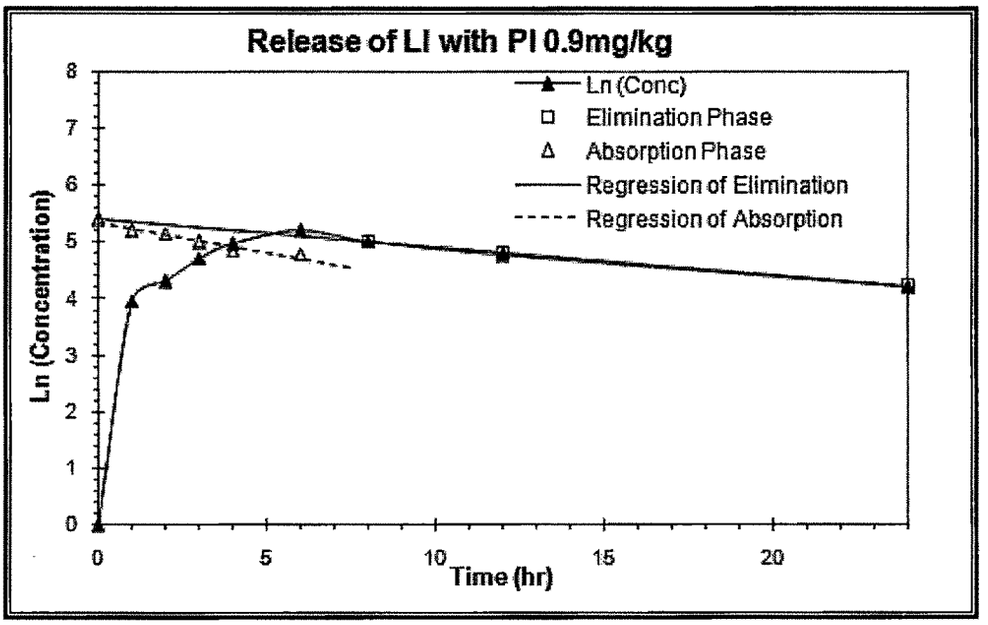


Figure 3.3.8 The Natural log of mean plasma concentrations of LI with PI 0.9 mg/kg versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.



Thus different concentrations of PI were administered to rats with LI. PI significantly increases mean plasma concentration of LI. Same as AT enhancement PI also increase bioavailability of LI with low concentration i.e. 0.09 mg/kg (5 mg human dose) of PI. PI causes 1.55 fold permeation enhancement of LI. An enhancement in absorption could be due to following mechanism,

(a) PI can increases splanchnic blood flow, decreased hydrochloric acid secretion, delay in gastric emptying and an alteration of membrane dynamics which aid in efficient permeability through membranes (8). As described in section 2.4.5 it could modulate membrane dynamics and its easy partitioning ability, increases small intestinal surface, and assisting efficient penetration through epithelial barriers (9). Thus enhanced absorption of LI may be due to brush broader mechanism of PI.

(b) LI absorbs in jejunum pH through peptide transporters (10), PI which is inhibitor of P-glycoprotein and CYP3A4 (11) may be having some action on peptide transporter which can lead increase in permeation of LI.

It is observed that as the concentration of PI increasing, enhancement effect on absorption of LI is decreasing. Thus, absorption enhancement found to be highest with lowest concentration. Thus PI is most effective in lowest concentration.

Table 3.3.7 The pharmacokinetic parameters of LI after a single oral dose of LI, to rat (each group 3 rats), in absence and presence of each of three concentrations of PI.

