



CHAPTER 7
SUMMARY AND CONCLUSION

The pulmonary route is a promising alternative for delivering the peptide and protein drugs. Many drugs that are poorly absorbed from other mucosal sites are well absorbed from the lung. This route of administration offers a number of advantages over the conventional gastrointestinal pathway including large surface area, extensive vasculature, easily permeable membrane and low intra-cellular and extra-cellular enzymatic activity.

Insulin and calcitonin were selected as model polypeptide drugs, because of their large molecular size, well established method of analysis, great therapeutic utility and known pharmacokinetic and pharmacodynamic parameters. Insulin a well-known peptide drug used for the treatment of diabetes mellitus. Calcitonin is a peptide hormone that lowers plasma calcium concentration in mammals by diminishing the rate of bone resorption. It is used in the treatment of Paget's disease and established post-menopausal osteoporosis. The bioavailability of these peptides is always less than 10 % through the non-invasive routes other than pulmonary route. Proteolytic enzymes present in the lungs results in their degradation and the absorptive alveolar membrane excludes absorption of such hydrophilic macromolecules. The deposition of these drugs to the alveolar region where the absorption takes place is also very less. Many approaches have been used to overcome these barriers and increase the pulmonary bioavailability of these drugs.

In vivo pulmonary absorption studies for selection of absorption promoters to increase pulmonary bioactivity of insulin and calcitonin was carried out in swiss albino male rats. Initially, influence of pH was studied on absorption of insulin through pulmonary route followed by influence of penetration enhancers, protease inhibitors and combinations thereof. For studying influence of absorption promoters on pulmonary insulin absorption, intratracheal formulations having different pH (7.4, 5.0, 6.0 and 3.5) were prepared followed by using absorption promoters and their combinations at optimized pH. Absorption promoters studied were penetration enhancers (sodium caprylate, sorbiton trioleate, oleic acid sodium salt, sodium tauroglycocholate) and protease inhibitors (bacitracin, bestatin and chymostatin) in citrate buffer pH 3.5. The prepared intratracheal formulations in solution were administered in anesthetized rats by intratracheal instillation method.

100 μ l of the formulation solution was instilled through a tube inserted in the trachea by a 500 μ l of micro glass syringe. Blood samples (100 μ l) were withdrawn at - 30, 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min through the lateral tail vein. The potency of insulin permeating in the *in vivo* study was estimated by comparing in given conditions the hypoglycemic effect it produces with that produced by the known quantity of drug in animals. The blood glucose reduction by insulin in blood was estimated by GOD/POD method using glucose estimation kit. This analytical method yielded serum glucose concentration in the range of 20 - 350 mg/dL with 2 % precision. The method is linear up to 500 mg/dL. The use of quality control serum of known blood glucose concentration checks both, the instrument and the reagent functions together. The mean percent blood glucose reductions (PBGR) were calculated from the amount (mg/dL) of blood glucose measured. The AUC was calculated and the relative pulmonary bioactivity of the formulations in compared to subcutaneous route was calculated. For that five subcutaneous formulations containing 0.50 IU/kg, 0.75 IU/kg, 1.0 IU/kg, 1.25 IU/kg, and 1.50 IU/kg of insulin, respectively were prepared and administered subcutaneously and AUC calculated from percent blood glucose reduction time curve. The relative pulmonary bioactivity of the insulin formulation having pH 3.5 was 43.20% \pm 2.48%, and was found to be decrease to 21.63% \pm 1.28%, 14.57% \pm 1.51%, and to 11.36% \pm 1.27% respectively with increase in pH (5.0, 6.0 and 7.4 respectively). The concentration of oleic acid sodium salt used in the insulin formulation was 0.5% with relative bioactivity of 61.90% \pm 3.21% and of the sodium tauroglycocholate was 0.3% with relative bioactivity of 67.09% \pm 3.23%. Both of these enhancers were used in combination, where the concentration of oleic acid sodium salt and sodium tauroglycocholate was 0.2% and 0.1% respectively and the bioactivity obtained was 79.25% \pm 4.31%. Even though the concentration of individual penetration enhancers was reduced more than 50%, bioactivity increased significantly. Similarly, the protease inhibitors bestatin and chymostatin were co-administered with insulin; the bioactivity obtained was 95.51% \pm 4.77% compared to incorporation of bestatin and chymostatin individually (max bioactivity of 69.84% \pm 3.02%). When penetration enhancers - oleic acid sodium salt (0.2%) and sodium tauroglycocholate (0.10%) and protease inhibitors - bestatin (0.02%) and chymostatin (0.04%) were used in combination in insulin formulation in citrate buffer pH 3.5, significant increase in relative

