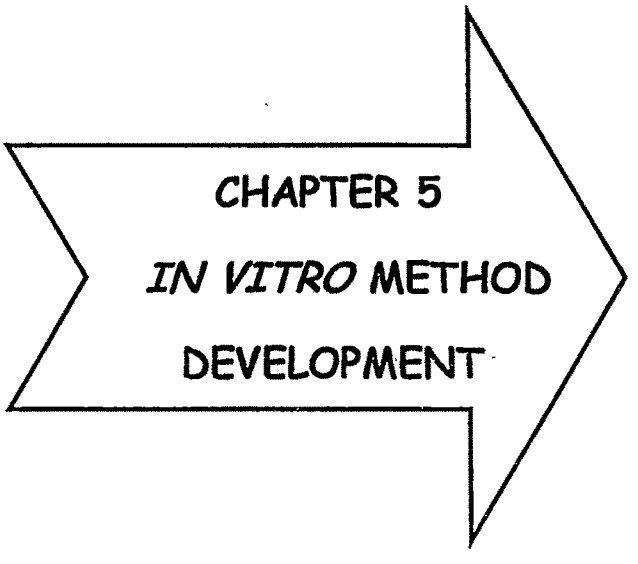

CHAPTER 5
IN VITRO METHOD
DEVELOPMENT

Currently, protein and peptide drug delivery is a challenging work for the formulation scientists [1,2]. One major challenge in pulmonary drug delivery is that the epithelial cell uptake and transport mechanisms of pulmonary barrier are not well understood.

In vitro models employed for drug transport studies allow rapid screening of a number of compounds and ability to control a large number of variables. They also help in elucidating drug transport mechanisms. A disadvantage associated with the employment of these *in vitro* models is that they do not exactly mimic the behavior of living tissue present *in vivo* conditions. However they are employed for the primary transport studies.

Various *in vitro* methods including isolated perfused lung, lung slices, tissue minces, homogenates and isolated cell systems have been employed to study the transport mechanism of peptide molecules across the alveolar membrane [3,4]. Even cell culture techniques developed for *in vitro* estimation of peptide drug permeation there is no established cell lines exist for the terminally differentiated type I alveolar epithelial cell, which comprise the primary absorptive barrier of alveoli [5-8]. The primary cultures of alveolar epithelium are viable only for 7-8 days, a period of time, which includes an initial 4-5 days period of differentiation into squamous type I morphology. Such cultures lack in surfactant and basement membrane endothelium. In isolated perfused lung systems sampling of the alveolar space is difficult due to extensive structural subdivision of this compartment [9-11]. The complex architecture of the mammalian lung has precluded mounting of planar sheets of the tissue in diffusion chamber systems used for other epithelial tissues, such as gastro-intestinal, transdermal and nasal mucosa [12-14].

The amphibian lung resembles mammalian lung morphologically and physiologically possessing similar composition and dimension of the air-blood barrier, surfactant production by primary epithelial cells and high transepithelial resistance [15]. Transports of several model hydrophilic and hydrophobic compounds like mannitol, inulin, leucine, and antipyrine have been reported and tissue viability can be maintained over 4 h in the *in vitro* [16].

We demonstrated the frog lung mounted in the diffusion chamber might provide a physiologically relevant *in vitro* system for studies on transport of insulin and calcitonin. The system may also be used to investigate the effects of formulation components such as pH; penetration enhancers like sodium tauroglycocholate, oleic acid sodium salt and dimethyl β-cyclodextrin; protease inhibitors like bestatin, chymostatin and bacitracin and combination thereof, which may alter the permeability of drug through the epithelial layer. It may help the formulation scientists to develop potent formulations and reduces the burden of carrying out a large number of *in vivo* experiments.

In this present work alveolar membrane of frog lung mounted in the diffusion chamber employed to study the permeation of insulin and calcitonin.

The permeation of insulin and calcitonin alone and with the absorption promoters, which are proven more effective in the *in vivo* studies were studied. The penetration enhancers sodium tauroglycocholate, oleic acid (cis 9-ocatadecanoic acid) sodium salt and protease inhibitors bestatin, and chymostatin were selected from *in vivo* absorption studies of insulin. The penetration enhancers sodium tauroglycocholate, dimethyl β-cyclodextrin and protease inhibitors bacitracin, and chymostatin were selected from *in vivo* absorption studies of calcitonin carried out in chapter 4.

5.1 EQIUPMENTS AND 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, USA). Citric acid anhydrous (extra pure) and sodium tauroglycocholate was purchased from SD Fine-Chem Ltd (Boisar, India). Oleic acid (cis 9-ocatadecanoic acid) sodium salt, Dimethyl β-cyclodextrin, bestatin, bacitracin, and chymostatin were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

BCA - kit (Genei Pvt.Ltd, Bangalore, India), Magnetic stirrer (Remi Electronics, Mumbai, India), Diffusion cell, Clinical chemistry analyzer RA 50 (Miles Inc.Diagnostics Division, Elkhart, IN, USA), Calibrated pipettes and other glassware of borosil.

5.2 REAGENTS

The following reagents were prepared,

1. 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.
2. 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.
3. Frog ringer's solution contains sodium chloride 6.5 gm/L, potassium chloride 0.14 gm/L, calcium chloride 0.12 gm/L, sodium dihydrogen phosphate 0.05 gm/L, glucose 1.5 gm/L and sodium bicarbonate 0.4 gm/L.
4. 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.
5. Acetate buffer pH 3.9 was prepared by dissolving anhydrous sodium acetate 1.48 mol and acetic acid 4.87 mol) was prepared in water for injection.

5.3 CONSTRUCTION OF DIFFUSION ASSEMBLY FOR *IN VITRO* STUDY

For the *in vitro* study a vertical type of diffusion cell was developed. A borosil tube having outer diameter of 1.26 cm was taken and was cut so that it becomes hollow (open at both ends). The cut end was rounded and polished. The area available for diffusion was 1.25 cm^2.

Frogs were anesthetized by keeping in ice for 5-10 minutes and a ventral incision was made to expose the lung. Lungs were excised by severing the tracheoglottis

after filling each lung with approximately 3-4 mL of ringer's solution. It was made into a sheet for mounted in the diffusion cell. The alveolar membrane mounted in the diffusion cell shown in the Figure 5.1.

5.4 PREPARATION OF FORMULATIONS

Freshly prepared formulations were used throughout the study.

Porcine insulin crystalline powder at a concentration of 3.0 mg/mL was dissolved in phosphate buffer pH 7.4 (In1). Another solution of insulin was made in citrate buffer pH 3.5. 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 solution 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 3.0 mg/mL of insulin and the required concentration of absorption promoters. The compositions of the insulin formulations prepared for the *in vitro* studies are recorded in Table 5.1 (In1- In9).