Pharmacokinetic parameters	**LI**	**LI + PI 0.09 mg/kg**	**LI + PI 0.45 mg/kg**	**LI + PI 0.9 mg/kg**
Absorption rate constant, K_a (hr^{-1})	0.10	**0.14**	0.13	0.11
Elimination rate constant, K_{el} (hr^{-1})	0.05	**0.05**	0.05	0.05
Time required for maximum plasma concentration, T_{max} (hr)	6.00	**6.00**	6.00	6.00
Maximum plasma concentration, C_{max} (ng/ml)	125.95	**219.92**	199.39	182.86
Plasma half life, $T_{1/2}$ (hr)	14.11	**15.19**	14.21	13.75
Area under curve at 24hr, $AUC_{(0-24)}$ (ng hr/ml)	1950.15	**3020.62**	2826.59	2634.18
Area under curve at infinite time, $AUC_{(0-\infty)}$ (ng hr/ml)	2920.66	**4826.47**	4336.51	3963.56
Area under curve at 24hr, $AUMC_{(0-24)}$ (ng hr^2/ml)	20583.38	**32998.74**	30647.89	28347.02
Volume of distribution, V_d (ml)	2.3	**1.2**	1.3	1.5
Mean residence time, MRT (hr)	10.55	**10.92**	10.84	10.76
Total clearance rate, TCR (ml/hr)	0.11	**0.04**	0.05	0.06
Relative Bioavailability (%)	1.00	**1.55**	1.44	1.35

Pharmacokinetic data are Mean values $\pm$ SD (n=3).

3.3.4.3 *Pharmacokinetic Study of LI with GA*

Plasma samples collected from rats were analyzed using proposed reverse phase HPLC method and drug plasma concentration values were determined from calibration curve. Mean plasma concentrations of LI in each of rats i.e. control rat and rats treated with each of three concentrations of GA are shown in Table 3.3.8. Average plasma drug concentrations versus time profiles in presence of each concentrations of GA are represented in Figure 3.3.9, Figure 3.3.10 and Figure 3.3.11. It is clearly observed that plasma drug concentrations with each concentrations of GA is highly significant ($p < 0.001$) and increased than control LI.

Table 3.3.8 The mean plasma concentrations of LI after administrations of LI, LI with three concentrations of GA.

Time (hr)	Observed Mean ± SD Plasma Concentrations of LI (ng/ml) (n = 3)			
	LI (Control)	LI + GA 0.0225 mg/kg	LI + GA 0.1125 mg/kg	LI + GA 0.225 mg/kg
0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.00	53.14 ± 3.12	105.33 ± 3.93	91.39 ± 4.08	83.14 ± 1.83
2.00	72.92 ± 3.10	171.16 ± 3.69	150.16 ± 1.90	149.86 ± 4.42
3.00	92.86 ± 7.04	224.57 ± 6.49	227.24 ± 4.13	182.83 ± 5.77
4.00	112.83 ± 8.50	291.84 ± 1.04	300.34 ± 5.20	224.45 ± 6.80
6.00	125.95 ± 6.89	237.93 ± 4.39	259.96 ± 1.80	183.04 ± 1.13
8.00	103.04 ± 4.15	198.65 ± 2.79	189.98 ± 3.79	149.36 ± 6.62
12.00	89.36 ± 4.98	134.32 ± 6.22	137.88 ± 2.55	111.92 ± 4.91
24.00	47.68 ± 3.61	98.41 ± 2.14	101.62 ± 3.00	67.68 ± 3.32

Figure 3.3.12, Figure 3.3.13, and Figure 3.3.14 represents the natural log of mean plasma concentrations of LI with time plot for absorption and elimination rate constant. Each Figure indicates no much change in elimination rate constant. Table 3.3.9 compares various pharmacokinetic parameters of LI with and without GA administration. It is found that C_{max}, $AUC_{0\text{-}24\ hr}$, $T_{1/2a}$, were increased; decrease in T_{max}, whereas no much difference observed in K_{el}, $T_{1/2el}$, and K_a. Relative bioavailability of LI with GA is almost 2 fold more than the pure drug LI.

Figure 3.3.9 The Mean plasma concentrations versus time profile following a single oral administration of LI, and LI in presence of GA 0.0225 mg/kg. Each value is Mean ± SD of three determinations.