bioactivity of $155.60\% \pm 5.19\%$ was observed. In neutral pH, the insulin molecule is typically associated to form hexameric units, a process called fibrillation. When the pH decreases, then fibrillation decreases and the insulin molecules exist only in monomeric form. Insulin is more stable at acidic pH, and the monomeric form absorbs rapidly in the alveolar region of the lungs. Hence, acidic pH was observed to favor higher penetration of insulin through the alveolar membrane. The bile salts and fatty acid salts act by reverse micellar binding with subsequent formation of hydrophilic channels in the tight junction. The change in paracellular path and formation of hydrophilic channels result in an increase in transepithelial flow. Bile salts also enhance the absorption by binding Ca^{2+} to increase paracellular permeability and by inhibiting proteases like amino peptidases. The sodium tauroglycocholate used as enhancer was less irritating and its absorption profile also more acceptable. The surface of a wide variety of mammalian cell types including lung are rich in a group of proteolytic enzymes, like aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, dipeptidases, omega peptidases. These enzymes are responsible for the hydrolysis of peptide drugs administered to the lungs. Bestatin is an amino-peptidase inhibitor and chymostatin is serine protease inhibitor. . Hence, combination of these protease inhibitors inhibits wide variety of enzymes involved on degradation of insulin.

Like insulin, for the study of influences of absorption promoters on pulmonary calcitonin absorption, intratracheal formulations were prepared. Intratracheal formulations prepared containing different pH (6.0, 3.9, and 2.8) and absorption promoters and their combinations including oleic acid sodium salt, sodium tauroglycocholate, dimethyl β -cyclodextrin, dodecyl maltoside, bacitracin, bestatin, amastatin and chymostatin in acetate buffer pH 3.9. For calculating the relative pulmonary bioactivity in compared to subcutaneous route, subcutaneous formulations were also prepared. Five subcutaneous formulations containing 0.50 IU/Kg, 1.0 IU/Kg, 3.0 IU/Kg, 4.0 IU/Kg and 6.0 IU/Kg of calcitonin, respectively were prepared. The intratracheal formulations administered by intratracheal instillation method in anesthetized rats. The serum was separated from the blood samples and the calcium content was determined. The potency of calcitonin is estimated by comparing in given conditions the hypocalcaemic effect it produces with that produced by the International Reference preparation of salmon calcitonin