Calcitonin was dissolved in acetate buffer pH 6.0 at a concentration of 3.0 mg/mL (C1). Another solution was made in acetate buffer pH 3.9. 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 3.0 mg/mL of calcitonin and the required concentration of absorption promoters. The compositions of the formulations prepared for the *in vitro* studies are recorded in Table 5.2 (C1 - C9).

For studying the influence of excipients/absorption promoters used in the *in vitro* insulin formulations, six control formulations were prepared, two were phosphate buffer pH 7.4, citrate buffer pH 3.5, and other four formulations contained the absorption promoters individually (sodium tauroglycocholate, oleic acid sodium salt, bestatin, and chymostatin) in citrate buffer pH 3.5. The concentrations of the

absorption promoters in the control formulations were equal to the concentrations of these used in the insulin formulations of *in vitro* study.

For studying the influence of excipients/absorption promoters used in the *in vitro* calcitonin formulations, Five control formulations were prepared, one was acetate buffer pH 3.9 and other four formulations contained the absorption promoters individually (sodium tauroglycocholate, dimethyl β-cyclodextrin, chymostatin and bacitracin) in acetate buffer pH 3.9. The concentrations of the absorption promoters in the control formulations were equal to the concentrations of these used in the calcitonin formulations for *in vitro* study.

5.5 *IN VITRO* PERMEATION EXPERIMENTS

Frogs were anesthetized by keeping in ice for 10 minutes and a ventral incision was made to expose the lung. Lungs were excised by severing the tracheoglottis after filling each lung with approximately 3 - 4 mL of ringer's solution. The planar sheet of lung was mounted on the diffusion chamber so as to expose 1.25 cm^2 surface area of the alveolar membrane bathed in frog ringer's solution. The receptor compartment containing 10 mL of frog ringer's solution was continuously gassed with the 95% carbogen, and the temperature was equilibrated to 22°C ± 2°C (45 min) and maintained throughout the diffusion studies. Tissues were continuously short-circuited with an automatic voltage clamp.

1.0 mL of the formulation of insulin/calcitonin was taken on the surface of the membrane and 0.5 mL of the sample was collected from the receptor compartment at every 30-min interval. After each withdrawal, an equal volume of buffer was added to receptor compartment. The samples were collected over a period of –30 to 300 min for insulin and samples were collected over a period of –30 to 240 min for calcitonin.

5.6 ASSAY METHOD

The amount of insulin and calcitonin permeated through the alveolar membrane mounted *in vitro* was estimated by Bicinchoninic acid (BCA) method [17]. This

assay is based on the color change resulting from the strong complex formed between Cu^+ and BCA. Cu^+ is produced by oxidation of cysteine, tyrosine, cystine and tryptophan residues, which is temperature independent. The reaction of peptide bonds with Cu^+ is temperature dependent. After adding the BCA reagent the solution was incubated at 60°C for about 30 min. This analytical method yielded protein drug concentration in the range of 0.2 μg - 300 μg. The amount of drug was directly read from the analyzer by measuring the absorbance at λ_{max} 561.5 ± 0.5 nm and the results (cumulative percent permeation) are shown in Fig 5.2 and Fig. 5.3.

5.7 DATA ANALYSIS

Each permeation study was repeated 6 time and mean values along with the ± SD were recorded. Linear regression analysis was done for finding the correlation between the cumulative AUC of the drug *in vivo* and the cumulative drug permeated *in vitro*. The *in vitro – in vivo* correlation factor (r^2) was calculated from the regressed line of the graph.

5.8 RESULTS AND DISCUSSION

In Table 5.1 and 5.2, the compositions of formulations used for the *in vitro* studies are recorded. Formulations In1-In9 and C1-C9 were prepared using different classes of absorption promoters like pH, penetration enhancers, protease inhibitors and combinations thereof. Fig. 5.2 shows the cumulative percent permeation of insulin through the alveolar membrane of the frog lung mounted in the diffusion chamber over a period of 300 min. Insulin formulation (In1) at pH 7.4 shows cumulative permeation of 7.4% ± 2.2% and the pH of the formulation become slightly acidic (pH 3.5: In2), then cumulative permeation of the drug increases to 24.4% ± 4.4%. The penetration enhancers oleic acid sodium salt (In3) and sodium tauroglycocholate (In4) 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 (In5) shows 52.4% ± 4.6%

permeation. The formulations with protease inhibitors like bestatin (In6), chymostatin (In7), and combination of bestatin with chymostatin (In8) causes increase in bioactivity 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 with insulin (In9) the cumulative permeation obtained was 81.6% ± 3.8%.

In Fig. 5.3 the cumulative percent permeation of calcitonin over a period of 240 min was indicated. In calcitonin formulation (C1) the cumulative permeation was 6.0% ± 2.1% at pH 6.0 and increases to 17.9% ± 2.6% at acidic pH 3.9 (C2). Sodium tauroglycocholate (C3) increases calcitonin permeation from 17.9% ± 2.6% to 23.8% ± 2.5% and dimethyl β-cyclodextrin (C4) to 26.5% ± 3.4% and incorporation of both sodium tauroglycocholate and cyclodextrin (C5) causes increase in drug permeation of 63.7% ± 4.0%. The calcitonin formulations containing chymostatin (C6), bacitracin (C7), and combination of chymostatin with bacitracin (C8) 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 (C9), the cumulative permeation obtained was 86.7% ± 4.4%.

From chapter 4, the percentage blood glucose reduction measured over a period of 0-300 min after administration of insulin formulations and the percentage blood calcium reduction measured over a period of 0-240 min after administration of calcitonin by intratracheal instillation were shown in Table 5.3 and Table 5.4. 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 (ie, minimum 20% percent blood glucose reduction and maximum 80% percent blood glucose reduction) depending on absorption promoters used. Like insulin, different doses of calcitonin were used, otherwise some may not give any response and some may become excess dose. The pharmacodynamics parameter AUC was calculated from the percent blood glucose reduction/percent blood calcium reduction values of Table 5.3 and Table 5.4.

$$AUC = C_1+C_2 \times [(t_2-t_1)\div 2] /100 \quad \text{.....(e.q. 5.1)}$$

C_1 – Percent blood glucose reduction/percent blood calcium reduction at time t_1

C_2 – Percent blood glucose reduction/percent blood calcium reduction at time t_2

t_1, and t_2 are subsequent sampling time interval of 30 minutes from 0-300 min for insulin and 0-240 min for calcitonin.

