4. Summary & Discussions

Bioavailability is the significant factor in affecting the systemic availability of a drug in a dosage form which has led to continuous development and expansion of novel drug delivery system. The market potential of novel drug delivery system is less due to its high cost of manufacturing and lack of long stability. While oral route of administration is always preferred route if systemic effect is desired. Oral route is also most common route for administration. For patient oral formulations are convenient as it is easy to handle and administer in correct dose. In addition, manufacturers usually have a preference for oral formulations owing to their low costs of production comparison to novel drug delivery systems.

Chapter 1 of thesis exemplifies introduction to the problem. There are many biopharmaceutical and pharmacokinetics factors which are responsible for poor bioavailability of drugs. Various techniques used for bioavailability enhancement are mentioned in introduction. Of these all available techniques new and promising concept of administering natural bioenhancer with poor permeable drugs is studied.

Thus it is hypothesized that natural bioenhancers when administered with poor bioavailable drug show improvement in drug bioavailability. In the present work, this new concept of incorporating bioenhancer was used to improve bioavailability pattern of poor permeable BCS class III drugs such as atenolol (AT) and lisinopril (LI). Objectives of the work achieved and profiles of AT and LI are pointed out in introduction part. The hypothesis was studied by using three concentrations of two different natural bioenhancers such as piperine (PI) and glycyrrhizic acid ammonium salt (GA).

In the present study binary systems of drug-bioenhancers were prepared with three different methods. The binary systems were further formulated in oral single powder dosage form. Permeability/bioavailability was determined by various methods which have been accepted by FDA such as *ex vivo* permeation experiments with excised animal intestinal tissue, *In vivo* studies in animals and *In vitro* permeation experiments across epithelial cell monolayers (Caco-2 cells).

Chapter 2 is designated to bioenhancement of atenolol. Wherein subchapters give information about different methods used to evaluate the permeability and bioavailability of atenolol in presence and absence of piperine and glycyrrhizic acid ammonium salt. Various methods such as *ex vivo, in vitro* Caco-2 cell line and *in vivo* methods were used to evaluate permeability/bioavailability of atenolol. It illustrated materials and methods for each study.

Brief overview of each study for bioenhancement of atenolol (AT)

Atenolol-Piperine binary system

Three different methods such as physical mixture (PM), solvent evaporation (SE) method and kneading method (KE) were used to prepare the AT – PI binary systems with three weight ratios.

Each binary system of PM, SE and KE methods were analyzed for FTIR, DSC and *ex-vivo* permeation study. Spectrophotometric analytical method was developed to analyse AT in presence of PI in simulated fluid, AT was measured at 273.6 nm. Each binary system of PM, SE and KE methods were assessed for *ex-vivo* permeation study using goat intestine as intestinal membrane, with 3.80 cm^2 area containing specific diffusion cell at 37±1 °C. The purpose of carrying out permeation studies was to get discriminatory results for AT permeation pattern through intestinal membrane. The values of permeation coefficient were determined for each binary system of all three methods.

Results of DSC and FTIR of each binary systems of PM, SE and KE methods suggests that there is no physical interaction between AT and PI. The results of *ex-vivo* permeation of binary system of PM, SE and KE method showed incredible increase in permeation coefficient of AT in presence of PI than without PI (i.e pure AT). However KE method did not show increase in AT permeation to the extent that previous binary systems of PM and SE method showed.

The binary system with AT-PI (5:1) ratio shows good permeation enhancement of AT with each three method PM, SE and KE method. Binary system with AT-PI (5:2) ratio shows maximum permeation enhancement (2 fold more than pure drug AT) with PM method.

While results of permeation studies suggest that PM method with each ratio shows good bioenhancement than SE and KE method.

Atenolol- Glycyrrhizic acid ammonium salt binary system

Two methods PM and SE method were used to prepare AT – GA binary systems at three different ratios, where GA was taken as 1 %, 5 % and 10 % of w/w of AT dose.

Each binary system of PM and SE methods were analyzed for FTIR, DSC and *ex-vivo* permeation study. First derivative spectrophotometric analytical method was developed to analyse AT in presence of GA in simulated fluid, AT was measured at 258.1 nm. Each binary system of PM and SE methods were assessed for *ex-vivo* permeation study using goat intestine as intestinal membrane, with 3.80 cm^2 area containing specific diffusion cell at 37±1 °C. The values of permeation coefficient were determined for each binary system of both methods.