or a reference preparation calibrated in International Units. This analytical method yielded serum calcium concentration in the range of 1.0 - 50.0 mg/dL with 2 % precision. The use of quality control serum containing known concentration of calcium checks, both the instrument and the reagent functions together. The AUC was calculated and the relative pulmonary bioactivity of the formulations was calculated. The pulmonary bioactivity of the calcitonin at pH 3.9 was $53.9\% \pm 2.8\%$, and was found to be decrease to $21.0\% \pm 1.5\%$, and to $12.0\% \pm 1.3\%$ respectively with formulations of pH 6.0 and 2.8. The concentration of sodium tauroglycocholate in the calcitonin formulation was 0.5% with relative bioactivity of $72.0\% \pm 2.7\%$ and dimethyl β -cyclodextrin was 1.0% and the relative bioactivity of $79.2\% \pm 3.9\%$. Both of these enhancers in combination used, where the concentration of sodium tauroglycocholate and dimethyl β -cyclodextrin was 0.2% and 0.3% respectively and the relative bioactivity obtained was $102.9\% \pm 5.3\%$. The formulations individually containing the protease inhibitors chymostatin (0.05%) and bacitracin (0.03%) gave relative bioactivity of $83.0\% \pm 2.1\%$ & $87.0\% \pm 3.9\%$ respectively. When both of these inhibitors (0.04% and 0.02% of chymostatin and bacitracin respectively) added in combination, the relative bioactivity was increased significantly to $111.9\% \pm 4.4\%$. When penetration enhancers - sodium tauroglycocholate (0.2%), dimethyl β -cyclodextrin (0.3%) and protease inhibitors - chymostatin (0.04%), bacitracin (0.02%) were used in combination with calcitonin in isotonic acetate buffer pH 3.9, significant increase in relative bioactivity of $139.1\% \pm 7.3\%$ was obtained. Calcitonin is reported to be more stable at acidic pH. Lowering the pH also causes paracellular permeability, possibly by displacing Ca^{2+} from the tight junction. Hence, acidic pH was observed to favor higher penetration of insulin/calcitonin through the alveolar membrane. The bile salts and fatty acid salts act by various mechanisms discussed under insulin. Dimethyl β -cyclodextrin has a direct effect on membrane and enhances drug absorption by binding with the membrane components that serve as a barrier to calcitonin transport. It transiently opens the tight junction by extraction of membrane cholesterol. Furthermore it protects calcitonin from enzymatic degradation by molecular encapsulation or releases membrane proteins from the membrane, which may have resulted in inactivation of the proteolytic enzymes. Dimethyl β -cyclodextrin was reported to be safe in nasal absorption of drugs.

Chymostatin has more effect on inhibition of enzymes degrading calcitonin. The inhibitory activity of bacitracin might be non-specific as a peptide substrate and is not always specific. When combination of these chymostatin and bacitracin were used, they inhibit wide variety of enzymes involved on degradation of calcitonin. Control formulations contain the absorption promoters were studied and the interference was found to be below 1% by the buffer or penetration enhancers or protease inhibitors.

To develop potential formulation and reduce the burden of carrying out a large number of *in vivo* experiments, we demonstrated the frog lung mounted in the diffusion chamber provides a physiologically relevant *in vitro* system for studies on transport of insulin and calcitonin. For the development of *in vitro* method, formulations of insulin were prepared using selected absorption promoters from *in vivo* studies. Porcine insulin crystalline powder was dissolved in phosphate buffer pH 7.4 and citrate buffer pH 3.5 and the concentration of insulin was 3.0 mg/mL. Solutions of oleic acid sodium salt, sodium tauroglycocholate, bestatin and chymostatin were prepared in citrate buffer pH 3.5. These solutions were added separately and in combination to the insulin in citrate buffer pH 3.5. Before addition, both the solutions were diluted with the buffer of same pH to 1.0 mL of the final solutions containing the 3.0 mg/mL of insulin and the absorption promoters. *In vitro* method development for calcitonin, the drug was dissolved in acetate buffer pH 6.0 and acetate buffer pH 3.9 and the concentration of calcitonin was 3.0 mg/mL. Solutions of dimethyl β -cyclodextrin, sodium tauroglycocholate, bacitracin and chymostatin were prepared in acetate buffer pH 3.9. These solutions were added separately and in combination to the calcitonin in acetate buffer pH 3.9. Before addition, both the solutions were diluted with the buffer of same pH to 1.0 mL of the final solutions containing the required concentration of calcitonin and the absorption promoters. Frogs were anesthetized by kept in ice and a ventral incision was made to expose the lung. Lungs were excised by severing the tracheoglottis and each filled with approximately 3 – 4 ml ringers solution. The planar sheet of lung was mounted in the diffusion chamber. Tissues were bathed with frog ringer's solution. The reservoir compartment was continuously gassed with the carbogen 95% and the temperature maintained was 22°C. After mounting was over, tissues were equilibrated for 45 min and then transport studies begun.