From the AUC values obtained for each time interval the cumulative AUC was calculated.

In Fig. 5.4 and 5.5 the cumulative percent permeation of insulin formulations (for a period of 0-300min) and calcitonin formulations (for a period of 0-240min) were plotted respectively against their cumulative AUC obtained *in vivo*. The correlation factors obtained were recorded in Table 5.5.

The *in vitro* control studies show that the interference of the excipients, sodium tauroglycocholate, oleic acid sodium salt, dimethyl β-cyclodextrin, bestatin, chymostatin and bacitracin used was found to be below 1.0 %. The concentration of each of the absorption promoters in the *in vivo* control formulations was equal to the maximum concentration of these used in intratracheal formulations.

We studied the transport of insulin and calcitonin across alveolar membrane of frog lung mounted in the diffusion chamber, to determine whether this would be a suitable system for demonstrating factors controlling absorption of insulin or calcitonin molecules across pulmonary epithelium. Although structural subdivision of amphibian lungs is much less extensive than the alveolar organization seen in the mammalian organ, cellular composition and overall dimensions of the air blood barrier are very similar. In addition both possess surfactant layer, which covers the alveolar epithelial cells and prevents collapse of the lung during end respiration. The only difference between mammalian and amphibian lung was, a distinct cell (type II alveolar epithelium cells) is responsible for surfactant production, while the alveolar lining where gas exchange occurs is comprised largely of Type I (squamous) epithelial cells in mammals but a single epithelial cell type is responsible for both the functions in amphibian.

The *in vitro* study results suggest that the absorption promoters used plays a significant role in the drug permeation. The penetration enhancers increases drug permeation by various reported mechanisms like binding with Ca^{2+} or displacing the Ca^{2+} from tight junction to increase paracellular permeability or reverse micellar binding with subsequent formation of hydrophilic channels.[26-29] When more than a single enhancer used in the formulation they act through different mechanisms and enhances the drug permeation. The slightly acidic pH provides maximum stability of both insulin and calcitonin. The protease inhibitor causes inhibition effect on various proteases (aminopeptidases, dipeptidases, carboxypeptidases, dipeptitidyl peptidases, and omega peptidases) in the alveolar membrane of the lung.[30] Like penetration enhancers, more than one protease inhibitors used, they inhibit variety of proteolytic enzymes and causes increase in permeation. When combination of both the penetration enhancers and protease inhibitors were used in slightly acidic pH, then cumulative percent drug permeation obtained was higher due to partial inhibition of the proteolytic enzymes of lungs, and dilation of tight junction of alveolar membrane.

The *in vivo – in vitro correlation factor* obtained for all the formulations was 0.99 (for both insulin and calcitonin). This 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.

****

COMPOSITION OF INSULIN FORMULATIONS FOR *IN VITRO* STUDY

Insulin formulations[§]	Composition		
	pH	Penetration enhancer	Protease inhibitor
In1	7.4	--	--
In2	3.5	--	--
In3	3.5	Oss 0.5%	--
In4	3.5	Stg 0.3%	--
In5	3.5	Oss0.2% + Stg 0.1 %	--
In6	3.5		Bes 0.03%
In7	3.5		Chy 0.05%
In8	3.5		Bes 0.02% + Chy 0.04%
In9	3.5	Oss0.2% + Stg 0.1 %	Bes 0.02% + Chy 0.04%

[§] Concentration of insulin was 3.0/mg in all the formulations; Oss, oleic acid sodium salt; Stg, sodium tauroglycocholate; Bes, bestatin; Chy, chymostatin.

TABLE 5.1

COMPOSITION OF CALCITONIN FORMULATIONS FOR *IN VITRO* STUDY

Calcitonin formulations[¶]	Composition		
	PH	Penetration enhancer	Protease inhibitor
C1	6.0	--	--
C2	3.9	--	--
C3	3.9	Stg 0.5%	--
C4	3.9	β-cd 1.0%	--
C5	3.9	Stg 0.2 % + β-cd 0.3%	--
C6	3.9		Chy 0.05%
C7	3.9		Bac 0.03%
C8	3.9		Chy 0.04% + Bac 0.02%
C9	3.9	Stg 0.2 % + β-cd 0.3%	Chy 0.04% + Bac 0.02%

[¶] Concentration of calcitonin was 3.0/mg in all the formulations; Stg, sodium tauroglycocholate; β-cd, dimethyl β-cyclodextrin; Chy, chymostatin; Bac, bacitracin.

TABLE 5.2

BLOOD GLUCOSE REDUCTION AFTER INTRATRACHEAL ADMINISTRATION OF INSULIN IN RATS In1 – In9 ARE FORMULATIONS OF INSULIN CONTAINING DIFFERENT ABSORPTION PROMOTERS.

Formula-tion	Dose (IU/Kg)	Blood glucose reduction (%)									
		30min	60min	90min	120min	150min	180min	210min	240min	270min	300min
In1	7.0	40.3	46.6	76.1	59.4	35.5	20.3	12.7	13.1	10.9	4.6
In2	3.0	63.9	63.1	60.9	79.8	46.4	30.7	37.8	27.9	25.0	10.4
In3	2.5	64.1	78.6	70.0	74.6	53.5	54.6	29.5	42.4	23.0	6.5
In4	1.5	51.4	56.3	59	60.1	45.8	35.4	25.1	10.7	9.7	5.9
In5	1.0	43.9	50.3	53.9	56.3	41.4	29.9	19.9	10.4	7.1	4.9
In6	1.5	51.3	55.4	58.0	60.9	45.7	34.1	24.9	11.3	10.9	7.1
In7	1.5	47.8	55.4	60.1	62.3	45.2	34.3	29.1	18.4	13.3	9.7
In8	1.0	48.6	52.3	56.9	57.9	43.6	33.2	23.1	10.6	8.9	5.8
In9	1.0	63.7	68.2	71.2	73.9	67.3	53.9	40.3	31.9	19.3	13.0

TABLE 5.3

BLOOD CALCIUM REDUCTION AFTER INTRATRACHEAL ADMINISTRATION OF CALCITONIN IN RATS. C1 – C9 ARE FORMULATIONS OF CALCITONIN CONTAINING DIFFERENT ABSORPTION PROMOTERS.