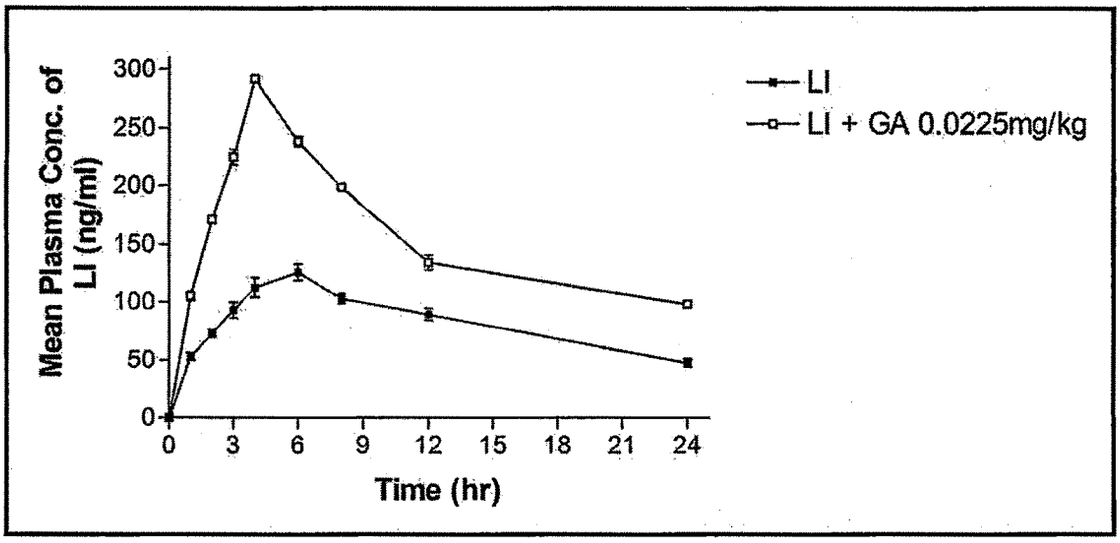


Figure 3.3.10 The Mean plasma concentrations versus time profile following a single oral administration of LI, and LI in presence of GA 0.1125 mg/kg. Each value is Mean ± SD of three determinations.

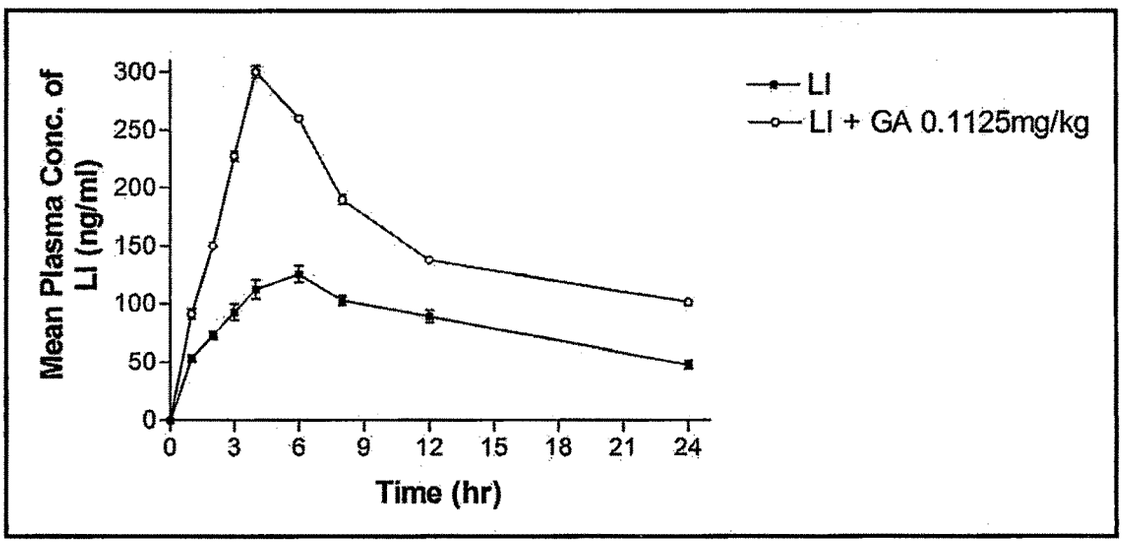


Figure 3.3.11 The Mean plasma concentrations versus time profile following a single oral administration of LI, and LI in presence of GA 0.225 mg/kg. Each value is Mean ± SD of three determinations.