Tissues were continuously short-circuited with an automatic voltage clamp. 1.0 ml of the formulations of insulin or calcitonin was taken on the surface of the membrane and samples were collected from the receptor side reservoir in every 30 min interval. After sampling, an equal volume of buffer was added back to maintain constant volume. The samples were collected over a period of 0-300 min for insulin formulations and 0-240 min for calcitonin formulations. The amount of insulin and calcitonin transported through the alveolar membrane mounted *in vitro* was estimated by Bicinchoninic acid (BCA) method using protein assay kit and the amount of drug permeated was determined and results were correlated with *in vivo* cumulative AUC of the formulations. This analytical method yielded protein drug concentration in the range of 0.2 µg – 300 µg with 2 % precision. The absorbance was measured at λ_{\max} 561.5 ± 0.5 nm.

Insulin formulation at pH 7.4 shows cumulative permeation of 7.4% ± 2.2% and the pH of the formulation become slightly acidic (pH 3.5), and then cumulative permeation of the drug increases to 24.4% ± 4.4%. The penetration enhancers oleic acid sodium salt and sodium tauroglycocholate increases insulin permeation from 24.4% ± 4.4% to 32.4% ± 4.7% and 39.9% ± 4.4% respectively. Insulin formulation having combination of sodium tauroglycocholate and oleic acid sodium salt shows 52.4% ± 4.6% permeation. The formulations with protease inhibitors like bestatin, chymostatin, and combination of bestatin with chymostatin causes increase in permeation of insulin to 40.1% ± 4.4%, 40.6% ± 4.3%, 57.3% ± 3.3%, respectively. When both the penetration enhancers and protease inhibitors used in combination in the insulin the cumulative permeation obtained was 81.6% ± 3.8%. In calcitonin formulation the cumulative permeation was 6.0% ± 2.1% at pH 6.0 and increases to 17.9% ± 2.6% at acidic pH 3.9. Sodium tauroglycocholate (C3) increases calcitonin permeation from 17.9% ± 2.6% to 23.8% ± 2.5% and dimethyl β-cyclodextrin to 26.5% ± 3.4% and incorporation of both sodium tauroglycocholate and cyclodextrin causes increase in drug permeation of 63.7% ± 4.0%. The calcitonin formulations containing chymostatin, bacitracin, and combination of chymostatin with bacitracin shows permeation of 27.8% ± 3.0%, 29.5% ± 3.5%, 67.3% ± 3.3%, respectively. When combination of penetration enhancers and protease inhibitors at acidic pH 3.9 were used in calcitonin formulation, the cumulative permeation obtained was 86.7% ± 4.4%. When the

cumulative permeation of insulin and calcitonin formulations (for a period of 0-300min) was plotted against their *in vivo* cumulative AUC, the correlation factor obtained was 0.99. Control formulations containing the absorption promoters were studied and the interference was found to be below 1 % by the buffer components or penetration enhancers or protease inhibitors used in the formulations.

A dry powder inhaler formulation of promising formulations were developed and drug lung deposition was established using *in vitro* techniques. For insulin the potent formulation having higher pulmonary bioactivity was lyophilized. For calcitonin, as the dose of the drug very less, lactose was incorporated and lyophilized to increase the bulk content of the potent formulation. The lyophilized powder mass was size reduced by lab-scale attrition method. The carrier lactose of different grades (Respitose, LactoHale) was incorporated individually and in combination to impart flow property, deaggregation and maximum deposition of the powder. The *in vitro* deposition of the dry powder formulations was estimated by twin stage glass impinger and Anderson cascade impactor. The fine particle fraction of the developed formulations was determined. The *in vitro* deposition of insulin studied in twin stage impinger and cascade impactor was estimated by HPLC method. The *in vitro* deposition of calcitonin studied in twin stage impinger was estimated by HPLC method. The linearity of the method was found to be 1.0 (r^2 value).

At a flow rate of 30 l/min, insulin deposited from the formulations containing Respitose[®] 45 μ m, Respitose[®] 25 μ m and LactoHale was 24.9%, 28.9% and 35.1% respectively. As the size of the carrier decreases the deposition of drug increases. The deposition was further increases to 40.3% and 45.1% respectively of the formulations containing LactoHale in combination with Respitose[®]25 μ m and Respitose[®]45 μ m. When carriers of larger particle sizes with multimodal particle sizes distribution were used along with lesser size lactose carriers the deaggregation of the drug particles were improved and the deposition increases proportionately. The same was observed when the formulations were aerosolized at other flow rates of 60 l/min and 90 l/min. At all the three flow rates the deposition of drug was found to be same in case of formulation with lactose of median size 39.3 μ m (Respitose[®]45 μ m) and in formulation with Respitose[®]25 μ m the