Formu	Dose	Blood calcium reduction (%)							
lation	(IU/Kg)	30min	60min	90min	120min	150min	180min	210min	240min
C1	12.0	18.0	27.2	37.2	36.0	25.2	22.8	17.6	13.0
C2	3.0	13.9	17.0	29.3	24.1	20.7	16.9	12.0	6.6
C3	3.0	16.8	26.5	35.7	34.0	24.6	21.9	16.8	13.6
C4	3.0	17.9	29.1	39.0	38.0	27.4	24.1	18.2	15.6
C5	1.0	14.0	23.9	32.4	30.8	22.0	19.5	14.8	12.4
C6	3.0	19.3	30.0	41.8	39.9	28.0	25.1	19.4	15.6
C7	3.0	20.2	32.5	43.4	41.6	29.0	26.2	20.2	16.6
C8	1.0	16.9	25.5	34.0	33.5	23.7	22.0	15.8	12.9
C9	1.0	20.3	31.7	43.0	41.5	29.6	26.7	20.0	16.5

TABLE 5.4

IN VITRO-IN VIVO CORRELATION OF INSULIN AND CALCITONIN FORMULATIONS

Insulin	Insulin Formulations	In1	In2	In3	In4	In5	In6	In7	In8	In9
	In vitro-in vivo correlation factor (r^2)	0.991	0.990	0.989	0.993	0.994	0.986	0.990	0.994	0.992
Calcitonin	Calcitonin Formulations	C1	C2	C3	C4	C5	C6	C7	C8	C9
	In vitro-in vivo correlation factor (r^2)	0.993	0.998	0.993	0.997	0.990	0.996	0.994	0.992	0.997

TABLE 5.5

IN VITRO PERMEATION APPARATUS

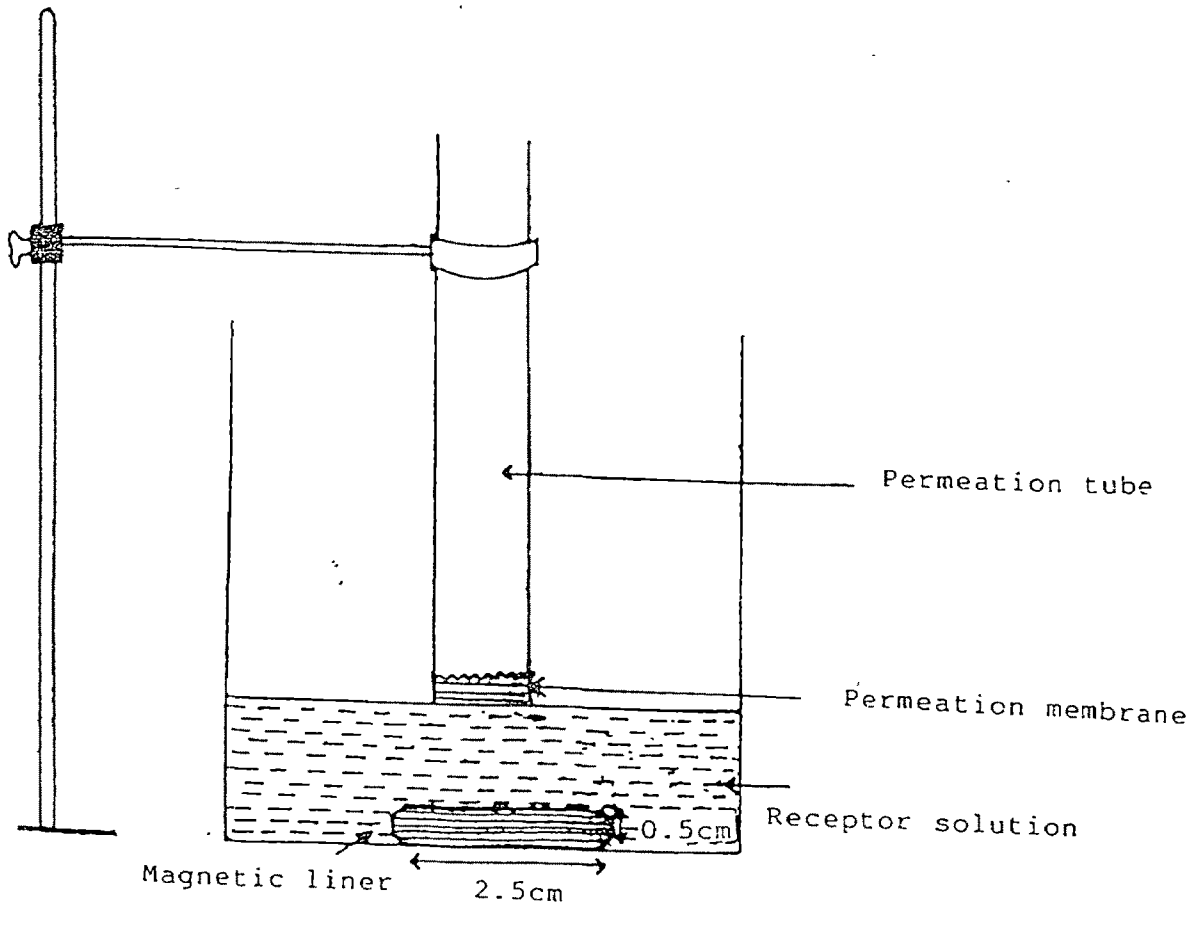


FIGURE 5.1

IN VITRO CUMULATIVE PERCENT PERMEATION OF INSULIN THROUGH THE ALVEOLAR MEMBRANE OF THE FROG LUNG. VALUES REPRESENTS MEAN ± SD (n=6). In1 – In9 ARE FORMULATIONS OF INSULIN CONTAINING DIFFERENT ABSORPTION PROMOTERS.

