CHAPTER 4

IN VIVO PULMONARY ABSORPTION STUDIES FOR SELECTION OF ABSORPTION PROMOTERS

The *in vivo* studies represent the most important stage for the development of formulations for pulmonary delivery. *In vivo* studies allows both pharmacokinetics and pharmacodynamics of the drug. Synthetic *in vitro* membrane models have not been established before the *in vivo* studies begins, as the texture, cell components, membrane enzymes does not match with the artificial cellophane membranes. Obviously, there are significant differences between synthetic membrane and the highly complex alveolar membrane in the biological system.

The ideal way to study the pulmonary delivery potential of a compound is to perform the actual study in man. However, the cost and complexity of studies *in vivo* in man are immense. Therefore, preclinical studies are needed to determine the potential toxicity and efficacy/bioavailability before clinical studies are undertaken.

Before it is possible to test novel approaches to pulmonary delivery in man, it is necessary to perform initial experiments in animal models. In the present study rats were used for *in vivo* evaluation. The hypoglycemic activity of insulin and hypocalcemic activity of calcitonin is studied in rats, rabbits, mice and cats. Rats are preferred because they being omnivorous resemble humanbeings nutritionally and they are easy to handle. The basal blood glucose level as well as blood calcium level remains more or less same. The normal blood glucose level in rat (non-diabetic) was 80 mg – 120 mg/dL and the blood calcium was 8.0 – 10.0 mg/dL that is similar to value found in humans. The blood glucose reduction after insulin administration is the direct indication of the amount of drug absorbed. The blood calcium reduction in blood is the indication of the amount of calcitonin absorbed. Measuring the amount of drug in the biological fluid is more cumbersome which required sophisticated analytical methods but measuring their pharmacological effect is easier i.e. measuring the blood glucose reduction and blood calcium reduction respectively of insulin & calcitonin.

The use of various absorption promoters can promote the permeation of the drug across the alveolar membrane. Absorption promoters may be pH, osmolarity, ionic strength etc. and penetration enhancers and protease inhibitors. Penetration enhancer is a compound that alters the biological membrane (skin/alveolar

membrane/others) as a barrier to flux of a desired permeant. The key to finding an enhancer is to compare the flux across the biological membrane of the permeant at the same thermodynamic activity in a vehicle with the putative enhancer and a standard vehicle such as an aqueous solution. For sometime it has been recognized that surface-active agent such as soaps can enhance the permeation. Apart from detergents, dimethyl sulfoxide (DMSO) is perhaps the most widely known penetration enhancers. Bile salts, non-ionic surfactants, cyclodextrins and polysaccharides are reported to be less toxic to biological membrane. The toxicity of penetration enhancers directly proportional to their concentration used. Toxicity point of view, it is well known fact that reduced concentration of penetration enhancer is safer to use.

The surface of a wide variety of mammalian cell types, including lung, is rich in a group of proteolytic enzymes that includes aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, dipeptidases, and omegapeptidases [1]. These enzymes are responsible for the hydrolysis of peptide drugs administered to the lungs. One can use protease inhibitors like bacitracin, bestatin, chymostatin, etc., to overcome the enzyme action in the lung.

These investigations endeavored to achieve pulmonary bioactivity comparable to that found with the parenteral route of peptide drug delivery. Insulin, a well-known polypeptide drug is used for diabetes mellitus. A non-injectable form of regular insulin would make it easier for patients to take insulin more frequently and thereby lower their risk of long-term diabetes complications. Calcitonin is a 32 amino acid polypeptide mainly secreted by the thyroid C-cells. It lowers plasma calcium concentration in mammals by diminishing the rate of bone resorption and used in the treatment of Paget's disease and established post-menopausal osteoporosis. At present, insulin, calcitonin and other peptides of high molecular weight and low lipid solubility are been administered parenterally. Recently, nasal spray dosage form of calcitonin is marketed in various countries shows great variability in bioavailability (3 - 30 %; PDR, 1997). The obstacles in delivering the selected peptide drugs through pulmonary route are proteolytic enzymes present in the lung, muco-cilliary clearance, tight junction texture of absorptive alveolar membrane and poor deposition in the absorptive alveolar area of lung. In this

investigation attempt was made to study the effect of pH, penetration enhancers (oleic acid sodium salt / dimethyl β-cyclodextrin / dodecyl maltoside / sodium caprylate / sorbitan trioleate / sodium tauroglycocholate), protease inhibitors (bestatin/amastatin/bacitracin/chymostatin) and combination of these absorption promoters on the pulmonary absorption of insulin and calcitonin in rats. It was hypothesized that combination of absorption promoters acting through different mechanism may enhance the pulmonary drug absorption synergistically. It may result in increase of bioactivity of the drug to manifold. It may reduce the required dose of insulin and calcitonin and reduce the concentration of individual absorption promoters. When the concentration of the absorption promoters reduced, it is expected that toxicity associated with them at higher concentrations would also been reduced. The present studies demonstrated the selection of absorption promoters to increase pulmonary bioactivity of insulin and calcitonin by *in vivo* studies in rats.

4.1 PREPARATION OF FORMULATIONS FOR *IN VIVO* STUDIES

For the evaluation of absorption promoters, solution of porcine insulin and solution of salmon calcitonin with various absorption promoters and their combinations were prepared.

4.1.1 MATERIALS

Insulin porcine (25.5 IU/mg) was donated by Sarabhai Chemicals (Vadodara, India). Salmon calcitonin (6123 IU/mg) was kindly gifted by Novartis Pharma AG, Basel (Switzerland). Citric acid anhydrous (extra pure), sodium tauroglycocholate, sorbitan trioleate, and sodium caprylate were purchased from SD Fine-Chem Ltd (Boisar, India). Dimethyl β-cyclodextrin, dodecyl maltoside, oleic acid (cis 9-ocatadecanoic acid), sodium salt, bacitracin, bestatin, amastatin, chymostatin, and urethane were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

4.1.2 EQIUPMENTS

Clinical chemistry analyzer RA 50 (Miles Inc.Diagnostics Division, Elkhart, IN, USA), cooling centrifuge (Remi Instruments, Mumbai, India), calibrated pipettes 0.1 ml, 0.2 ml, 0.5 ml, 1.0 ml, 5.0 ml, 10.0 ml and other glasswares.

4.1.3 REAGENTS

The following reagents were used:

1. Glucose assay kit (Bayer Diagnostics India Ltd).
2. Phosphate buffer pH 7.4 was prepared by dissolving potassium dihydrogen phosphate 35.44 mmol and sodium hydroxide 20.56 mmol in water for injection. The ionic strength (μ) was 0.056.
3. Phosphate buffer pH 6.0 was prepared by dissolving potassium dihydrogen phosphate 50 mmol, sodium hydroxide 5.6 mmol in water for injection. The ionic strength (μ) was 0.056.
4. Phosphate buffer pH 5.0 was prepared by dissolving potassium dihydrogen phosphate 50 mmol, potassium hydroxide 6.0 mmol in water for injection. The ionic strength (μ) was 0.056.
5. Citrate buffer pH 3.5 was prepared by dissolving citric acid monohydrate 16.53 mmol and disodium hydrogen phosphate 17.8 mmol in water for injection. The ionic strength (μ) was 0.056.
6. Calcitonin assay kit (Bayer Diagnostics India Ltd, India).
7. Acetate buffer pH 6.0 was prepared by dissolving ammonium acetate 1.49 mol and glacial acetic acid 0.08 mol in water for injection. The ionic strength (μ) was1.49.
8. Acetate buffer pH 3.9 was prepared by dissolving anhydrous sodium acetate 1.48 mol and acetic acid 4.87 mol in water for injection. The ionic strength (μ) was1.49.
9. Acetate buffer pH 2.8 was prepared by dissolving anhydrous sodium acetate 1.31mol and glacial acetic acid 72.62 mol in water for injection. The ionic strength (μ) was1.49.

4.1.4 METHOD

Formulations were freshly prepared throughout the studies. For the study of influences of absorption promoters on pulmonary insulin and calcitonin absorption, intratracheal formulations were prepared. For calculating the relative pulmonary bioactivity in compared to subcutaneous route, subcutaneous formulations were also prepared. Control formulations were prepared to study the effect of excipients/absorption promoters on blood glucose/blood calcium level, which are used in the intratracheal formulations.

a) SUBCUTANEOUS FORMULATIONS

For subcutaneous administration of insulin, 5 formulations of porcine insulin were prepared. The drug was dissolved in citrate buffer pH 3.5, and the solutions were diluted with the same buffer to 100 µL of the final solutions 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.

For subcutaneous administration of calcitonin, five formulations of salmon calcitonin were prepared. The drug was dissolved in acetate buffer pH 3.9 and the solutions were diluted with the same buffer to 100 µL of the final solutions 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.

b) INTRATRACHEAL FORMULATIONS

Porcine insulin crystalline powder was dissolved in phosphate buffer pH 7.4, 6.0, and 5.0 and citrate buffer pH 3.5. These solutions were diluted in a buffer of the same pH, and the final volume was adjusted to the required dose of insulin being instilled (Table 4.1: F1-F4). Solutions of sodium caprylate, sorbitan trioleate, oleic acid sodium salt, sodium tauroglycocholate, bacitracin, bestatin, and chymostatin were prepared in citrate buffer pH 3.5. These solutions were added separately or in combination to the insulin in citrate buffer pH 3.5. The solutions were diluted with the buffer of the same pH to 100 µL of the final solutions containing the required

dose of insulin and the concentration of absorption promoters (Table 3: F5-F34). The compositions of all the formulations prepared are recorded in Table 4.1.

Calcitonin was dissolved in acetate buffer pH 6.0, pH 3.9 and 2.8. These solutions were diluted in the buffer of same pH and the final volume was adjusted to the required dose of calcitonin being instilled (Table 4.7: F#1- F#3). Solutions of oleic acid sodium salt, sodium tauroglycocholate, dimethyl β-cyclodextrin, dodecyl maltoside, bacitracin, bestatin, amastatin and chymostatin were prepared in acetate buffer pH 3.9. These solutions were added separately or in combination at three concentration levels (a, b, c) to the calcitonin in acetate buffer pH 3.9. Before addition, both the solutions were diluted with the buffer of same pH to 100 µL of the final solutions containing the required dose of calcitonin and the concentration of absorption promoters. The compositions of the intratracheal formulations prepared are recorded in Table 4.8 – 4.10.

c) CONTROL FORMULATIONS

Nine control formulations were prepared to study the influence of excipients/absorption promoters used in intratracheal formulations of insulin. Two were phosphate buffer pH 7.4 and citrate buffer pH 3.5, and the other 7 formulations contained the absorption promoters individually in citrate buffer pH 3.5. The concentration of each of the absorption promoters in the control formulations was equal to the maximum concentration of these used in intratracheal formulations.

Another nine control formulations were prepared to study the influence of excipients/absorption promoters used in intratracheal formulations of calcitonin. Out of nine, one was acetate buffer pH 3.9 and other eight formulations contained the absorption promoters individually in acetate buffer pH 3.9. The concentration of the absorption promoters in the control formulations was equal to the maximum concentrations of these used in the intratracheal formulations.