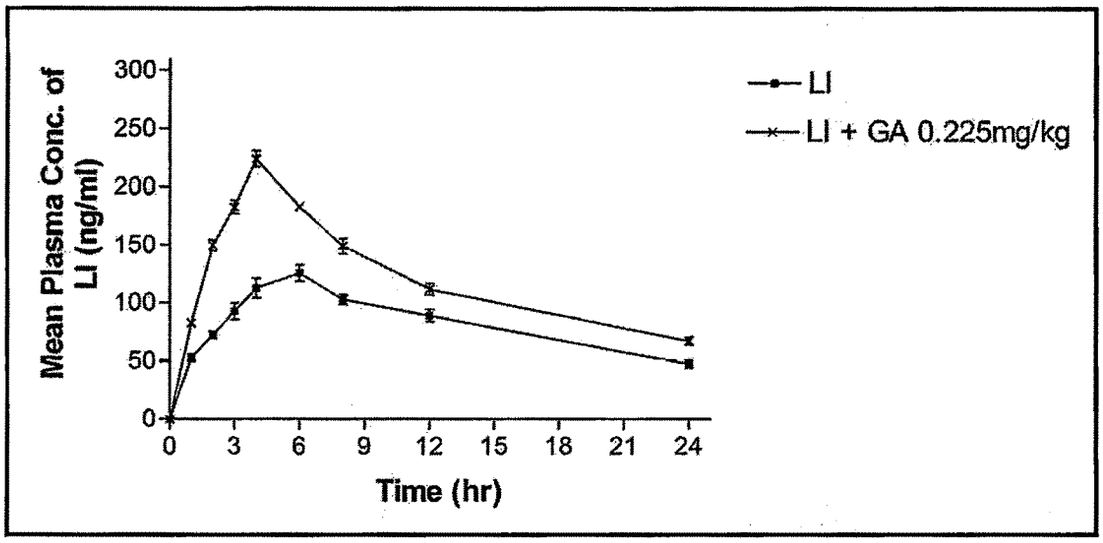


Figure 3.3.12 The Natural log of mean plasma concentrations of LI with GA 0.0225 mg/kg versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.

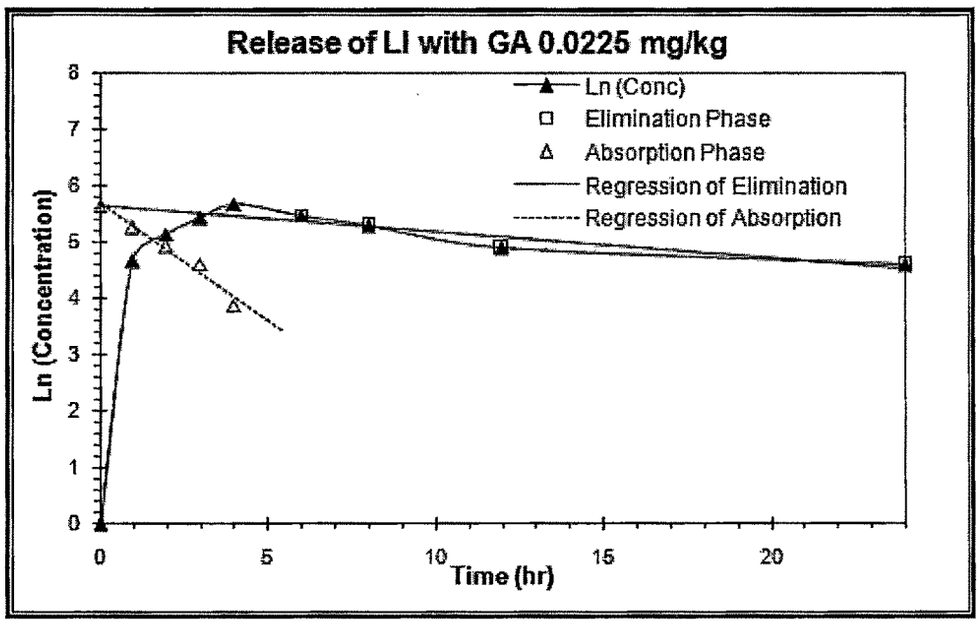


Figure 3.3.13 The Natural log of mean plasma concentrations of LI with GA 0.1125 mg/kg versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.