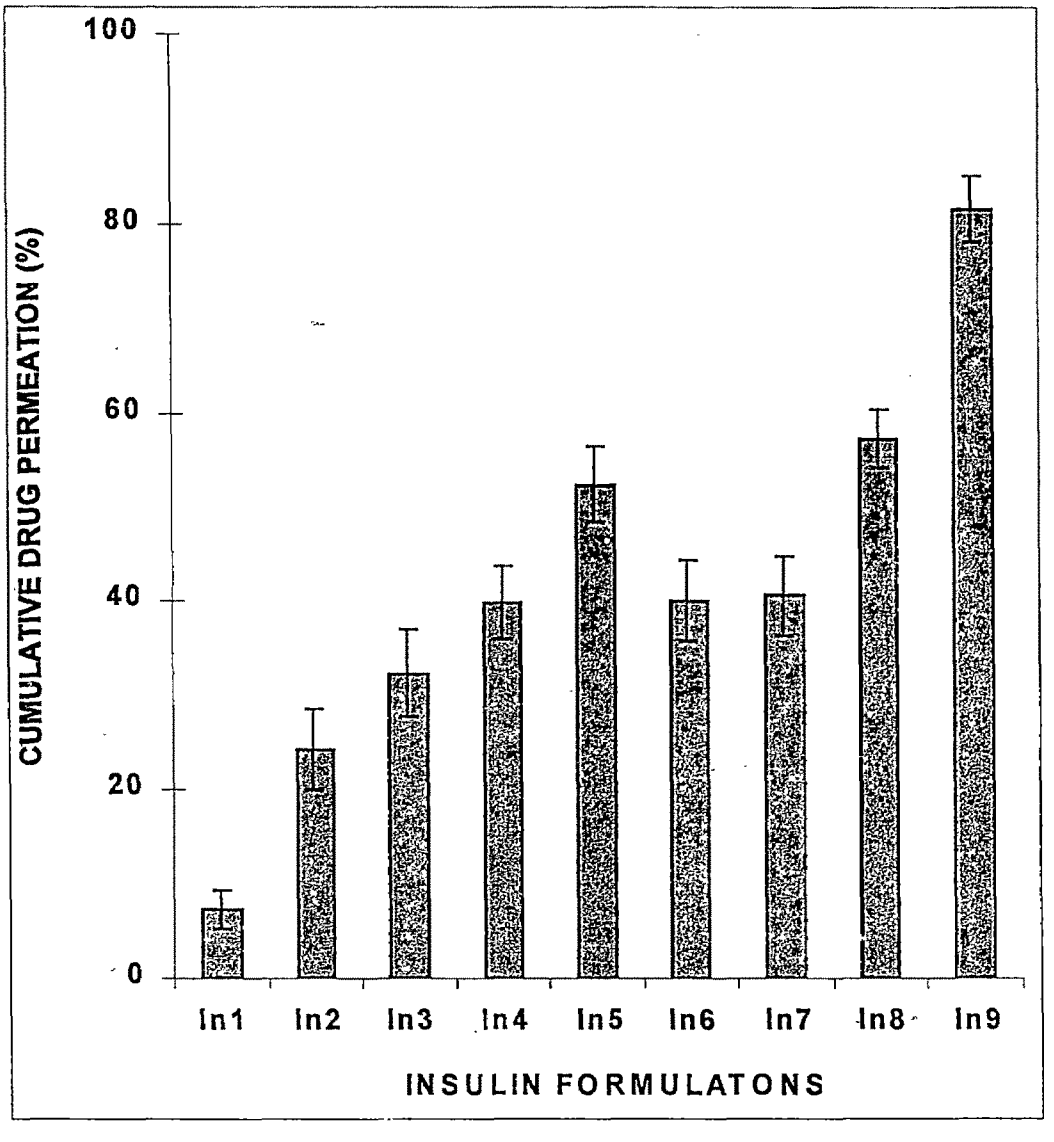


FIGURE 5.2

IN VITRO CUMULATIVE PERCENT PERMEATION OF CALCITONIN THROUGH THE ALVEOLAR MEMBRANE OF THE FROG LUNG. VALUES REPRESENTS MEAN ± SD (n=6). C1 – C9 ARE FORMULATIONS OF CALCITONIN CONTAINING DIFFERENT ABSORPTION PROMOTERS.

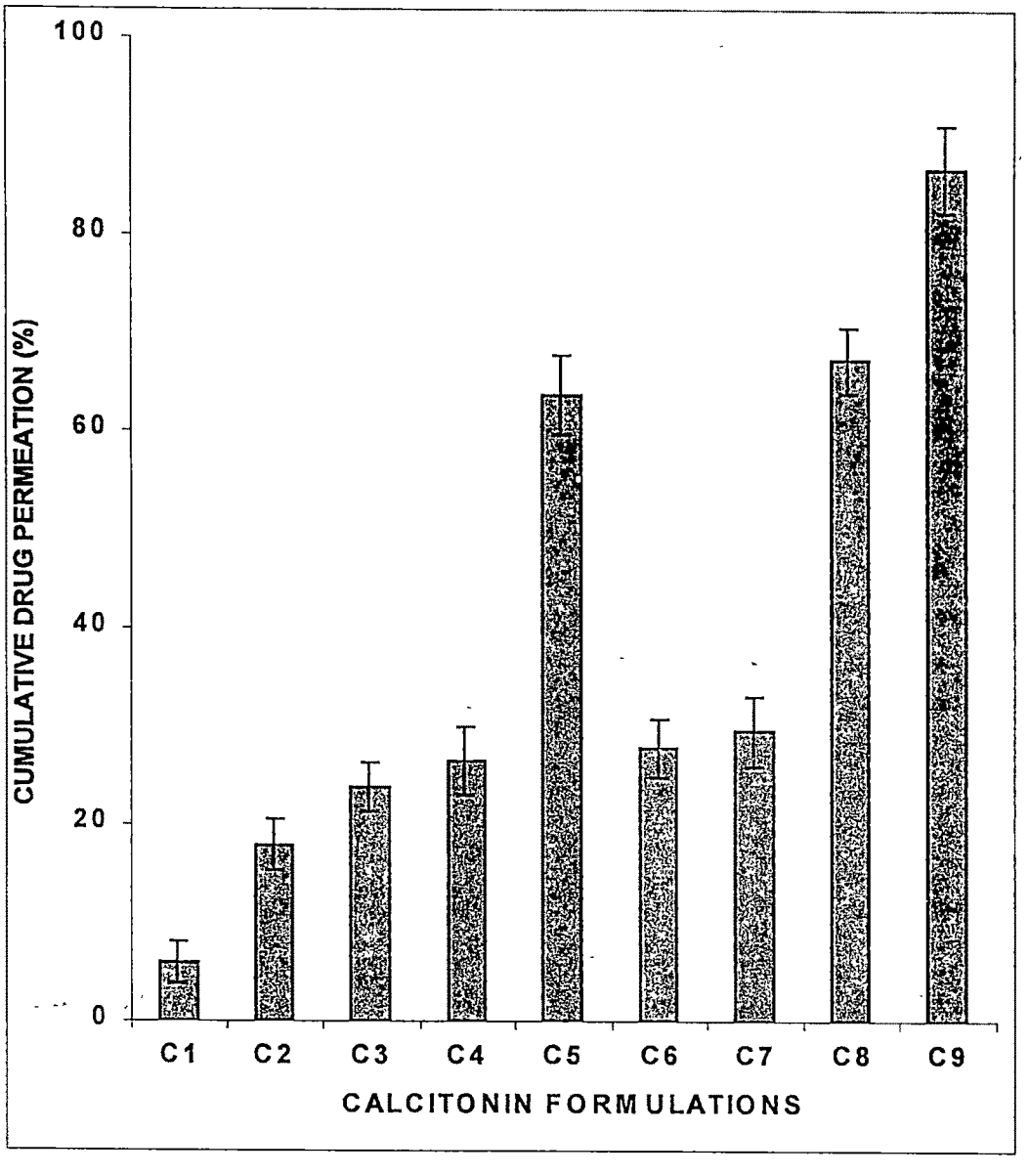
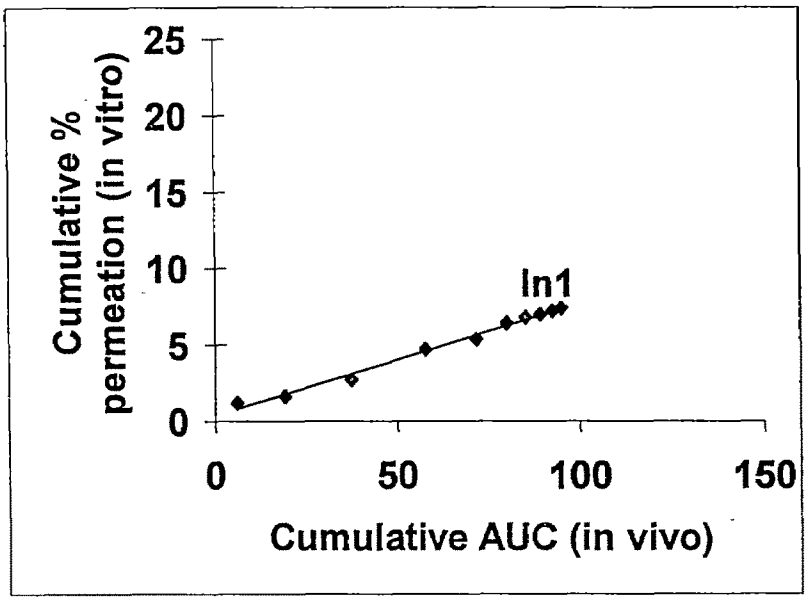