4.2 SELECTION OF ANIMALS AND EXPERIMENTAL DESIGN

Animal experiments were approved by the Social Justice & Empowerment Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Government of India, New Delhi. Animals were included in the study on the basis of randomization selection technique. The following procedure was adopted before starting the animal studies:

1. Six male albino rats (Swiss strain) weighing between 220 and 230 g were included in each group.
2. Rats were housed in large polypropylene cages at air-conditioned temperatures (22°C-24°C), normal hygiene, and normal diet at a 12-hour light/dark cycle, and water was given ad libitum.
3. Rats in each group were subjected to the experiments on the same day at the same time.
4. Animals were fasted overnight (16 hours) before study. However, water was allowed ad libitum.

4.3 SUBCUTANEOUS ADMINISTRATION

Rats were anesthetized by means of an intraperitoneal injection of urethane (120 mg/100 g). The femoral vein was catheterized using silicone tubing (0.02-mm internal diameter and 0.05-mm outer diameter), and the patency of the catheter was confirmed by slowly flushing the cannula with 200 µL of heparinized saline.

Increasing doses of insulin formulations prepared in citrate buffer pH 3.5 (0.50 IU/kg, 0.75 IU/kg, 1.0 IU/kg, 1.25 IU/kg, and 1.50 IU/kg) were administered subcutaneously to each animal group. These doses were chosen because a linear dose-response relationship was seen. The doses above this range resulted in hypoglycemia or fluctuation in peak response time. Blood samples (100 µL) were withdrawn at –60, –30, 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 minutes through the lateral tail vein. Following each sampling, an equal amount of saline was injected through the catheter tube attached to the femoral vein.

Increasing doses of calcitonin formulations prepared in acetate buffer pH 3.9 (0.50 IU/Kg, 1.0 IU/Kg, 3.0 IU/Kg, 4.0 IU/Kg, and 6.0 IU/Kg) were administered subcutaneously to each animal group. These doses were chosen, because linear relationship in dose-response was seen between 0.5 – 4.0 IU/Kg. Blood samples (100 µl) were withdrawn at – 60, – 30, 0, 30, 60, 90, 120, 150, 180, 210 and 240 min through the lateral tail vein. After each sampling, equal amount of saline was injected through the catheter tube, attached with the femoral vein.

4.4 INTRATRACHEAL SOLUTION INSTILLATION

After the animals were anesthetized, intratracheal instillation was performed as reported by Enna and Schanker [2]. The rats were placed on a heating blanket thermostatically controlled at 37°C via rectal probe. Before the surgery (–60 minutes of instillation), 1 blood sample was collected from the tail vein. The trachea was exposed by blunt dissection of the sternohyoideus muscle, and a small midline incision was made over the trachea between the fifth and sixth tracheal rings using a 20-gauge needle. The trachea was cannulated with polyethylene (PE) 200 tubing (5-7 cm) with the tip positioned approximately at the tracheal bifurcation. The PE 50 (10-15 cm) tubing connected to a glass Hamilton syringe (Waters, Mumbai, India) was inserted into the cannula and advanced to the bifurcation of the trachea. The femoral vein was catheterized using silicone tubing (0.02-mm internal diameter and 0.05-mm outer diameter), and the patency of the catheter was confirmed by slowly flushing the cannula with 200 µL of heparinized saline.

For the study of insulin absorption, exactly 100 µL of the intratracheal insulin formulation was instilled through a tube inserted in the trachea by a 500-µL glass syringe. Blood samples (100 µL) were withdrawn at –30, 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 minutes through the lateral tail vein.

For the study of calcitonin absorption, 100 µL of the intratracheal calcitonin formulation was instilled through a tube inserted in the trachea by a 500 µL of

micro glass syringe. Blood samples (100 µL) were withdrawn at – 30, 0, 30, 60, 90, 120, 150, 180, 210 and 240 min through the lateral tail vein.

After each sampling, an equal amount of saline was injected through the catheter tube, inserted in the femoral vein. Insulin syringe was used for the withdrawal of blood samples from the tail vein and transferred to micro-centrifuge tube. The absence of reflexes, and breathing rate was visually monitored throughout the experiment. At the end of experiments, the rats were euthanized with an overdose of anesthesia and exsanguinated.

4.5 ASSAY METHOD

The blood samples were allowed to clot for 10 minutes and centrifuged at 4000 to 5000 rpm for about 5 minutes in cold centrifuge at 0°C (C-94, Remi Instruments, Mumbai, India). The serum was separated out using a micropipette and transferred to siliconized 1.0-mL Eppendorf tubes. It was refrigerated at 0°C to 4°C until completion of the study for subsequent glucose/calcitonin estimation.

The glucose content was measured by the glucose oxidase–peroxidase method [3]. The analysis is based on the enzyme-catalyzed reaction of glucose with molecular oxygen, followed by a second reaction that produces an intense red color. The color intensity is proportional to the amount of oxidized glucose in the sample. This analytical method yielded a serum glucose concentration in the range of 20 to 350 mg/dL with 2% precision. The mean percent blood glucose reduction (PBGR) was calculated from the amount (mg/dL) of blood glucose measured.

The calcitonin was estimated by measuring its hypocalcemic effect in the blood. The ionized calcium content was measured using a calorimetric method based on the complexation with o-cresolphtalein complexone, in alkaline medium. The color intensity developed was proportional to the amount of calcium in the sample. This analytical method yielded serum calcium concentration in the range of 1.0 - 20.0 mg/dL with 2% precision. The calcium present in the samples was stable for a period of week/more.

4.6 PHARMACODYNAMIC ANALYSIS

The $AUC_{0\text{-}300\ min}$ (area under the blood glucose reduction–time curve) of both subcutaneously administered insulin and intratracheally instilled insulin formulations was calculated by the trapezoidal rule [4].

The maximum percent blood glucose reduction ($PBGR_{max}$) and the time to attain each $PBGR_{max}$ ($T.PBGR_{max}$) were determined from PBGR-time curves.

The amount (mg/dL) of blood calcium reduction by each of the formulation was measured (mg/dL) over a period of –30, 0, 30, 60, 90, 120, 150, 180, 210, and 240min. From this values the percentage blood calcium reduction (PBCR) was calculated over this period and their mean was calculated which is recorded as mean PBCR (Table 4.7 - Table 4.10). The $AUC_{0\text{-}240min}$ (area under blood calcium reduction - time curve) of both subcutaneously administered calcitonin and intratracheally instilled calcitonin formulations were calculated by trapezoidal rule.

The relative percent pulmonary bioactivity (F*) with respect to subcutaneously administered insulin was calculated by,

$$F^* = \frac{AUC_{0\text{-}300\ min}\ \text{Intratracheal Route} \times \text{Subcutaneous Dose}}{AUC_{0\text{-}300\ min}\ \text{Subcutaneous Route} \times \text{Intratracheal Dose}} \times 100 \ldots (\text{Eq.4.1})$$

The relative percent pulmonary bioactivity (F*) with respect to subcutaneously administered calcitonin was calculated by,

$$F^* = \frac{AUC_{0\text{-}240\ min}\ \text{Intratracheal Route} \times \text{Subcutaneous Dose}}{AUC_{0\text{-}240\ min}\ \text{Subcutaneous Route} \times \text{Intratracheal Dose}} \times 100 \ldots (\text{Eq.4.2})$$

4.7 STATISTICAL ANALYSIS

Each experiment was conducted on a group of 6 rats, and the mean and SD of the 6 values was recorded.

Linear regression analysis was done for finding the correlation among the responses (mean blood glucose reduction for insulin and mean blood calcium reduction for calcitonin) of different doses of subcutaneously administered insulin and calcitonin were shown in Figure 4.1 and Fig. 4.5. Tukey's multiple comparisons test [5] Devore, Farnum, 1999) was applied individually between the formulations having same dose of drug and same absorption promoters at three different concentrations. From each three formulations compared, either one or two were chosen which are significant. Further, from the significant formulations, the formulation with the smaller concentration of absorption promoter was selected. The method of selection for insulin formulations described in Table 4.2 and for calcitonin formulations in Table 4.6.

4.8 RESULTS AND DISCUSSION

The subcutaneous formulations of insulin in doses of 0.50 IU/kg, 0.75 IU/kg, 1.0 IU/kg, 1.25 IU/kg, and 1.50 IU/kg were administered subcutaneously, and the PBGR over a period of 30 to 300 minutes is shown in Figure 4.1. The PBGR was found to be continuously increasing up to 120 minutes and then decreasing up to 300 minutes for all the doses of insulin administered. A linear dose-response relationship was observed ($r^2 = 0.96$). The pharmacodynamic parameters of the subcutaneously administered insulin are recorded in Table 4.3. The $T.PBGR_{max}$ observed was 120 minutes for all the doses of insulin, and the $PBGR_{max}$ was found to increase linearly with respect to dose. The $AUC_{0\text{-}300\ min}$ (7689 ± 369, 8958 ± 403, 10 617 ± 548, 12 754 ± 634, and 14 349 ± 624) was also found to increase in proportion to the dose (0.5-1.5 IU/kg, respectively) administered.

The compositions of various formulations of insulin prepared for intratracheal administration are recorded in Table 4.1. F1 to F4 have 4 different pHs (7.4, 6.0, 5.0, and 3.5) but no other absorption promoters. F5 to F25 have 7 individual absorption promoters (penetration enhancers and protease inhibitors) at 3 concentration levels each. F26 to F31 contain a combination of penetration enhancers or a combination of protease inhibitors at 3 concentration levels. F32 to F34 have a combination of penetration enhancers and protease inhibitors. F5 to

F34 have the same pH: 3.5. Doses of insulin incorporated in the formulations were kept between 1.0 IU/kg to 7.0 IU/kg to avoid hypoglycemia and death and to obtain measurable response (i.e. minimum 20% PBGR and maximum 80% PBGR) depending on absorption promoters used. Exactly 100 μL of these formulations were intratracheally instilled, and the mean PBGRs are recorded (Table 4.1). Tukey's multiple comparisons test [12] was applied individually between the formulations having the same dose of insulin and the same absorption promoters at 3 different concentrations (Table 4.2). Formulations having a smaller concentration of absorption promoters with significantly high PBGR were selected by this method. The choice of the formulation(s) were narrowed by comparing the mean PBGR of the formulations of possible pair-wise in the testing group of formulations and further narrowed by selecting the formulation with smaller concentration. The pharmacodynamic parameters of these selected formulations along with other formulations (F1-F4 and F32-F34) are recorded in Table 4.4 (S1-S16).

Figure 4.2 shows the influence of pH on PBGR after intratracheal administration of insulin formulations (F1-F4), where the calculated PBGR values corresponding to a dose of 3.0 IU/kg insulin were plotted. The maximum reduction at all sampling points was observed in the formulation with pH 3.5 even though it had the lowest dose of insulin (3.0 IU/kg). The pulmonary bioactivity of the formulation (F4) having pH 3.5 was 43.20% ± 2.48% and was found to be decrease to 21.63% ± 1.28%, 14.57% ± 1.51%, and 11.36% ± 1.27%, respectively, with increase in pH (F1-F3 in Table 4.4). In neutral pH, the insulin molecule is typically associated to form hexameric units, a process called fibrillation. When the pH decreases, fibrillation decreases and the insulin molecules exist in only monomeric form [6,7]. Insulin is more stable at acidic pH, and the monomeric form absorbs rapidly in the alveolar region of the lungs [8,9]. 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 through the alveolar membrane and the pH of subsequent formulations (F5-F34) was 3.5.