Results of DSC and FTIR of each binary systems of PM, SE and KE methods suggests that there is no physical interaction between AT and GA. The results of *ex-vivo* permeation of binary system of PM and SE method showed increase in permeation coefficient of AT in presence of GA than without GA (i.e pure AT). Specifically binary systems with AT: GA (1:0.05) ratio of PM and SE method cause 2.5 fold increase in the permeation of AT.

Atenolol transport studies across Caco-2 Cell monolayers

The Caco-2 cells were obtained from ECACC used at passages 7-13 for all the studies. Cells were cultured, seeded with seeding density 1 X 10^5 cells/cm^2 and used for transport studies between 21 and 28 days. The integrity of the monolayer was checked by measuring TEER after each week till transport studies done. Transport studies were performed using HBSS/HEPES buffer pH 7.4. Permeation enhancement effect of different concentration of EDTA (standard bioenhancer) was studied on D-[1-^{14}C] Mannitol. The optimum concentration amongst six concentration of EDTA (1 mM, 2 mM, 4 mM, 8 mM, 16 mM and 24 mM) obtained after permeation studies, was selected as a standard concentration in AT permeation study. The bioenhancement effect of PI on permeation of AT was determined with five concentrations (1 µM, 10 µM, 50 µM, 100 µM and 250 µM) of PI. While

bioenhancement effect of GA was measured with 0.01 mM, 0.05 mM, 0.1 mM, 2.5 mM and 24 mM concentrations of GA. The amount of drug (^{3}H radiolabelled) transported was determined from radioactivity content in withdrawn samples using scintillation counter. P_{app} values and transport enhancement ratios (R) were calculated and statistically differentiated.

The results of [^{14}C] Mannitol transport studies EDTA with 8 mM concentration was selected as optimised concentration as it does almost twice increment in permeation of [^{14}C] Mannitol than control. It causes decrease in % TEER which suggests opening of paracellular pathways.

The transport study results of AT permeation with PI clearly indicate that higher concentrations of PI did not show increase in permeation of AT. While lower concentrations, 1, 10 and 50 µM of PI cause increase in P_{app} values and enhancement ratio of AT than control AT. The enhancement with each mentioned concentrations of PI is significantly higher and different ($p < 0.05$) than control. Even there is reduction in the % TEER of monolayer in presence of bioenhancer PI. Thus all results suggests that PI cause increase in permeation of AT though Caco-2 cell monolayer is due to paracellular openings and inhibiting P-glycoprotein.

The transport study results of AT permeation with GA show that 0.01 and 0.05 mM concentrations of GA increase the P_{apps} values and enhancement ratio. Both the concentrations of GA are decreasing % TEER values. It may be increasing permeation via its surfactant like action and opening paracellular route. The higher concentrations of GA are decreasing permeation of AT.

All the concentrations of PI and GA used for AT permeation studies were very less but important in knowing molecular mechanism responsible for enhancement of atenolol.

Pharmacokinetics studies of Atenolol

To understand the achievement of bioavailability enhancement, pharmacokinetic studies were performed in rats. Binary systems with three ratio of bioenhancer of PM method were

studied for *in vivo* behaviour in rats. The study investigated pharmacokinetics of AT in rats in presence and absence of PI and GA.

The protocol 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.

HPLC method for determination of AT in presence of both bioenhancer in plasma was developed. Method was validated for accuracy, precision, selectivity and recovery studies.

The extraction efficiency was calculated by adding known amount of AT (50, 100 and 500 ng/ml; n = 6 per concentration) or internal standard (Metoprolol 50 µl, 4 µg/ml) to 100 µl of blank rat plasma. AT and IS were extracted into 4 ml diethyl ether.

The control (pure AT 2.5 mg/kg), AT with three weight ratio of PI and AT with three weight ratio of GA was administered into the oral cavity of each rats. Total seven groups of rats were undertaken for study. Blood samples were collected from the retro orbital plexus of rat at 0, 0.5, 1, 2, 3, 6, 8, and 24 hrs after administration. Plasma samples collected from rats were analyzed using reverse HPLC method and AT plasma concentration values were determined from calibration curve.