FIGURE 5.3

PROFILES OF CORRELATION BETWEEN IN VITRO INSULIN PERMEATION IN FROG ALVEOLAR MEMBRANE AND IN VIVO ABSORPTION IN RAT LUNG. In1 – In9 ARE FORMULATIONS OF INSULIN CONTAINING DIFFERENT ABSORPTION PROMOTERS



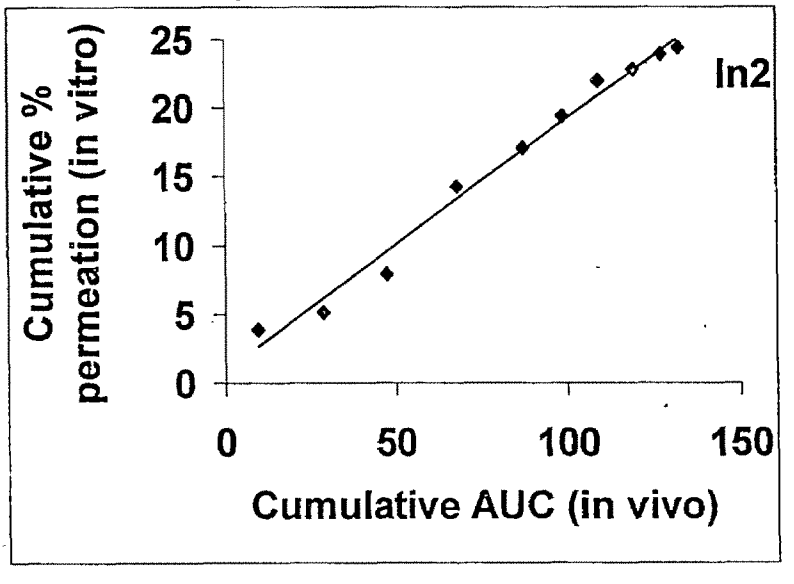
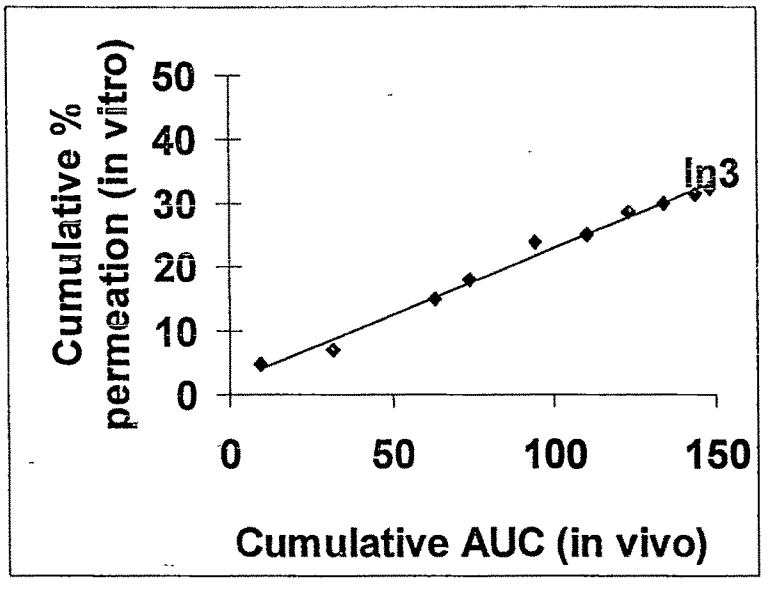


FIGURE 5.4


Cumulative % permeation (in vitro)
50
40
30
20
10
0
0
50
100
150
Cumulative AUC (in vivo)
In3