Figure 4.3 shows the influence of different absorption promoters on PBGR after intratracheal administration of selected insulin formulations (Table 4.4: S5-S11),

where the calculated PBGR values corresponding to a dose of 1.0 IU/kg insulin were plotted. In Figure 4.4 the influence of combinations of absorption promoters on PBGR of the selected insulin formulations (Table 4.2: S12-S16) is shown. The formulations with oleic acid sodium salt (F13) and sodium tauroglycocholate (F15) as penetration enhancer showed more effect on PBGR in comparison to sodium caprylate (F6) and sorbitan trioleate (F9). The protease inhibitors bestatin (F21) and chymostatin (F25) showed more effect on PBGR compared to bacitracin (F19). Hence, oleic acid sodium salt, sodium tauroglycocholate, bestatin, and chymostatin were chosen for the combination studies of absorption promoters at pH 3.5.

The concentration of oleic acid sodium salt in F13 was 0.5% with bioactivity of 61.91% ± 3.21% and of sodium tauroglycocholate was 0.3% in F15 with bioactivity of 67.09% ± 3.23% (Table 4.4). Both of these enhancers were used in F27, 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 ± 4.31%. Even though the concentration of individual penetration enhancers was reduced by more than 50%, bioactivity increased significantly. This synergistic increase may be due to more than 1 mechanism involved in enhancing drug absorption. The bile salts and fatty acid salts act by reverse micellar binding with subsequent formation of hydrophilic channels in the tight junction [10,11]. 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+} [12] to increase paracellular permeability [13] and by inhibiting proteases like aminopeptidases [14]. The sodium tauroglycocholate used as enhancer was less irritating and its absorption profile more acceptable [15].

Similarly, when the protease inhibitors bestatin and chymostatin were coadministered with insulin (F31), the bioactivity obtained was 95.51% ± 4.77% compared to incorporation of bestatin (F21: 67.24% ± 2.11%) and chymostatin (F25: 69.84% ± 3.02%) individually. The surface of a wide variety of mammalian cell types, including lung, are rich in a group of proteolytic enzymes that includes aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, dipeptidases, and omegapeptidases [16]. These enzymes are responsible for the

hydrolysis of peptide drugs administered to the lungs. Bestatin is an aminopeptidase inhibitor, and chymostatin is a serine protease inhibitor. When a combination of these protease inhibitors was used, they inhibited a wide variety of enzymes involved in the degradation of insulin. Hence, the drug degradation was highly protected from the proteolytic enzymes and an increase in bioactivity was observed. The protease and peptidase inhibitors act through inhibition of proteolytic enzymes, and some of them are already approved as therapeutic agents [15].

When the penetration enhancers oleic acid sodium salt (0.2%) and sodium tauroglycocholate (0.10%) and the protease inhibitors bestatin (0.02%) and chymostatin (0.04%) were combined in an insulin formulation (F34) in citrate buffer pH 3.5, a significant increase in bioactivity of 155.60% ± 5.19% was observed (Table 4.4). Higher bioactivity of formulations with combinations of absorption promoters may be due to degradation of insulin in the subcutaneous tissue [17,18], partial inhibition of the proteolytic enzymes of lungs, and dilation of the tight junction of the alveolar membrane.

The dose of insulin used in the formulations was found to affect the consistency of PBGR profile significantly. The doses of F26 to F34 are 1.0 IU/kg and the $T.PBGR_{max}$ was found to be 120 minutes for all the formulations (Table 4.4). In Figure 6, the PBGR of these formulations increases up to 120 minutes and decreases from 120 to 300 minutes and resembles the subcutaneous PBGR-time profile (Figure 4.1). In formulations with high doses of insulin (1.5 IU/g or more; F1-F25), the $T.PBGR_{max}$ was found to be inconsistent, and interanimal variation of $T.PBGR_{max}$ was 20% to 57%. The PBGR values of these formulations fluctuate significantly, as seen in Figures 4.2 and 4.3. It is difficult to assign a specific reason for these fluctuations in glucose levels noticed with higher insulin doses (1.5 IU/kg and above). However, these fluctuations may be due to the body's preventive mechanism in which when the blood glucose level drops below a certain point, the glucose supply from the body's glycogens is triggered and glucose levels again go down because insulin is present in the required concentration. The PBGR-time profile was more consistent for the formulations having a combination of absorption promoters. This increased consistency may be

due to the reduction in insulin dose and combination of absorption promoters present in substantially lower concentration, contributing significantly to the safety, efficacy, and reproducibility of the formulations' pharmacological response.

In a group of animals, a surgical operation similar to intratracheal instillation of formulations was performed but no formulations were administered. The glucose levels were monitored over a period of 5 hours. The variation in blood glucose level was found to be within ± 5%. It may be due to the physiological variation observed in basal glucose level. Of the 9 control solutions studied, 7 contained the absorption promoters used in this study, and the remaining 2 contained phosphate buffer pH 7.4 and citrate buffer pH 3.5. The variation in PBGR was found to be within ± 5%.

Fig. 4.5 depicts the PBCR (Percent Blood Calcium Reduction)-time profiles of subcutaneously administered calcitonin at doses of 0.5 IU/Kg, 1.0 IU/Kg, 3.0 IU/Kg 4.0 IU/Kg and 6.0 IU/Kg. In Table 4.5 the pharmacodynamic parameters were recorded. The mean PBCR (15.1% ± 1.1%, 20.6% ± 0.9%, 33.2% ± 1.4% and 39.3% ± 1.5%) for doses 0.5 – 4.0 IU/Kg were found to be linearly related with the increasing doses of calcitonin formulations ($r^2 = 0.99$). The $AUC_{0\text{-}240min}$ of these formulations (3543 ± 258, 4769 ± 208, 7634 ± 322 and 8885 ± 339) were also found to increase proportionately with the dose ($r^2 = 0.99$). At a dose of 0.5 IU/Kg and 1.0 IU/Kg $PBCR_{max}$ of 25% - 32% was observed at 90-min post administration, and then blood calcium level increased gradually to reach the baseline level. Calcitonin formulations of 3.0 IU/Kg and 4.0 IU/Kg decreased the calcium (Ca) level until 150 min post administration and then increases. A maximum of 48.9% ± 1.7% PBCR was observed at 4.0 IU/Kg dose, after that increasing in dose (6.0 IU/Kg) does not caused further reduction in Ca level. It may be due to saturation of calcitonin binding receptors.

100 µL of the intratracheal formulations of calcitonin were instilled intratracheally and their mean PBCR were recorded in Table 4.6. The mean PBCR of formulations having same dose of calcitonin and same absorption promoters at three different concentrations (a, b, c) were compared by Tukey's multiple

comparisons test. Formulation having smallest concentration of each absorption promoters with highest PBCR was selected by this method. The choice of the formulation(s) were narrowed by comparing the mean PBCR of the formulations of possible pair-wise in each of the testing group of three formulations (a, b, c) and further narrowed by selecting the formulation with smaller concentration. The pharmacodynamic parameters of these selected formulations along with formulations of different pH were recorded in Table 4.7 to Table 4.10 and their PBCR shown in Fig. 4.6, 4.7 and 4.8. The influence of formulation variables is discussed below.

Fig. 4.6 shows the influence of pH on PBCR after intratracheal administration of calcitonin formulations (F#1 - F#3), where the calculated PBCR values corresponding to dose of 3.0 IU/Kg calcitonin were plotted. The maximum reduction at all sampling points was observed in formulation having pH 3.9. The pharmacodynamic parameters of these formulations were recorded in Table 4.7. The pulmonary bioactivity of the F#2 (pH 3.9) was 53.9% ± 2.8%, and was found to be decrease to 21.0% ± 1.5%, and to 12.0% ± 1.3% respectively with formulations of pH 6.0 and 2.8. Calcitonin is reported to be more stable at slightly acidic pH [19], and lowering the pH causes paracellular permeability possibly by displacing Ca^{2+} from the tight junction. Acidic pH was observed to favor higher penetration of calcitonin through the alveolar membrane when pH was reduced from 6.0 to 3.9. However, further reduction in the pH to 2.8 (F#3) leads to reduction in bioactivity from 53.9 ± 2.8 % to 12.0% ± 1.3%. This reduction in bioactivity may be due to degradation of calcitonin at pH 2.8. Hence, the pH of subsequent formulations (F#4 - F#15) was adjusted to pH 3.9.

Fig. 4.7 a & b shows the influence of various penetration enhancers and combination thereof on PBCR after intratracheal administration of calcitonin formulations. The penetration enhancers sodium tauroglycocholate (0.5%), dodecyl maltoside (0.1%) and dimethyl β-cyclodextrin (1.0%) were having maximum effect on PBCR when compared with oleic acid sodium salt (0.5%). Hence, for the combination study, the first three enhancers were chosen. The formulation with combination of dimethyl β-cyclodextrin and sodium tauroglycocholate (F#9) shows increase in PBCR, whereas formulation with

combination dimethyl β-cyclodextrin and dodecyl maltoside (F#8) shows decrease in PBCR. The pharmacodynamic parameters of these formulations were recorded in Table 4.8. The concentration of sodium tauroglycocholate in F#5 was 0.5% with bioactivity of 72.0% ± 2.7% and dimethyl β-cyclodextrin was 1.0% in F#7 with bioactivity of 79.2% ± 3.9%. Both of these enhancers used in combination in the F#9, where the concentration of sodium tauroglycocholate and dimethyl β-cyclodextrin was 0.2% and 0.3%, respectively and the bioactivity obtained was 102.9% ± 5.3%. Even though the concentration of individual penetration enhancers was reduced more than 50%, bioactivity increased significantly. This synergistic increase may be due to more than one mechanism involved in enhancing calcitonin absorption. Sodium tauroglycocholate acts 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. Non-ionic surfactants are been reported to be less toxic and reduction in the concentration of these absorption promoters demonstrated in these studies further makes them interesting for human use. 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 [20].

The formulation (F#8) having combination of β- cyclodextrin and dodecyl maltoside, shows decrease in bioactivity of 58.0% ± 5.3%. This was due to mutual inhibition of their ability to enhance systemic absorption of calcitonin. The formation of an inclusion complex between these enhancers lacks the ability to enhance the absorption of calcitonin and the same was reported for insulin delivery through nasal route [21].

The influence of protease inhibitors and their combination on PBCR of intratracheally administered calcitonin shown in Fig. 4.8 a & b. The formulation with chymostatin and bacitracin shows higher PBCR compared to bestatin and amastatin and hence, the first two were chosen for combination study. The pharmacodynamic parameters were recorded in Table 4.9. The F#12 & F#13 individually containing the protease inhibitors chymostatin (0.05%) and bacitracin (0.03%) gave relative bioactivity of 83.0% ± 2.1% & 87.0% ± 3.9% respectively. When both of these inhibitors (0.04% and 0.02% of chymostatin and bacitracin respectively) added in combination (F#14), the relative bioactivity was increased significantly to 111.9% ± 4.4%.