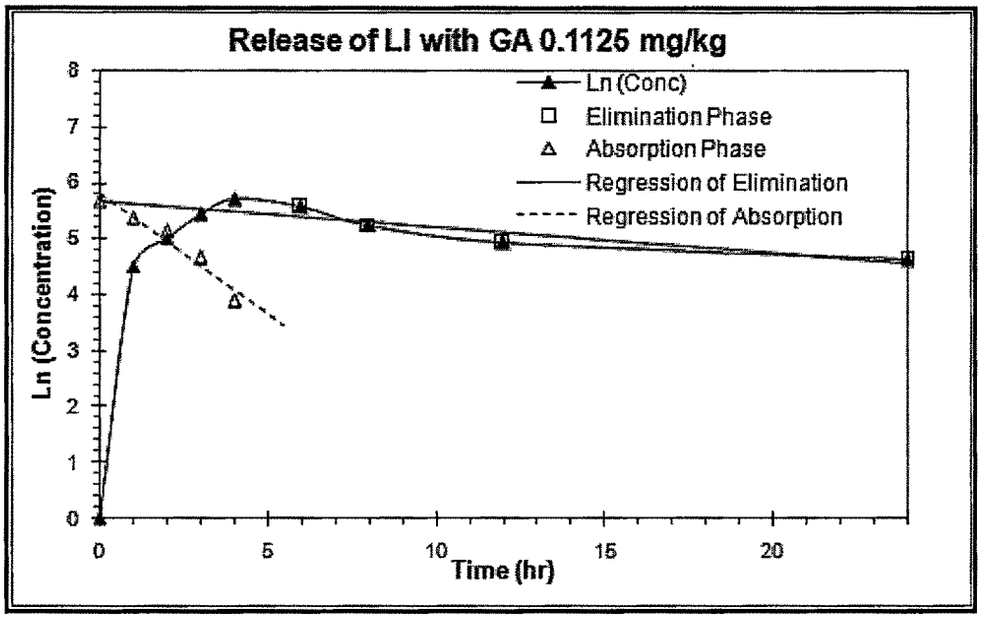


Figure 3.3.14 The Natural log of mean plasma concentrations of LI with GA 0.225 mg/kg versus time plot for determination of k_a and k_{el}. Each value is Mean ± SD of three determinations.