deposition was moderately increases as the flow rate increases. The difference in the deposition was not so much remarkable. But in case of LactoHale formulation and the other formulations with mixture of LactoHale and Respitose, where the lesser particle size lactose was used the deposition proportionately increases with the flow rates. This suggest that airflow rate of 30 l/min was sufficient to detach the respirable drug from the carriers of larger particle sizes (Respitose[®] 45µm and Respitose[®] 25µm). When carriers of smaller particle sizes were used then increase in airflow is necessary to detach the respirable fraction of drug. As like drug deposition the amount of lactose at lower stage of impinger increases proportionately with the decrease in the particle size of the carrier. In cascade impactor, at a flow rate of 30 l/min insulin deposited in the throat and the preseparator was found to be higher in case of Respitose[®] 45µm i.e. 32.1% and 14.1% respectively. When LactoHale and their combinations with Respitose were used then the drug deposition decreases. The order of drug and lactose deposited from the carrier in the throat and preseparator was as follows,

$$\text{Respitose}^{\text{®}} 45\mu\text{m} > \text{Respitose}^{\text{®}} 25\mu\text{m} > \text{LactoHale} > \text{LactoHale} + \text{Respitose}^{\text{®}} 25\mu\text{m} > \text{LactoHale} + \text{Respitose}^{\text{®}} 45\mu\text{m}$$

Similarly, the drug and lactose deposited in the stages 0-7 increases with the decrease in the particle size of the carrier. . The order of drug and lactose deposited from the carrier in the 0-7 stages was as follows,

$$\text{Respitose}^{\text{®}} 45\mu\text{m} < \text{Respitose}^{\text{®}} 25\mu\text{m} < \text{LactoHale} < \text{LactoHale} + \text{Respitose}^{\text{®}} 25\mu\text{m} < \text{LactoHale} + \text{Respitose}^{\text{®}} 45\mu\text{m}$$

The maximum deposition of 55.7% of insulin was obtained at 60 l/min in the formulation with carriers of combinations.

For calcitonin, at a flow rate of 30 l/min, the drug deposited from the formulations containing Respitose[®] 45µm, Respitose[®] 25µm was 27.8% and 29.9% respectively. As the size of the carrier decreases further, the deposition of drug decreases and the value obtained was 24.7 %. The deposition of calcitonin increases to 35.4% and 38.3% respectively of the formulations containing LactoHale in combination with Respitose[®]25µm and Respitose[®]45µm. When carriers of larger particle sizes with multimodal particle sizes distribution were used along with lesser size lactose carriers the deaggregation of the drug particles

were improved and the deposition increases proportionately. The same was observed when the formulations were aerosolized at other flow rates of 60 l/min and 90 l/min. At all the three flow rates the deposition of drug was found to be increases as the flow rate increases. The difference in the deposition was remarkable. This suggest that airflow rate of 30 l/min was not sufficient to detach the respirable drug from the carriers. (Respitose[®] 45 μ m, Respitose[®] 25 μ m and LactoHale). For higher deposition the size of carrier should be controlled and also flow has to be good enough to carry the drug particles deeper in the impactor. Lyophilized calcitonin powder mixture itself contains lactose of smaller particle sizes and when LactoHale incorporated, the flow properties decrease and the deposition also decreases. When wide range of lactose carrier incorporated with calcitonin, then the carrying of the drug particles becomes deeper and the deposition in the lower stage of impactor also higher. In both insulin and calcitonin the flow properties increases when the particle size of the carrier would be more. As the size decreases the flow also decreases.

CONCLUSION

Many biologically active peptides have been discovered recently and have attracted attention as new drugs. Low membrane permeability, inadequate stability, potential safety issues and relatively short half-lives of many of these protein and peptide therapeutics limits their potential since they can only be administered by injection. Development of suitable non-injectable routes of administration (e.g. low cost, reproducible and safe) could significantly enhance patient compliance thereby increasing the benefit to be derived from this novel therapeutics. The pulmonary route of administration offers a number of advantages over the conventional gastrointestinal pathway including large surface area, extensive vasculature, easily permeable membrane and low intra-cellular and extra-cellular enzymatic activity.