Little has been reported on calcitonin degrading enzymes and is mainly metabolized by serine protease enzymes. Bestatin and amastatin are amino-peptidase inhibitors. Chymostatin is serine protease inhibitor having more effect on inhibition of degradation of 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. Hence, the drug degradation was highly protected from the proteolytic enzymes and increase in bioactivity was observed. The protease and peptidase inhibitors act through inhibition of proteolytic enzymes and some of them are already approved as therapeutic agents.

The pharmacodynamics of the formulations containing combination of both penetration enhancers and protease inhibitors were recorded in Table 4.10. 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 incorporated with calcitonin in isotonic acetate buffer pH 3.9 (F#15), significant increase in relative bioactivity of 139.1% ± 7.3% was obtained. Higher relative bioactivity in comparison to subcutaneous administration may be due to degradation of calcitonin in the subcutaneous tissue, partial inhibition of the proteolytic enzymes of lungs, and dilation of tight junction of alveolar membrane.

A surgical operation was performed in a group of animals, similar to intratracheal instillation of formulations but without administration of any formulations. The blood calcium levels were monitored over a period of 5 hour. The variation in blood calcium level was found to be within ± 5%. It may be due to the physiological variation observed in basal calcium level. Nine control solutions were studied out of which eight contained the absorption promoters used in this study and the remaining one contained acetate buffer pH 3.9. The variation in PBCR was found to be within ± 5%.

The data from *in vivo* absorption studies of insulin reveal that the absorption promoters significantly affect the bioactivity of intratracheally administered insulin. This effect depends on the dose of insulin and the concentration and type of the absorption promoters used. The formulation (F34) developed with a combination of protease inhibitors and penetration enhancers in citrate buffer pH 3.5 showed the highest pulmonary bioactivity: 155.60% ± 5.19%.

The results of *in vivo* absorption studies of calcitonin have proven that pulmonary absorption of calcitonin can be enhanced by various factors including pH, absorption promoters and protease inhibitors. It depends on the concentration and type of the promoters used and the dose of calcitonin. The formulation (F#15), which contains combination of protease inhibitors and penetration enhancers in acetate buffer pH 3.9, showed highest relative bioactivity (139.1% ± 7.3%).

The selected combination of absorption promoters provided a synergistic effect. Nonionic surfactants and fatty acid salts have been reported to have low toxicity and reduction in the concentration of these absorption promoters demonstrated in these studies makes them even more interesting for human evaluation. Reduction in the concentration of selected individual absorption promoters is likely to have low chronic toxicity to the membrane. Higher bioactivity may decrease the dose of intratracheally-administered insulin/calcitonin, help to prevent systemic side effects, and reduce the cost of therapy.

Assessment of stability and reproducibility upon dosing by pulmonary devices and safety following chronic pulmonary administration has to be investigated prior to development. Findings of these investigations may help in development of dry powder inhalers (DPI) or metered dose inhalers (MDI) of insulin and calcitonin for efficient pulmonary delivery (chapter 6).

4.9 COMPARISON BETWEEN THE INFLUENCES OF ABSORPTION PROMOTERS ON INSULIN AND CALCITONIN ABSORPTION

Insulin at neutral or near to neutral pH the relative bioactivity was 11.36 ± 1.27% and for calcitonin 21.0 ± 1.55% through the pulmonary route. The molecular weight of insulin is higher than calcitonin i.e. 5778 and 3432 respectively of insulin and calcitonin. Due to higher in molecular weight of insulin the permeation is lesser in compare to low molecular weight calcitonin. The slightly acidic pH causes 43.2 ± 2.48% bioactivity in insulin and 53.9 ± 2.8% in calcitonin. The increase in the permeation of both the peptides were found to be about 32% and the influence of the pH was found to be same in insulin and calcitonin.

The penetration enhancer oleic acid sodium salt and sodium tauroglycocholate causes about 19% and 24% increase in relative bioactivity of insulin whereas the same two enhancers causes only about 7% and 18% respectively. When dimethyl β -cyclodextrin was used in calcitonin formulation, the maximum effect i.e 24% as compared to that of insulin formulations by sodium tauroglycocholate was achieved. The effect of penetration enhancers differs from drug to drug which may be due to molecular weight/amino acid composition/amino acid linkage. When combination of penetration enhancers were used the increase in relative bioactivity was 36% in insulin and 49% in calcitonin and this difference also due to their molecular sizes.

Bestatin and chymostatin causes increase in relative bioactivity of about 24% and 27% respectively in case of insulin. But, the same protease inhibitors cause less effect in case of calcitonin i.e. bestatin increases bioactivity of 6% and chymostatin

of 18%. In case of insulin, the protease inhibitor bacitracin having less effect (7.5% increase in relative bioactivity) and the same inhibitor having very good inhibiting effect in calcitonin i.e. contribute about 23% increase in relative bioactivity. The peptide drugs having specific site for proteolytic enzymes which degrades their structure and reduces their bioactivity. The protease inhibitors occupies the site where the proteolytic enzyme binds, hence their proteolytic activity was reduced and the protein drug protected from the degrading enzymes. Their activity is more site specific and competitive. The binding sites for the proteolytic enzymes of every protein drugs vary due to their different aminoacid skeleton. Bestatin and amastatin are aminopeptidase inhibitor, and chymostatin is a serine protease inhibitor. Calcitonin mainly metabolized by serine protease enzymes and chymostatin is serine protease inhibitor having more effect on inhibition of degradation of calcitonin. The inhibitory activity of bacitracin might be non-specific as a peptide substrate and is not always specific.

Hence, the influence of absorption promoters (like pH, penetration enhancers and protease inhibitors) on drug permeation through the alveolar membrane depends on the molecular weight of the protein drugs, amino acid sequence and their binding sites for proteolytic enzymes/protease inhibitors and the characteristic mechanisms of the promoters.

COMPOSITION OF INSULIN FORMULATIONS FOR INTRATRACHEAL ADMINISTRATION

For-mula-tion.	Dose - IU/Kg	Formulation Variables				Mean Blood Glucose Reduction (%)
		PH	Penetration enhancer	Protease	inhibitor	
F#1	7.0	7.4	----	----		31.94 ± 2.8
F#2	7.0	6.0	----	----		36.49 ± 3.8
F#3	6.0	5.0	----	----		44.98 ± 2.8
F#4	3.0	3.5	----	----		44.60 ± 2.7
F#5	2.5	3.5	Sodium caprylate 0.1%	----		43.05 ± 1.6
F#6	2.5	3.5	Sodium caprylate 0.5%	----		45.07 ± 1.0
F#7	2.5	3.5	Sodium caprylate 1.0%	----		45.09 ± 1.2
F#8	2.0	3.5	Sorbiton trioleate 0.1%	----		29.13 ± 1.2
F#9	2.0	3.5	Sorbiton trioleate 0.4%	----		32.28 ± 0.7
F#10	2.0	3.5	Sorbiton trioleate 0.7%	----		32.71 ± 1.1
F#11	2.5	3.5	Oss 0.1%	----		39.02 ± 1.7
F#12	2.5	3.5	Oss 0.3%	----		46.71 ± 1.4
F#13	2.5	3.5	Oss 0.5%	----		49.68 ± 0.9
F#14	1.5	3.5	Stg 0.1%	----		33.12 ± 1.2
F#15	1.5	3.5	Stg 0.3%	----		35.93 ± 0.7
F#16	1.5	3.5	Stg 0.5%	----		36.65 ± 0.9
F#17	2.0	3.5	----	Bacitracin 0.02%		31.36 ± 1.3

F#18	2.0	3.5	----	Bacitracin 0.05%	35.34 ± 1.2
F#19	2.0	3.5	----	Bacitracin 0.10%	37.44 ± 1.5
F#20	1.5	3.5	----	Bes 0.01%	29.74 ± 0.9
F#21	1.5	3.5	----	Bes 0.03%	36.05 ± 1.0
F#22	1.5	3.5	----	Bes 0.05%	36.25 ± 1.1
F#23	1.5	3.5	----	Chy 0.01%	28.58 ± 1.4
F#24	1.5	3.5	----	Chy 0.03%	35.34 ± 1.5
F#25	1.5	3.5	----	Chy 0.05%	37.56 ± 0.6
F#26	1.0	3.5	Oss 0.1% + Stg 0.05%	----	30.05 ± 1.0
F#27	1.0	3.5	Oss 0.2% + Stg 0.10%	----	31.80 ± 0.7
F#28	1.0	3.5	Oss 0.5% + Stg 0.30%	----	32.15 ± 1.0
F#29	1.0	3.5	----	Bes 0.01% + Chy 0.02%	29.82 ± 1.0
F#30	1.0	3.5	----	Bes 0.03% + Chy 0.05%	32.67 ± 0.8
F#31	1.0	3.5	----	Bes 0.02% + Chy 0.04%	34.59 ± 1.6
F#32	1.0	3.5	Oss 0.2% + Stg 0.1%	Bes 0.02%	42.54 ± 1.8
F#33	1.0	3.5	Oss 0.2% + Stg 0.1%	Chy 0.04%	44.26 ± 1.7
F#34	1.0	3.5	Oss 0.2% + Stg 0.1%	Bes 0.02% + Chy 0.04%	50.28 ± 1.8

*PBGR indicates percent blood glucose reduction; oss, oleic acid sodium salt; stg, sodium tauroglycocholate; bes, bestatin; chy, chymostatin.

TABLE 4.1

STATISTICAL DATA ANALYSIS

S.No.	Formulations for comparison	Distance between Mean PBGR of testing formulation T	Choice of formulation (Significant)	Selected Formula -tions*
S1	F#5, F#6 F#5, F#7 F#6, F#7	43.05 – 45.07 = 2.02 > 1.93 43.05 – 45.09 = 2.04 > 1.93 45.07 – 45.09 = 0.02 < 1.93	F#6 or F#7	F#6
S2	F#8, F#9 F#8, F#10 F#9, F#10	29.13 – 32.28 = 3.15 > 0.63 29.13 – 32.71 = 3.58 > 0.63 32.28 – 32.71 = 0.43 < 0.63	F#9 or F#10	F#9
S3	F#11, F#12 F#11, F#13 F#12, F#13	39.02 – 46.71 = 7.69 > 2.04 39.02 – 49.68 =10.66 > 2.04 46.71 – 49.68 = 2.97 > 2.04	F#13	F#13
S4	F#14, F#15 F#14, F#16 F#15, F#16	33.12 – 35.93 = 2.81 > 1.42 33.12 – 36.65 = 3.53 > 1.42 35.93 – 36.65 = 0.72 < 1.42	F#15 or F#16	F#15
S5	F#17, F#18 F#17, F#19 F#18, F#19	31.36 – 35.34 = 3.98 > 1.98 31.36 – 37.44 = 6.08 > 1.98 35.34 – 37.44 = 2.10 > 1.98	F#19	F#19
6	F#20, F#21 F#20, F#22 F#21, F#22	29.74 – 36.05 = 6.31 > 0.60 29.74 – 36.25 = 6.51 > 0.60 36.05 – 36.25 = 0.20 < 0.60	F#21or F#22	F#21
S7	F#23, F#24 F#23, F#25 F#24, F#25	28.58 – 35.34 = 6.76 > 1.90 28.58 – 37.56 = 8.98 > 1.90 35.34 – 37.56 = 2.92 > 1.90	F#25	F#25
S8	F#26, F#27 F#26, F#28 F#27, F#28	30.05 – 31.80 = 1.75 > 1.36 30.05 – 32.15 = 2.10 > 1.36 31.80 – 32.15 = 0.35 < 1.36	F#27 or F#28	F#27
S9	F#29, F#30 F#29, F#31 F#30, F#31	29.82 – 32.67 = 2.85 > 1.77 29.82 – 34.59 = 4.77 > 1.77 32.67 – 34.59 = 1.92 > 1.77	F#31	F#31

* Selected formulation having smaller concentration of absorption promoters.