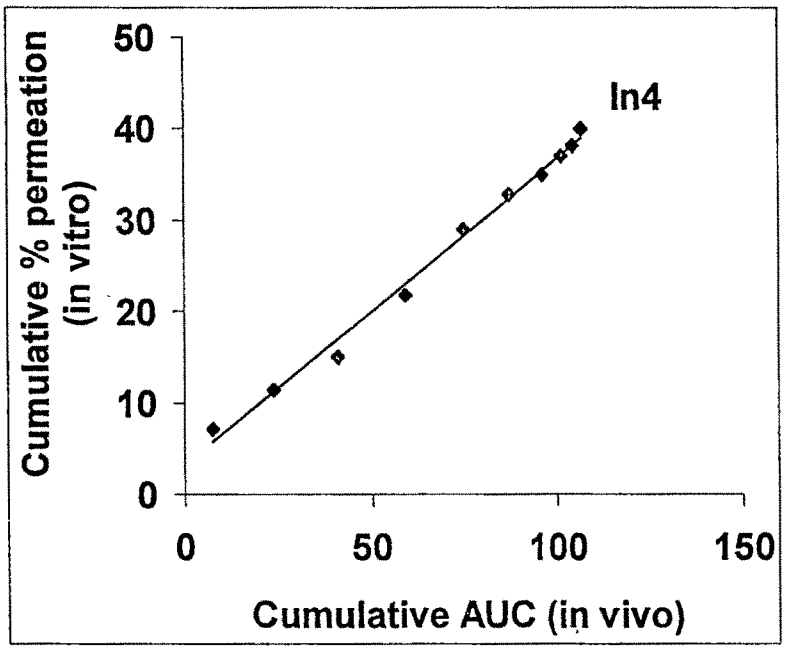
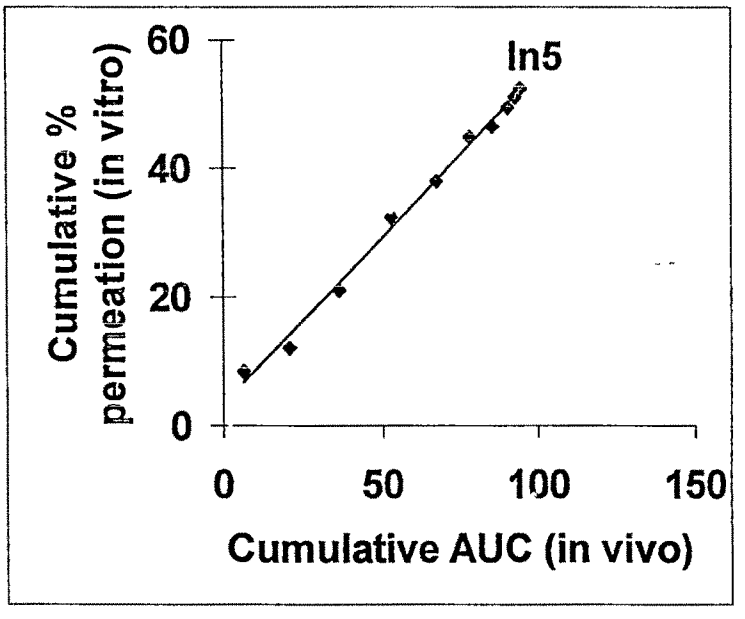

Cumulative % permeation (in vitro)
50
40
30
20
10
0
0
50
100
150
Cumulative AUC (in vivo)
In4

FIGURE 5.4


In5
Cumulative % permeation (in vitro)
60
40
20
0
0
50
100
150
Cumulative AUC (in vivo)

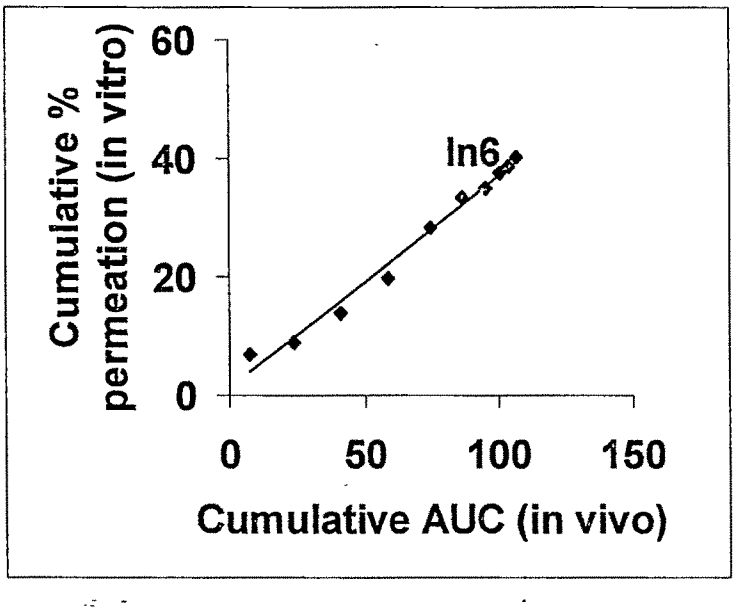
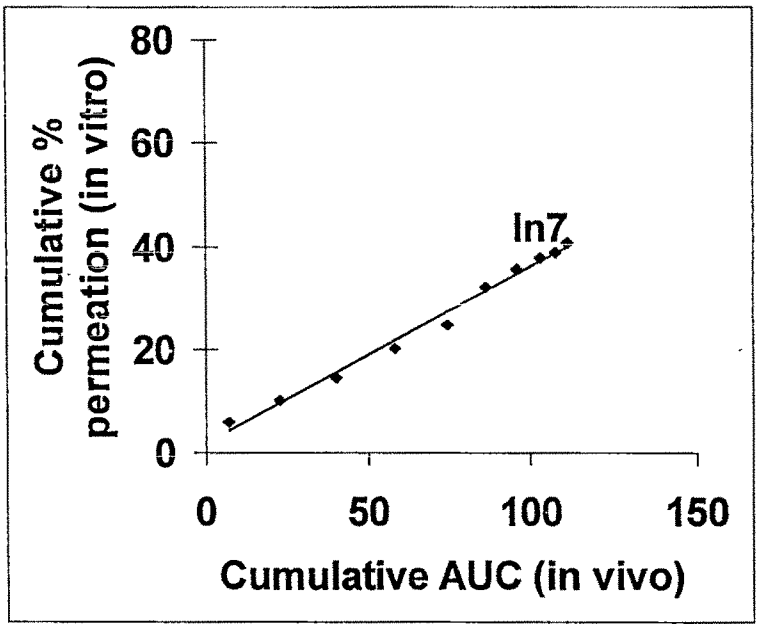

In6
Cumulative % permeation (in vitro)
60
40
20
0
0
50
100
150
Cumulative AUC (in vivo)

FIGURE 5.4


Cumulative % permeation (in vitro)
80
60
40
20
0
In7
0
50
100
150
Cumulative AUC (in vivo)