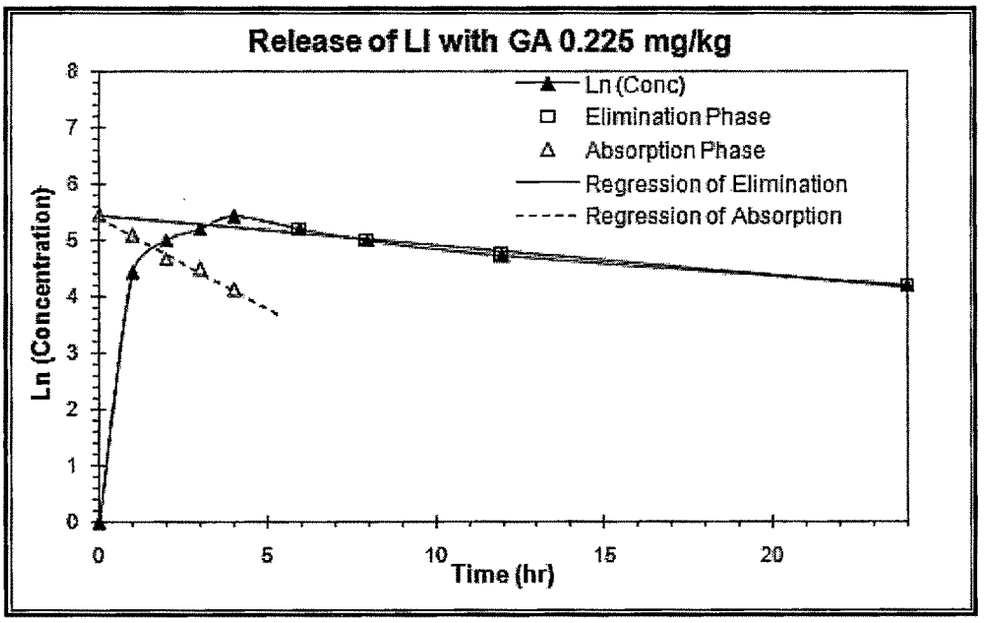


Table 3.3.9 The pharmacokinetic parameters of LI after a single oral dose of LI, to rat (each group 3 rats), in absence and presence of each of three concentrations of GA.

Pharmacokinetic parameters	**LI**	**LI + GA 0.0225mg/kg**	**LI + GA 0.1125mg/kg**	**LI + GA 0.225mg/kg**
Absorption rate constant, K_a (hr^{-1})	0.10	0.42	**0.42**	0.32
Elimination rate constant, K_{el} (hr^{-1})	0.05	0.05	**0.05**	0.05
Time required for maximum plasma concentration, T_{max} (hr)	6.00	4.00	**4.00**	4.00
Maximum plasma concentration, C_{max} (ng/ml)	125.95	291.84	**300.34**	224.45
Plasma half life, $T_{1/2}$ (hr)	14.11	14.92	**14.89**	13.14
Area under curve at 24hr, $AUC_{(0-24)}$ (ng hr/ml)	1950.15	3675.69	**3721.91**	2868.13
Area under curve at infinite time, $AUC_{(0-\infty)}$ (ng hr/ml)	2920.66	5795.19	**5905.83**	4151.65
Area under curve at 24hr, $AUMC_{(0-24)}$ (ng hr^2/ml)	20583.38	37561.67	**38424.04**	28549.25
Volume of distribution, V_d (ml)	2.3	0.72	**0.70**	0.93
Mean residence time, MRT (hr)	10.55	10.22	**10.33**	9.95
Total clearance rate, TCR (ml/hr)	0.11	0.03	**0.03**	0.05
Relative Bioavailability (%)	1.00	1.88	**1.91**	1.47

Pharmacokinetic data are Mean values $\pm$ SD (n=3).

Thus it is found that different concentrations of GA were administered to rats with LI, significantly increase mean plasma concentration of LI. Maximum bioenhancement of LI (1.91 fold than pure LI) was found with 0.1125 mg/kg of GA (i.e. AT: GA 1:0.05 ratio). This enhancement pattern is similar to AT enhancement by GA. It has been described in section 2.4.5 that GA hydrolysed (Figure 2.4.15) to glycyrrhetinic acid (GTA – aglycon of GA). GTA is very active and cause enhancement of absorption of many anti-cancer, anti-fungal and antibiotics (12). It is clearly observed that GTA has played role in enhancement of absorption of LI may be acting on transport carrier or some unknown cause (13).

It is observed from that two concentrations level of GA increasing absorption of LI. While highest concentration of GA, cause less enhancement than lower concentrations. These results are found to be similar absorption pattern as found in AT absorption enhancement by GA.

3.3.5 Conclusion

The results pharmacokinetic data suggests that both bioenhancers PI and GA effectively cause increment in mean LI plasma concentration. Both bioenhancers PI and GA administration with LI are causing increment in the area under the curve and absorption. They don't affect elimination rate. These results support results obtained in previous studies of *ex vivo* permeation. Both bioenhancers are more effective in lower concentrations.