TABLE 4.2

PHARMACODYNAMIC PARAMETERS AFTER SUBCUTANEOUS ADMINISTRATION OF INSULIN IN RATS

Various Parameters	Dose of Insulin				
	0.5 IU/Kg	0.75 IU/Kg	1.0 IU/Kg	1.25 IU/Kg	1.5 IU/Kg
Mean PBGR (%)	25.76±1.3	30.06±1.4	35.69±1.9	43.01±2.4	48.43±2.2
$PBGR_{max}$ (%)	48.1± 0.9	54.1 ± 1.6	60.1 ± 2.0	66.5 ± 2.1	74.0 ± 2.8
$T.PBGR_{max}$ (min)	120	120	120	120	120
$AUC_{0\text{-}300min}$ (%. min)	7689±369	8958±403	10617±548	12754.5±634	14349±624

$PBGR_{max}$ – Maximum percent of blood glucose reduction; $T.PBGR_{max}$ – Time at which maximum percent of blood glucose reduction occurs; $AUC_{0\text{-}300min}$ – Area calculated under percent blood glucose reduction - time curve of subcutaneously administered insulin over 0 – 300 min.

TABLE 4.3

PHARMACODYNAMIC PARAMETERS AFTER INTRATRACHEAL ADMINISTRATION OF DIFFERENT FOEMULATIONS OF INSULIN

S.No.	Formula -tion.	$AUC_{0\text{-}300min}$ (%. min)	$PBGR_{max}$ (% Glu)	$T.PBGR_{max}$ (min)	F*
S1	F#1	9513±1031	76.0±0.8	90±30	11.36±1.27
S2	F#2	10825±1109	66.6±4.4	60±30	14.57±1.51
S3	F#3	13261±804	72.0±2.1	70±45	21.63±1.28
S4	F#4	13224±823	79.9±5.1	80±45	43.20±2.48
S5	F#6	13196±672	76.7±3.7	105±21	51.73±2.70
S6	F#9	9583±571	54.3±1.5	105±21	53.49±3.18
S7	F#13	14806±765	78.6±4.1	90±30	61.91±3.21
S8	F#15	10685±509	60.1±2.1	90±30	67.09±3.23
S9	F#19	10753±825	78.6±5.3	75±21	50.64±3.90
S10	F#21	10708±613	60.9±3.1	105±21	67.24±2.11
S11	F#25	11122±478	62.3±0.9	105±21	69.84±3.02
S12	F#27	9466±678	56.3±3.3	120±00	79.25±4.31
S13	F#31	10140±489	57.9±3.1	120±00	95.51±4.77
S14	F#32	12615±496	64.8±1.8	120±00	123.63±5.13
S15	F#33	13126±495	66.7±2.0	120±00	128.64±4.75
S16	F#34	14889±510	73.9±1.5	120±00	155.60±5.19

$AUC_{0\text{-}300min}$ – Area under blood glucose reduction - time curve of intratracheally administered insulin over 300 min, $PBGR_{max}$ – Maximum percentage of blood glucose reduction, F* - Percent relative pulmonary bioactivity, $T.PBGR_{max}$ – Time at which maximum blood glucose reduction occurs.

TABLE 4.4

PHARMACODYNAMIC PARAMETERS AFTER SUBCUTANEOUS ADMINISTRATION OF CALCITONIN IN RATS

	Dose of Calcitonin				
Parameters	0.5 IU/Kg	1.0 IU/Kg	3.0 IU/Kg	4.0 IU/Kg	6.0 IU/Kg
Mean PBCR (%)	15.1±1.1	20.6±0.9	33.2±1.4	39.3±1.5	39.6±1.5
$PBCR_{max}$ (%)	25.8±1.5	31.2±1.2	46.7±1.1	48.8±1.2	48.9±1.3
$T.PBCR_{max}$ (min)	90	90	150	150	150
$AUC_{0\text{-}240min}$ (%. Min)	3543±258	4769±208	7634±322	8885±339	8895±297

$PBCR_{max}$ – Maximum percent of blood calcium reduction; $T.PBCR_{max}$ – Time at which maximum percent of blood calcium reduction occurs; $AUC_{0\text{-}300min}$ – Area calculated under percent blood calcium reduction - time curve of subcutaneously administered calcitonin over 0 – 240 min.

TABLE 4.5

TABLE 4.6 STATISTICAL DATA ANALYSIS

Formulations for comparison	Distance between mean PBCR of testing formulation	Threshold Value (T)	Choice of formulations (Significant)	Selected formulations with smaller concentration of absorption promoter.
F#4:				
a with b	17.9 – 20.2 = 2.3 >	1.6		
a with c	17.9 – 21.1 = 3.2 >	1.6	b or c	b
b with c	20.2 – 21.1 = 0.9 <	1.6		
F#5:				
a with b	21.1 – 23.7 = 2.6 >	1.9		
a with c	21.1 – 24.7 = 3.6 >	1.9	b or c	b
b with c	23.7 – 24.7 = 1.0 <	1.9		
F#6:				
a with b	24.3 – 25.7 = 1.4 <	1.6	a or b	
a with c	24.3 – 25.5 = 1.3 <	1.6	or c	a
b with c	25.7 – 25.5 = 0.2 <	1.6		
F#7:				
a with b	23.4 – 24.8 = 1.4 >	1.2		
a with c	23.4 – 26.1 = 2.7 >	1.2	c	c
b with c	24.8 – 26.1 = 1.3 >	1.2		
F#8:				
a with b	19.2 – 20.0 = 0.8 <	1.3	a or b	
a with c	19.2 – 20.1 = 0.9 <	1.3	or c	a
b with c	20.0 – 20.1 = 0.1 <	1.3		
F#9:				
a with b	19.7 – 21.2 = 1.5 >	1.4		
a with c	19.7 – 21.7 = 2.0 >	1.4	b or c	b
b with c	21.2 – 21.7 = 0.5 <	1.4		
F#10:				
a with b	19.8 – 20.4 = 0.6 <	1.4	a or b	
a with c	19.8 – 20.9 = 1.1 <	1.4	or c	a
b with c	20.4 – 20.9 = 0.5 <	1.4		
F#11:				
a with b	18.5 – 20.2 = 1.7 >	1.6		
a with c	18.5 – 21.3 = 2.8 >	1.6	b or c	b
b with c	20.2 – 21.3 = 1.1 <	1.6		
F#12:				
a with b	24.1 – 26.0 = 1.9 >	1.3		
a with c	24.1 – 27.4 = 3.3 >	1.3	c	c
b with c	26.0 – 27.4 = 1.4 >	1.3		
F#13:				
a with b	26.8 – 28.7 = 1.9 >	1.6		
a with c	26.8 – 29.8 = 3.0 >	1.6	b or c	b
b with c	28.7 – 29.8 = 1.1 <	1.6		
F#14:				
a with b	21.3 – 23.0 = 1.7 >	1.5		
a with c	21.3 – 24.0 = 2.7 >	1.5	b or c	b
b with c	23.0 – 24.0 = 1.0 <	1.5		
F#15:				
a with b	26.2 – 28.7 = 2.5 >	2.0		
a with c	26.2 – 30.1 = 3.9 >	2.0	b or c	b
b with c	28.7 – 30.1 = 1.4 <	2.0		

PHARMACODYNAMICS OF CALCITONIN FORMULATIONS WITN DIFFERENT pH ADMINISTERED INTRATRACHEALLY IN RATS

Calcitonin Formul -ation	pH	Dose (IU/Kg)	Mean PBCR (%)	$PBCR_{max}$ (%)	AUC (%. Min)	Relative bioactivity (%)
F#1	6.0	12.0	24.6±1.7	37.2±1.7	5706±397	21.0±1.5
F#2	3.9	3.0	17.6±0.9	29.3±1.8	4122±211	53.9±2.8
F#3	2.8	12.0	13.9±1.5	24.0±0.9	3261±352	12.0±1.3

PBCR – Percent blood calcium reduction

TABLE 4.7

COMPOSITION AND PHARMACODYNAMICS OF THE CALCITONIN FORMULATIONS WITH PENETRATION ENHANCERS FOR INTRATRACHEAL ADMINISTRATION

Calcitonin Formula-tion	Composition	Dose (IU/Kg)	Selected Formu -lation*	Mean PBCR (%)	$PBCR_{max}$ (%)	AUC (%. min)	Relative bio -activity (%)
F#4-a	O-0.1%	3.0					
b	O-0.5%	3.0	b	20.2±0.9	31.1±0.9	4680±209	61.3±2.7
c	O-1.0%	3.0					
F#5-a	S-0.1%	3.0					
b	S-0.5%	3.0	b	23.7±1.1	35.7±1.4	5493±209	72.0±2.7
c	S-1.0%	3.0					
F#6-a	D-0.1%	3.0					
b	D-0.5%	3.0	a	24.3±0.9	36.3±0.4	5624±208	73.7±2.7
c	D-1.0%	3.0					
F#7-a	β-0.1%	3.0					
b	β-0.5%	3.0	c	26.1±1.3	39.0±0.9	6042±301	79.2±3.9
c	β-1.0%	3.0					
F#8-a	β-0.1%+ D-0.1%	3.0					
b	β-0.3%+ D-0.2%	3.0	a	19.2±0.8	29.0±0.7	4431±185	58.0±2.4
c	β-0.5%+ D-0.3%	3.0					
F#9-a	β-0.1%+ S-0.1%	1.0					
b	β-0.3%+ S-0.2%	1.0	b	21.2±1.1	32.4±0.4	4908±255	102.9±5.3
c	β-0.5%+ S-0.3%	1.0					

O – oleic acid sodium salt, S – sodium tauroglycocholate, D – dodecyl maltoside, β–dimethyl β cyclodextrin; PBCR – Percent blood calcium reduction.

* – Formulations selected by Tukey's test, having significant blood calcium reduction with smaller concentration of absorption promoters.