On observing different *in vivo* pharmacokinetic parameters it is clear that AT with PI 0.09 mg/kg (i.e. AT: PI 5:1 weight ratio) showed most improved bioavailability over AT with other two ratios of PI. It causes almost 3 fold increase in bioavailability.

AT with GA 0.1125 mg/kg (GA is 5 % w/w of AT i.e. AT: GA 1:0.05) showed more improved bioavailability when compared with pure AT 2.5 mg/kg administration. It is causing 4 fold increase in bioavailability of AT than pure AT.

Formulation development for Atenolol

Optimized concentration, based on all previous study of AT bioenhancement, was selected to formulate single dose oral powder. The binary system of AT – PI with physical mixture

(PM) method with AT: PI (5:1 weight ratio) was formulated as single dose oral powder form. It was evaluated for uniformity of content and angle of repose.

The binary system of AT – GA with physical mixture (PM) method with AT: GA 1:0.05 (i.e GA is 5 % w/w of AT) was formulated as single dose oral powder form. It was evaluated for uniformity of content and angle of repose.

These single dose oral powders were incorporated into *cachets* to avoid leaching and provide better storage conditions.

Thus in conclusion *chapter 2* represent the bioenhancement of AT with both bioenhancers PI and GA. Both natural bioenhancers improves the permeability and bioavailability of AT in its low concentrations. Higher concentrations did not show enhancement to the extent that low concentration showed. Thus the natural bioenhancers can be formulated in single dose oral powder form with low cost of manufacturing and good patient compliance and reduced dose.

Chapter 3 is designated to bioenhancement of Lisinopril. Wherein subchapters give information about the different methods used to evaluate permeability and bioavailability of lisinopril in presence and absence of piperine and glycyrrhizic acid ammonium salt. Methods such as *ex vivo* and *in vivo* studies were used to evaluate permeability/ bioavailability of lisinopril. It illustrated the materials and methods for each study.

Brief overview of each study for bioenhancement of lisinopril (LI)

Lisinopril-Piperine binary system

Two different methods such as physical mixture (PM) and solvent evaporation (SE) method were used to prepare the LI – PI binary systems with three weight ratios.

Each binary system of PM and SE methods were analyzed for FTIR, DSC and *ex-vivo* permeation study. Spectrophotometric analytical method was developed to analyse LI in

presence of PI in phosphate buffer pH 6.0, LI was measured at 258.4 nm. Each binary system of PM and SE methods were assessed for *ex-vivo* permeation study using goat intestine as intestinal membrane, with 3.80 cm^2 area containing specific diffusion cell at 37±1 °C. The purpose of carrying out permeation studies was to get discriminatory results for drug permeation pattern through intestinal membrane. The values of permeation coefficient were determined for each binary system of both methods.

Results of DSC and FTIR of each binary systems of PM and SE methods suggests that there is no physical interaction between LI and PI. The results of *ex-vivo* permeation of binary system of PM and SE method showed increase in permeation coefficient of LI in presence of PI than without PI (i.e pure LI). However, SE method did not show increase in drug permeation to the extent that binary systems of PM method showed. The binary system with LI-PI (2:2) ratio showed maximum permeation enhancement (3.67 fold) of LI with PM method.

Lisinopril- Glycyrrhizic acid ammonium salt binary system

Two methods PM and SE method were used to prepare LI – GA binary systems at three different ratios, where GA was taken as 1 %, 5 % and 10 % of w/w of LI dose.

Each binary system of PM and SE methods were analyzed for FTIR, DSC and *ex-vivo* permeation study. Colorimetry method was developed to analyse LI in presence of GA in phosphate buffer pH 6.0, LI was measured at 347.0 nm. Each binary system of PM and SE methods were assessed for *ex-vivo* permeation study using goat intestine as intestinal membrane, with 3.80 cm^2 area containing specific diffusion cell at 37±1 °C. The values of permeation coefficient were determined for each binary system of both methods.