In the *in vivo* studies of LI with PI, as same for AT, lowest concentration of PI (LI: PI 2:1 i.e. 0.09 mg/kg of PI) is sufficient enough to act as bioenhancer. It causes 1.55 fold increment in bioavailability. While in *ex vivo* studies It has been found that PI (LI: PI 2:2) is most effective to cause bioenhancement of LI. This suggests that for *in vivo* permeation lowest concentration of PI is effective as bioenhancer. Therefore binary system of PM method with PI ratio (LI: PI 2:1) was optimised for formulation development.

In the *in vivo* studies of LI with GA, (LI: GA 1:0.05 i.e. 0.1125 mg/kg of GA) is causing 1.91 fold increase in bioavailability of LI. Same ratio of LI: GA enhances permeation of LI in *ex vivo* permeation studies. Therefore binary system of PM method with GA ratio (LI: GA 1:0.05) was optimised for formulation development.

Thus like AT, PI and GA can be successfully utilized as bioenhancer for lisinopril too.

3.3.6 References

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3.4 FORMULATION DEVELOPMENT OF LISINOPRIL

In the present section, binary systems of lisinopril and both bioenhancers (PI and GA) of physical mixture (PM) method were formulated as single oral powder form. Various compatible excipients were used to formulate the oral powders. The prepared formulations were evaluated for,

- Appearance,
- Angle of repose
- Uniformity of content

As described in section 2.5, oral single dose powders were formulated for lisinopril-bioenhancers binary system of PM method. The advantage of single dose oral powder form is easy formulation and having quick absorption (1).

3.4.1 Materials

The Model drug lisinopril (LI) was procured as a gift sample from Wockhardt Ltd., Mumbai, India. Piperine (PI) and Glycyrrhizic acid ammonium salt (GA) were purchased from Sigma Aldrich Ltd., Mumbai, India. Direct compressible grade mannitol and Sodium lauryl sulphate obtained from Alembic Ltd., Baroda, India. Magnesium Stearate was purchased from Suvidhinath Lab, Baroda, India.

3.4.2 Formulation Development for LI-bioenhancer binary systems

Each of previous studies of bioenhancement of LI by PI suggests that in the physical mixture (PM) method with LI: PI (2:1) ratio shows maximum permeation compared to other two ratios. The studies of bioenhancement of LI by GA suggest that PM method with LI: GA (1:0.05 i.e GA is 5 % w/w of LI) ratio shows maximum permeation. This optimized binary system of LI-PI and LI-GA was selected to formulate single dose oral powder. These single dose oral powders were incorporated into *cachets* to avoid leaching and provide better storage conditions. The formula of powder is summarised in Table 3.4.1. Mannitol of direct compressible (DC) grade was used as diluent. Magnesium stearate was used as lubricant. Sodium lauryl sulphate was used to aid the dissolution of the powder.

Table 3.4.1 Formulation of single dose oral powder.

Drug/Excipients	LI-PI binary system	LI-GA binary system
PM of LI-PI/ LI-GA containing eq. to 10 mg LI (g)	0.300	0.210
Mannitol DC grade (g)	3.644	3.734
Magnesium Stearate (g)	0.036	0.036
Sodium Lauryl Sulphate (g)	0.020	0.020
Total filled weight per 20 cachet (g)	4.000	4.000

3.4.3 Evaluation Parameters

3.4.3.1 *Angle of Repose (Φ)* As per section 2.5.3.1.

3.4.3.2 Uniformity of Content

The drug eq. to 10 mg of the powder dosage was dissolved in 10 ml methanol. LI was determined using spectrophotometric method. For LI-PI binary system, LI was determined as method described in section 3.1.3.3 and for LI-GA binary system, as per section 3.2.3.3. Each characteristics of oral powder are summarized in Table 3.4.2.

Table 3.4.2 Properties of single dose oral powder of LI-PI and LI-GA binary systems.

Parameters	LI-PI binary system	LI-GA binary system
Appearance	White powder	White powder
Uniformity of Content	100.37 ± 0.56	99.36 ± 0.38
Angle of Repose (°) ± SD*	34.75 ± 2.63	33.83 ± 2.36

* Data represents the Mean ± SD (n = 3)

3.4.4 References

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