TABLE 4.8

COMPOSITION AND PHARMACODYNAMICS OF THE CALCITONIN FORMULATIONS WITH PROTEASE INHIBITORS FOR INTRATRACHEAL ADMINISTRATION

.Calci -tonin Formula-tion	Composition	Dose IU/Kg	Selected Formula -tion*	Mean PBCR (%)	$PBCR_{max}$ (%)	AUC (%. min)	Relative Bicacti -vity (%)
F#10-a	BES-0.01%	3.0					
B	BES-0.03%	3.0	a	19.8±0.9	30.0±1.7	4584±208	60.1±2.7
C	BES-0.05%	3.0					
F#11-a	A-0.01%	3.0					
B	A-0.03%	3.0	b	20.2±0.7	30.4±0.6	4668±208	61.2±2.7
C	A-0.05%	3.0					
F#12-a	C-0.01%	3.0					
B	C-0.03%	3.0	c	27.4±0.7	41.8±1.0	6339±162	83.0±2.1
C	C-0.05%	3.0					
F#13-a	B-0.01%	3.0					
B	B-0.03%	3.0	b	28.1±1.3	43.4±0.9	6642±301	87.0±3.9
C	B-0.05%	3.0					
F#14-a	C-0.02%+ B-0.01%	1.0					
B	C-0.04%+ B-0.02%	1.0	b	23.0±0.9	34±0.7	5336±209	111.9±4.4
C	C-0.05%+ B-0.05%	1.0					

BES – bestatin, A – amastatin, C – chymostatin, B – bacitracin; PBCR – Percent blood calcium reduction.

* - Formulations selected by Tukey's test, having significant blood calcium reduction with smaller concentration of absorption promoters

TABLE 4.9

COMPOSITION AND PHARMACODYNAMICS OF THE CALCITONIN FORMULATIONS WITH COMBINATION OF PENETRATION ENHANCERS AND PROTEASE INHIBITORS FOR INTRATRACHEAL ADMINISTRATION

Calcitonin Formu-lation	Composition	Dose (IU/Kg)	Selected Formu-lation*	Mean PBCR (%)	$PBCR_{max}$ (%)	AUC (%. min)	Relative bio activity (%)
F#15-a	β-0.1%+ S-0.1% + C-0.02%+ B-0.01%	1.0					
b	β-0.3%+ S-0.2% + C-0.04%+ B-0.02%	1.0	b	28.7±1.5	43.0±2.0	6632±347	139.1±7.3
c	β-0.5%+ S-0.4% + C-0.05%+ B-0.05%	1.0					

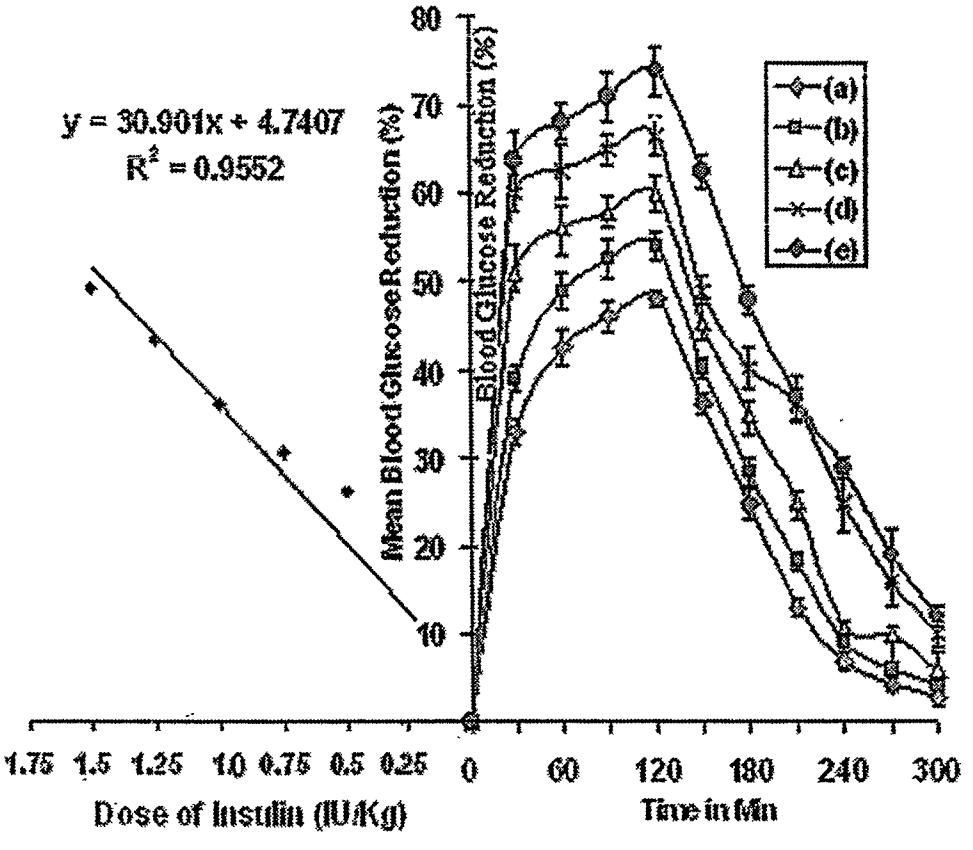
β – dimethyl β cyclodextrin, S – sodium tauroglycocholate, C – chymostatin, B – bacitracin;

PBCR – Percent blood calcium reduction.

* - Formulations selected by Tukey's test, having significant blood calcium reduction with smaller concentration of absorption promoters.

TABLE 4.10

PROFILES OF BLOOD GLUCOSE REDUCTION FOLLOWING SUBCUTANEOUS ADMINISTRATION *

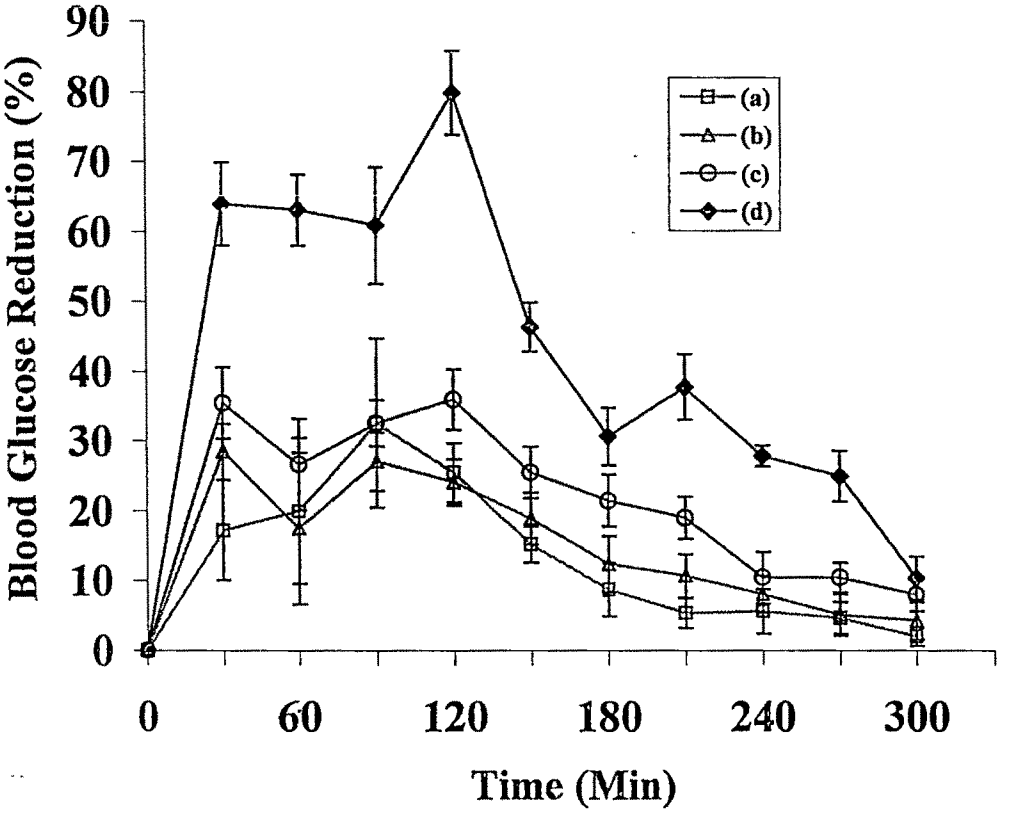


Key: *Values represent means ± SE (n = 6)

(a) 0.5 IU/Kg insulin, (b) 0.75 IU/Kg insulin, (c) 1.0 IU/Kg insulin, (d)1.25 IU/Kg insulin, and (e)1.5 IU/Kg insulin.

FIGURE 4.1

INFLUENCE OF pH ON BLOOD GLUCOSE REDUCTION-TIME PROFILE OF INTRATRACHEALLY ADMINISTERED INSULIN CALCULATED FOR THE DOSE OF 3.0 IU/Kg. VALUES REPRESENT MEANS ± SE (n = 6)

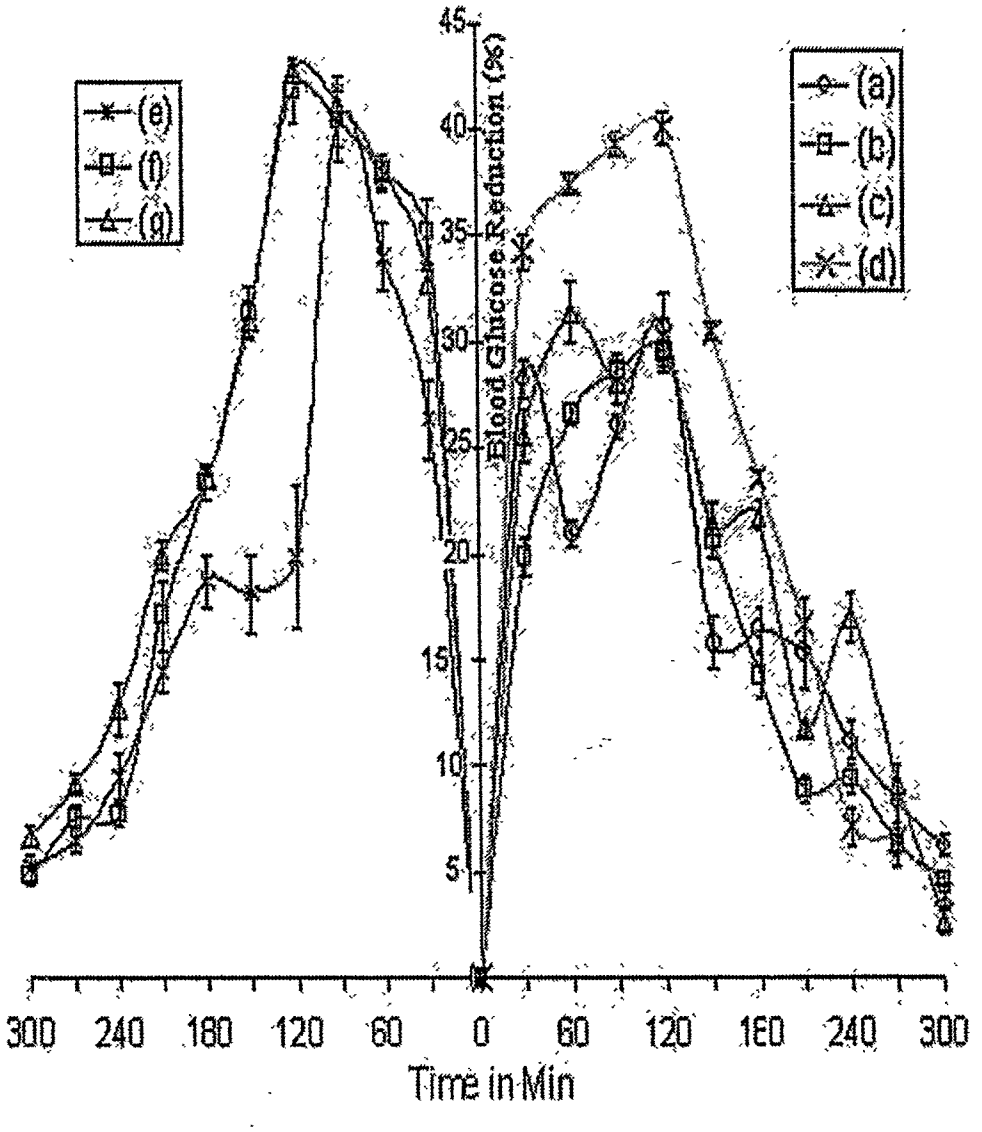


Key: *Values represent means ± SE (n = 6)

(a) pH 7.4, (b) pH 6.0, (c) pH 5.0 and (d) pH 3.5.