Attempt was made to study the influences of absorption promoters on pulmonary absorption of insulin and calcitonin, to develop a suitable *in vitro* method, and to develop dry powder inhaler formulations for delivering drug to the lung using suitable devices.

Insulin and calcitonin in solution with various absorption promoters and their combinations when administered in rats by intratracheal instillation shown variation in blood glucose/blood calcium, respectively, depending upon the influence of the enhancers on drug absorption. The blood glucose level/blood calcium level measured by readily available kits in the market. The data of these studies revealed that the absorption promoters significantly affect the bioactivity of intratracheally-administered insulin and calcitonin. It depends on the dose of drug and the concentration and type of the absorption promoters used. The insulin formulation developed with combination of protease inhibitors and penetration enhancers (bestatin - 0.02%, chymostatin - 0.04%, oleic acid sodium salt - 0.2%, sodium tauroglycocholate - 0.10%) in citrate buffer pH 3.5 have shown higher relative pulmonary bioactivity (compared to s.c.) of $155.60\% \pm 5.19\%$. The calcitonin formulation developed with combination of protease inhibitors and penetration enhancers (chymostatin - 0.04%, bacitracin - 0.02%, sodium tauroglycocholate - 0.2%, dimethyl β -cyclodextrin - 0.3%) in acetate buffer pH 3.9 shows relative pulmonary bioactivity of $139.1\% \pm 7.3\%$ (compared to s.c.). Selected combination of absorption promoters provided synergistic effect. Higher bioactivity may help in decreasing dose of intratracheally administered insulin and calcitonin and in avoiding systemic side effects and probably the cost of therapy. Reduction in the concentration of selected individual absorption promoters is likely to have low chronic toxicity to the membrane. The influence caused by absorption promoters (like pH, penetration enhancers and protease inhibitors) varies from insulin to calcitonin that depends on the molecular weight of the protein drug, amino acid sequence and their binding sites for proteolytic enzymes/protease inhibitors and the characteristic mechanisms of the promoters.

Even the *in vivo* data can give the real permeation data, for initial screening of the absorption promoters, an *in vitro* method has been developed using the alveolar membrane of frog lung and correlated with the *in vivo* data. The *in vivo* – *in vitro* correlation factor obtained for all the formulations was 0.99 (for both insulin and calcitonin), which proves that in both the *in vivo* and *in vitro* system the mechanism by which pH, penetration enhancers and protease inhibitors caused drug permeation through the membrane was found to be same. The amphibian lung

mounted in the diffusion chamber provides a physiologically relevant system for studying the transport of insulin, calcitonin and other peptide drugs.

Lyophilization after mixing with diluent/without diluent and size reduction by lab scale attrition method yields the required particle size range of particles for the effective drug deposition in the alveolar region of the lung. The proportion of carriers and their particle size used have significant effect on deaggregation and deposition. Both in insulin and calcitonin, when a mixture of different sizes of carrier used then deposition was increased due to improvement in dispersion and deaggregation of the drug from carrier. For insulin the FPF at a flow rate of 60 l/min was 55.3%, whereas for calcitonin it was only 43.5% at the same flow rate. The difference in the deposition may be due to the inherent property of the drug, molecular weight, and physical interaction with the carrier lactose. In insulin DPI, the use of LactoHale increases FPF but in case of calcitonin DPI, it decreases the FPF. This is because, the lyophilized calcitonin powder mixture itself contains lactose of smaller particle sizes and when LactoHale incorporated, the flow property decreases and the deposition also decreases. There should be a balance between the size of the carriers and their flow in the dry powder formulation for maximum deposition of the inhaled drug in the alveolar region

Issues to be investigated prior to be addressed before clinical use of the dry powder formulations of insulin and calcitonin developed in this investigation include assessment of long term stability and reproducibility upon dosing by pulmonary devices and assessment of safety after chronic pulmonary administration of the developed formulations.