Results of DSC and FTIR of each binary systems of PM and SE methods suggests that no physical interaction between LI and GA. The results of *ex-vivo* permeation of binary system of PM and SE method showed incredible increase in permeation coefficient of LI in presence of GA than without GA (i.e pure LI). The binary system with LI: GA (1:0.05) ratio of PM method shows maximum permeation enhancement (13.25 fold) of LI than control (LI without GA).

Pharmacokinetics studies of Lisinopril

The study investigated pharmacokinetics of LI in rats in presence and absence of PI and GA. The protocol 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.

HPLC method for determination of LI in presence of both bioenhancer in plasma was developed. Method was validated for accuracy, precision, selectivity and recovery studies.

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.

The control (pure LI 1 mg/kg), LI with three weight ratio of PI and LI with three weight ratio of GA was administered into the oral cavity of each rats. Total seven groups of rats were undertaken for study. Blood samples were collected from the retro orbital plexus of rat at 0, 1, 2, 3, 4, 6, 8, 12 and 24 hrs after administration. Plasma samples collected from rats were analyzed using reverse HPLC method and LI plasma concentration values were determined from the calibration curve.

On observing the different *in vivo* pharmacokinetic parameters it is clear that LI with PI 0.09 mg/kg (i.e. LI-PI 2:1 weight ratio) showed most improved bioavailability (1.55 fold) over LI with other two ratios of PI. LI with GA 0.1125 mg/kg (LI: GA 1:0.05) showed more improved bioavailability (1.91 fold) when compared with pure LI 1 mg/kg administration.

Formulation development for Lisinopril

Optimized concentration, based on all previous study of LI bioenhancement, was selected to formulate single dose oral powder. The binary system of LI – PI with physical mixture (PM) method with LI: PI (2:1 weight ratio) was formulated as single dose oral powder form. It was evaluated for uniformity of content and angle of repose.

The binary system of LI – GA with physical mixture (PM) method with LI: GA 1:0.05 (i.e GA is 5 % w/w of LI) was formulated as single dose oral powder form. It was evaluated for uniformity of content and angle of repose.

These single dose oral powders were incorporated into *cachets* to avoid leaching and provide better storage conditions.

Thus in conclusion *chapter 3* represent the bioenhancement of LI with both bioenhancers PI and GA. Both natural bioenhancers improves the permeability and bioavailability of LI in its low concentrations. Higher concentrations did not show enhancement to the extent that low concentration showed same as found for AT. Thus natural bioenhancers can be incorporated in formulation with low cost of manufacturing and good patient compliance and reduced dose.

Table 4.1 Comparison of various *ex vivo*, cell line studies and *in vivo* parameters for optimized binary systems of PM method.

	Piperine		**Glycyrrhizic acid ammonium salt**	
	AT-PI	LI-PI	AT-GA	LI-GA
FTIR & DSC	No Interaction	No Interaction	No Interaction	No Interaction
***Ex-vivo* permeation**				
P_{eff} x 10^{-5}cm/sec	2.506 ± 0.003	0.579 ± 0.01	4.393 ± 0.211	4.185 ± 1.2
Enhancement ratio	1.20	1.83	2.23	13.25
Cell line studies				
P_{app} x 10^{-6}cm/sec	11.01 ± 0.06	–	13.32 ± 0.14	–
Enhancement ratio	1.20		1.45	
***In vivo* studies**				
$AUC_{(0-24)}$ (ng hr/ml)	2448.77	3020.62	2984.70	3721.91
C_{max} (ng/ml)	290.69	219.92	310.02	300.34
T_{max} (hr)	2.00	6.00	2.00	4.00
MRT (hr)	7.20	10.92	7.14	10.33
K_a (hr^{-1})	-0.95	0.14	-0.69	0.42
K_e (hr^{-1})	0.08	0.05	0.10	0.05
% R. Bioavailability	3.31	1.55	4.03	1.91
Formulation properties				
Uniformity of Content	99.98 ± 0.04	100.37 ± 0.56	100.07 ± 0.14	99.36 ± 0.38
Angle of Repose	30.92 ± 2.02	34.75 ± 2.63	29.41 ± 1.57	33.83 ± 2.36

R. bioavailability: Relative bioavailability, AT: atenolol, LI: lisinopril, PI: piperine, GA: glycyrrhizic acid ammonium salt