FIGURE 4.2

EFFECTS OF PENETRATION ENHANCERS AND PROTEASE INHIBITORS ON BLOOD GLUCOSE REDUCTION-TIME PROFILE OF INTRATRACHEALLY ADMINISTERED INSULIN CALCULATED FOR THE DOSE OF 1.0 IU/Kg*.

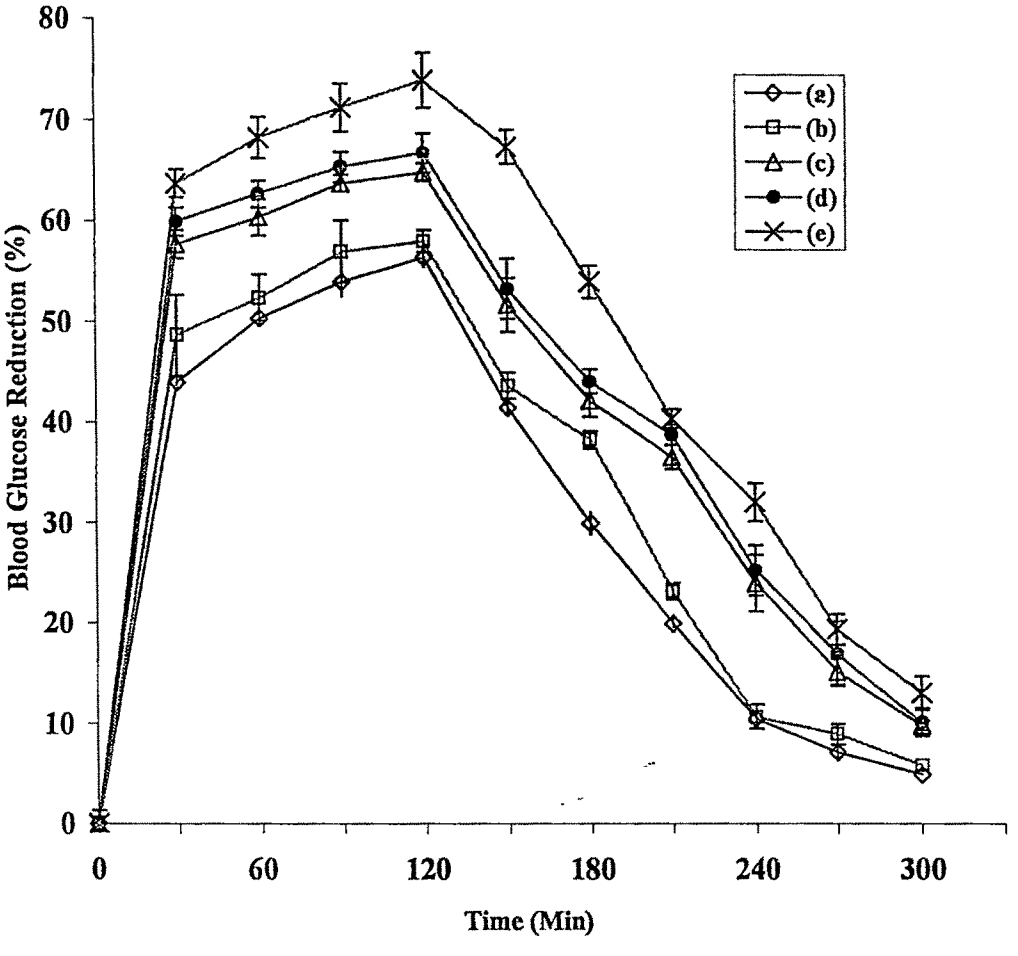


Key: *Values represent means ± SE (n = 6)

(a) sodium caprylate 0.5%, (b) sorbiton trioleate 0.4%, (c) oleic acid sodium salt 0.5%, (d) sodium tauroglycocholate 0.3%, bacitracin 0.10% (e), (f) bestatin 0.03%, and (g)chymostatin 0.05%

FIGURE 4.3

PROFILES OF BLOOD GLUCOSE REDUCTION FOLLOWING INTRATRACHEAL ADMINISTRATION OF 1.0 IU/Kg INSULIN WITH COMBINATIONS OF ABSORPTION PROMOTERS*.



Key: *Values represent means ± SE (n = 6)

(a) sodium tauroglycocholate 0.10% + oleic acid sodium salt 0.20%; (b) bestatin 0.02% + chymostatin 0.04%; (c) sodium tauroglycocholate 0.10% + oleic acid sodium salt 0.20% + bestatin 0.02%; (d) sodium tauroglycocholate 0.10% + oleic acid sodium salt 0.20% + chymostatin 0.04%; (e) sodium tauroglycocholate 0.10% + oleic acid sodium salt 0.20% + bestatin 0.02% + chymostatin 0.04%.

FIGURE 4.4

PROFILES OF BLOOD CALCIUM REDUCTION IN RATS FOLLOWING SUBCUTANEOUS ADMINISTRATION OF DIFFERENT DOSES OF CALCITONIN IN RATS. VALUES REPRESENT MEANS ± SD (n = 6)

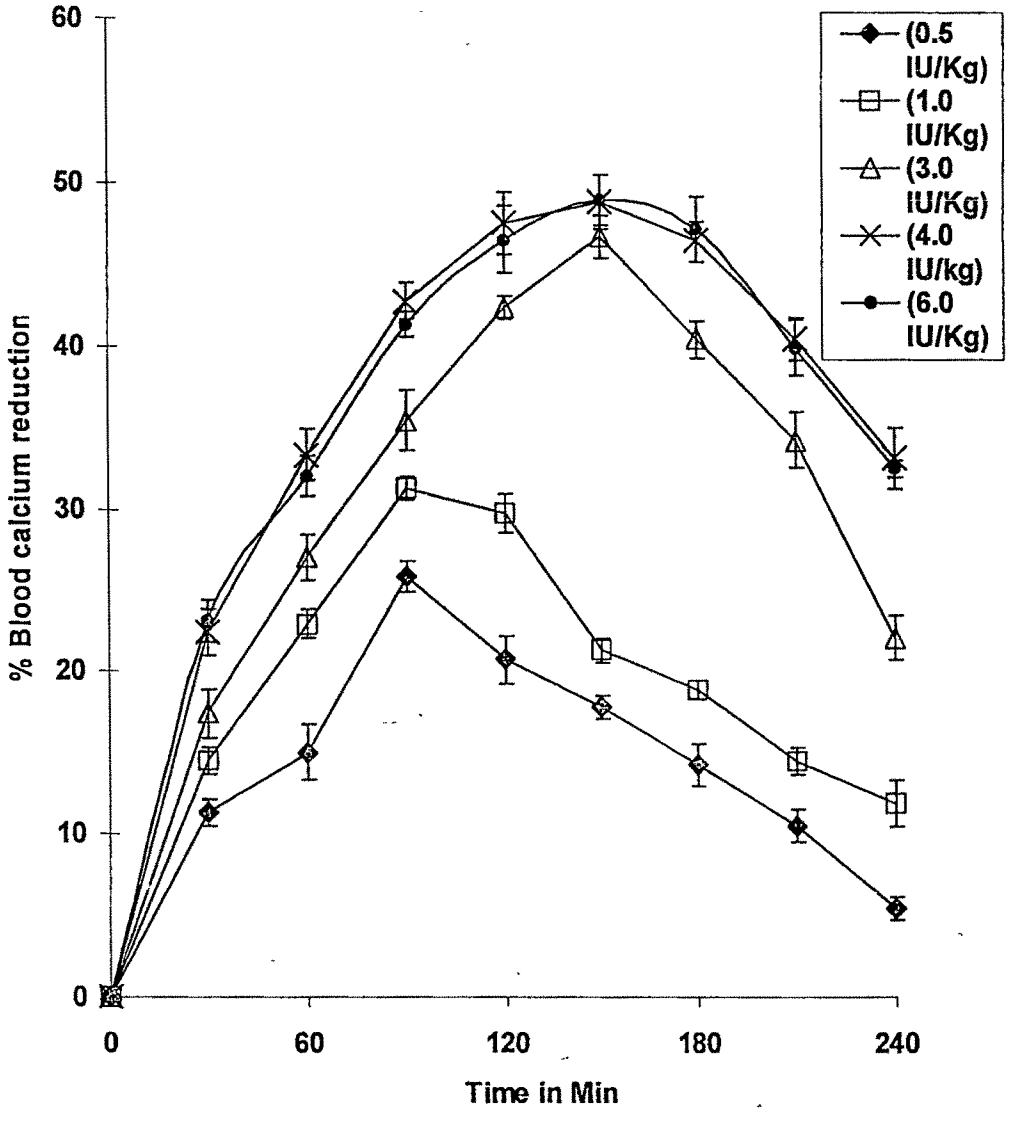
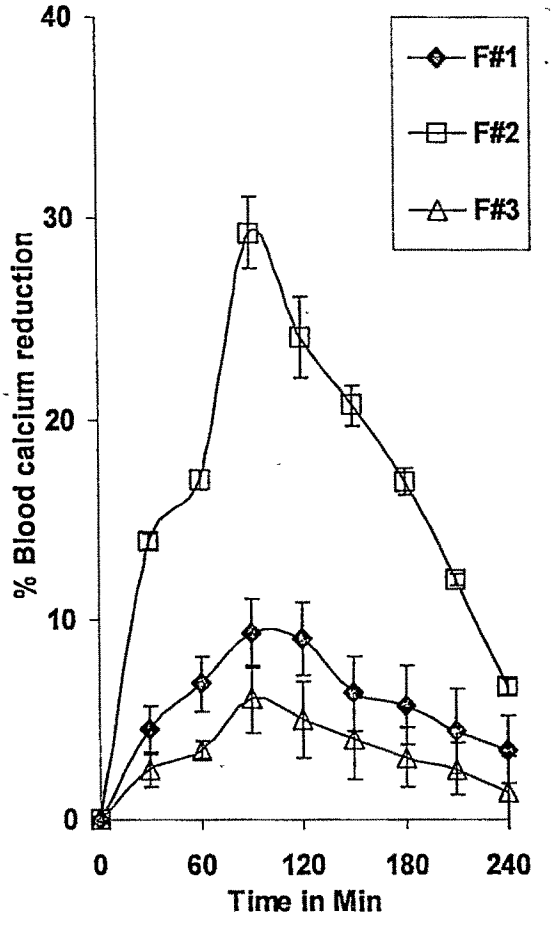


FIGURE 4.5

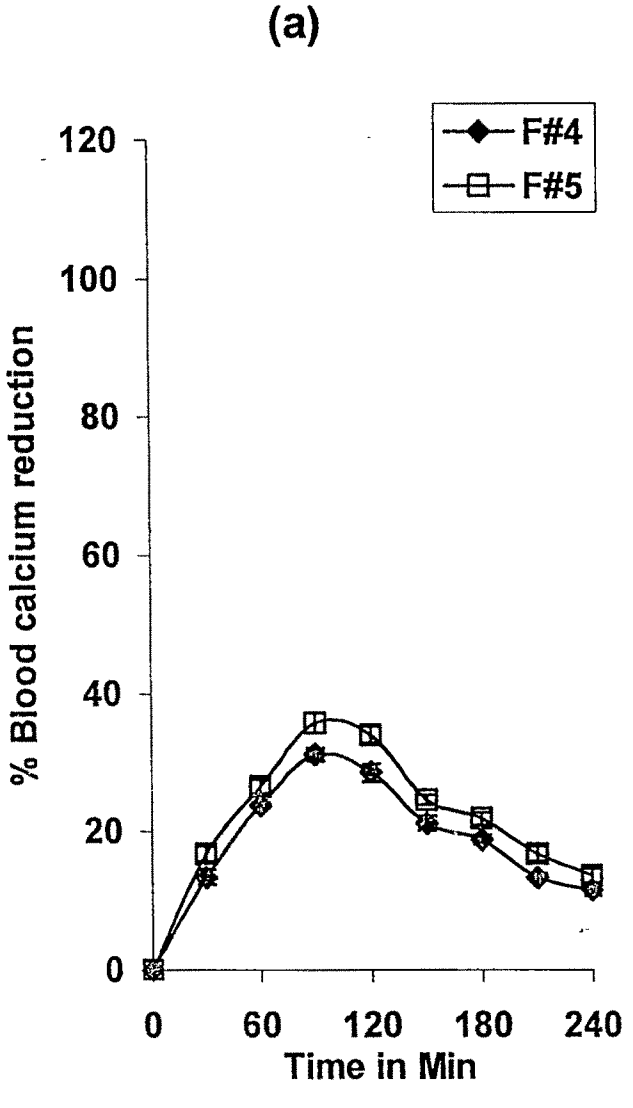
INFLUENCE OF pH ON BLOOD CALCIUM REDUCTION-TIME PROFILE OF INTRATRACHEALLY ADMINISTERED CALCITONIN CALCULATED FOR THE DOSE OF 3.0 IU/Kg. VALUES REPRESENT MEANS ± SD (n = 6)



Legends: (F#1) pH 6.0, (F#2) pH 3.9, and (F#3) pH 2.8.

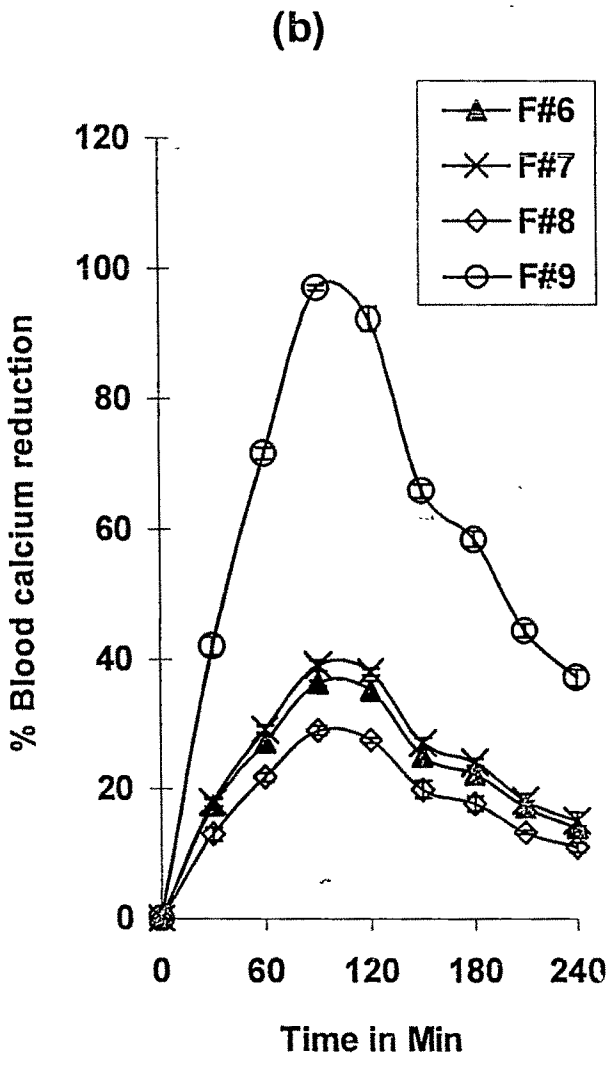
FIGURE 4.6

EFFECT OF PENETRATION ENHANCERS AND THEIR COMBINATIONS ON BLOOD CALCIUM REDUCTION-TIME PROFILE OF INTRATRACHEALLY ADMINISTERED CALCITONIN CALCULATED FOR THE DOSE OF 3.0 IU/Kg. VALUES REPRESENT MEANS ± SD (n = 6)



Legends: (F#4) oleic acid sodium salt 0.5%, (F#5) sodium tauroglycocholate 0.1%,

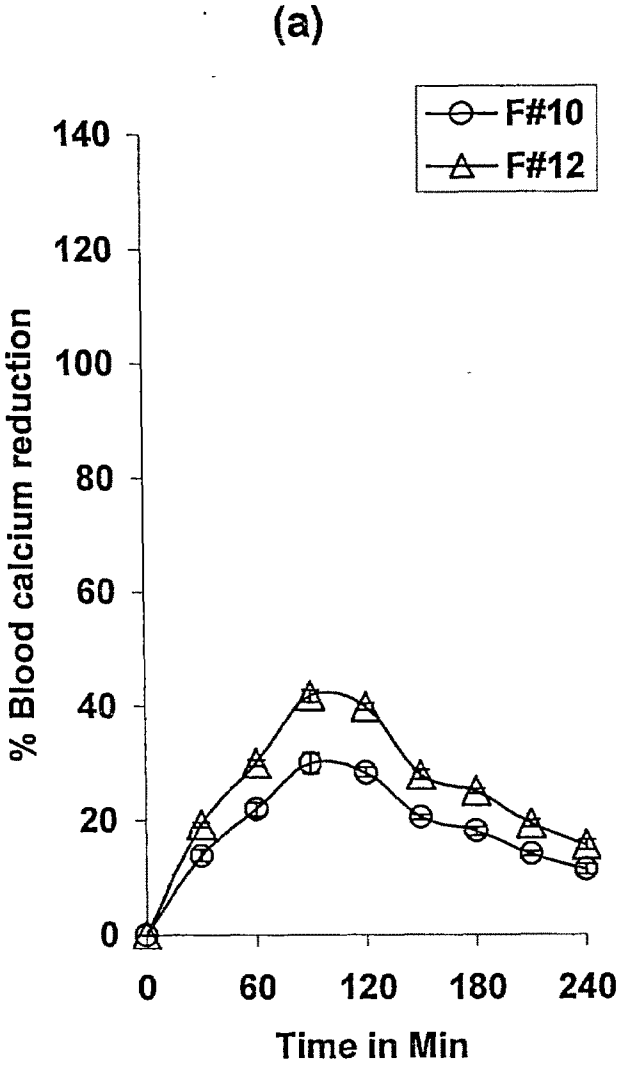
FIGURE 4.7



(F#6) dodecyl maltoside 0.1% and (F#7) dimethyl β-cyclodextrin 1.0%; (F#8) dimethyl β-cyclodextrin 0.1% + dodecyl maltoside 0.1%, (F#9) dimethyl β-cyclodextrin 0.3% + sodium tauroglycocholate 0.2%.

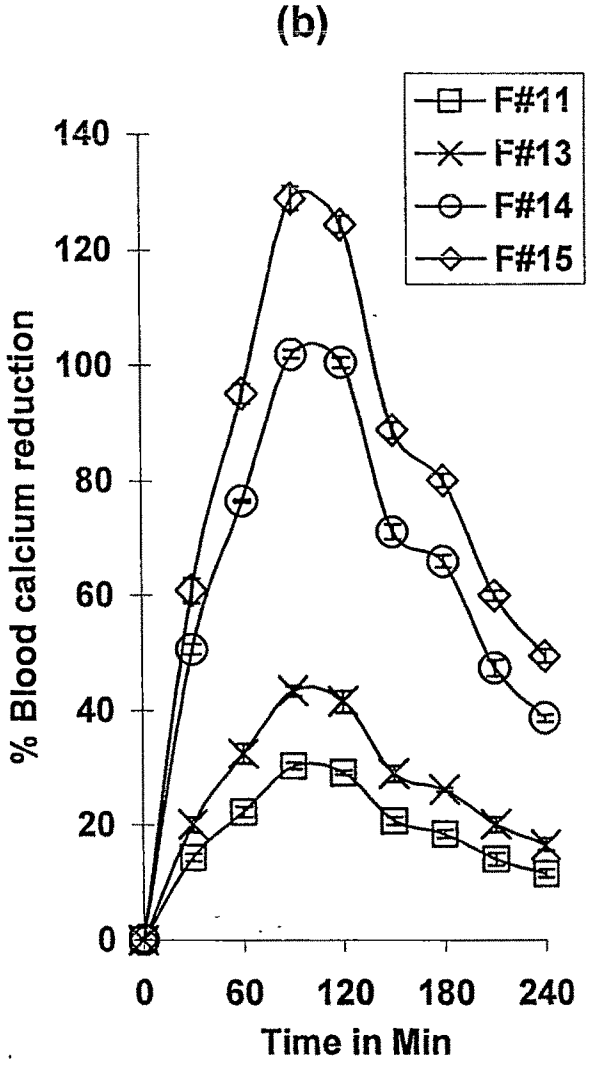
FIGURE 4.7

EFFECT OF PROTEASE INHIBITORS, THEIR COMBINATIONS AND COMBINATION OF PENETRATION ENHANCERS WITH PROTEASE INHIBITORS ON BLOOD CALCIUM REDUCTION-TIME PROFILE OF INTRATRACHEALLY ADMINISTERED CALCITONIN CALCULATED FOR THE DOSE OF 3.0 IU/Kg. VALUES REPRESENT MEANS ± SD (n = 6)



Legends: (F#10) bestatin 0.01%, (F#12) chymostatin 0.05%.

FIGURE 4.8



(F#11) amastatin 0.03%, (F#13) bacitracin 0.03%, (F#14) chymostatin 0.04% + bacitracin 0.02%; and (F#15) dimethyl-β-cyclodextrin -0.3% + sodium tauroglycocholate -0.2% + chymostatin -0.04% + bacitracin-0.02%

FIGURE 4.8

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