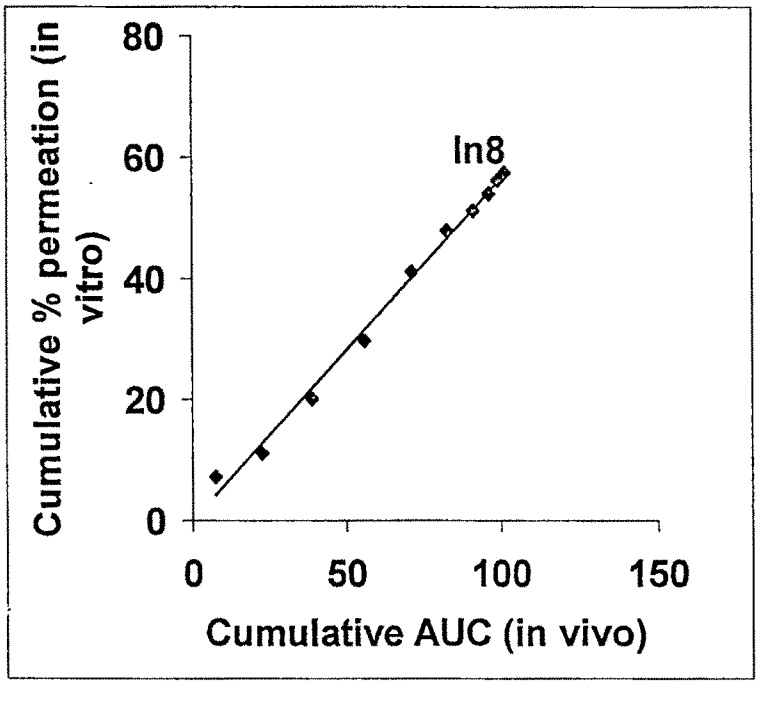

Cumulative % permeation (in vitro)
80
60
40
20
0
In8
0
50
100
150
Cumulative AUC (in vivo)

FIGURE 5.4

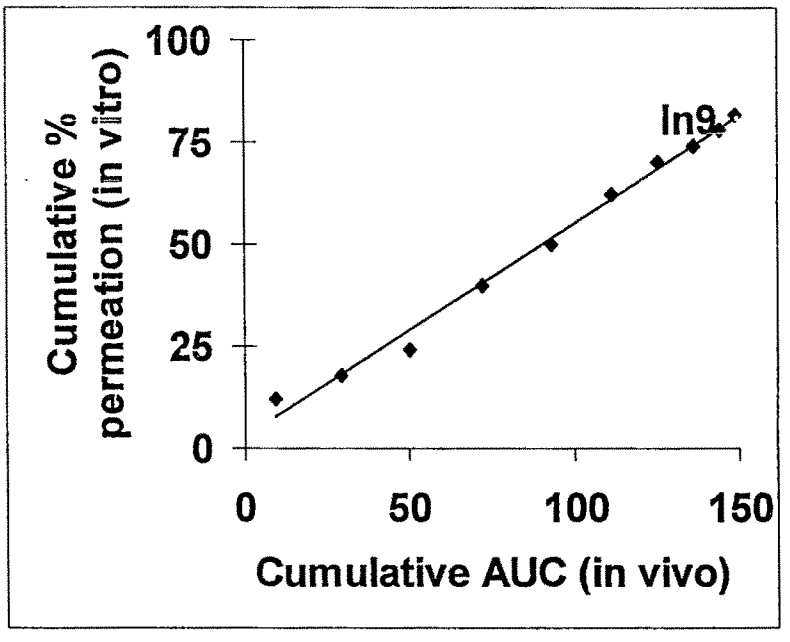
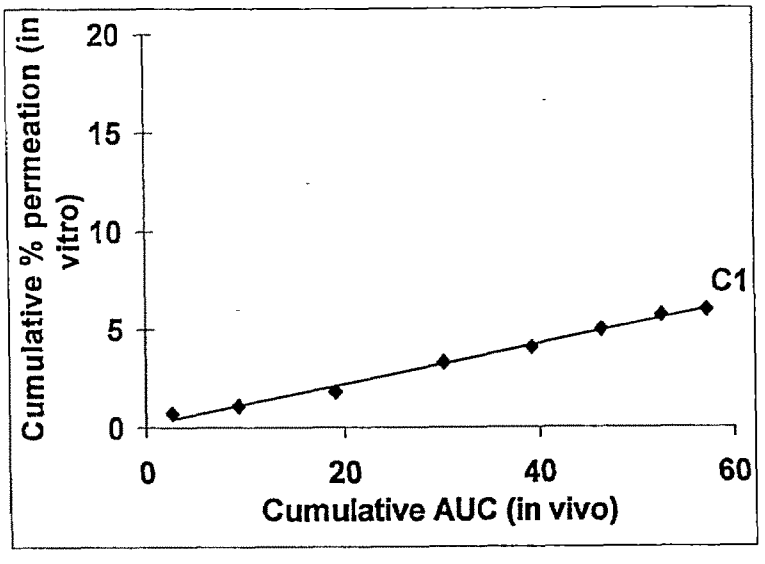

Cumulative % permeation (in vitro)
100
75
50
25
0
0
50
100
150
Cumulative AUC (in vivo)
In9

FIGURE 5.4

PROFILES OF CORRELATION BETWEEN IN VITRO INSULIN PERMEATION IN FROG ALVEOLAR MEMBRANE AND IN VIVO ABSORPTION IN RAT LUNG. C1 – C9 ARE FORMULATIONS OF CALCITONIN CONTAINING DIFFERENT ABSORPTION PROMOTERS



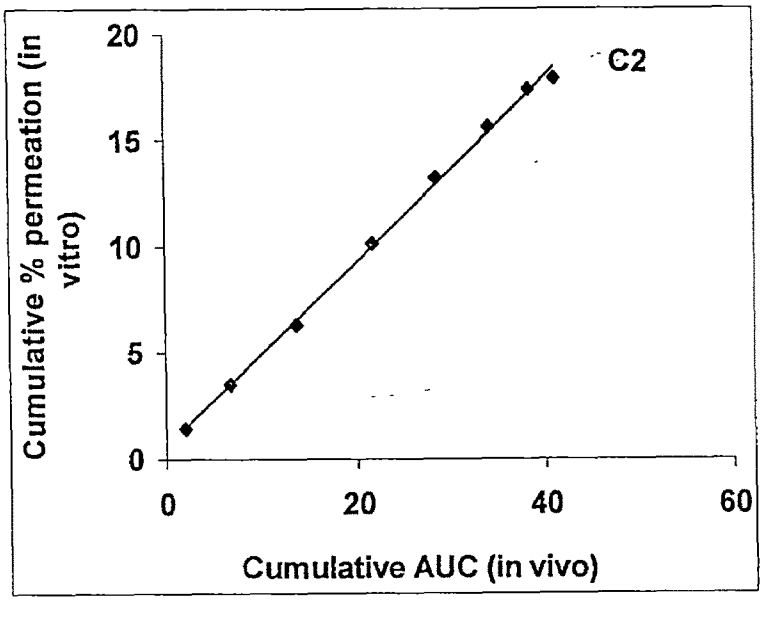
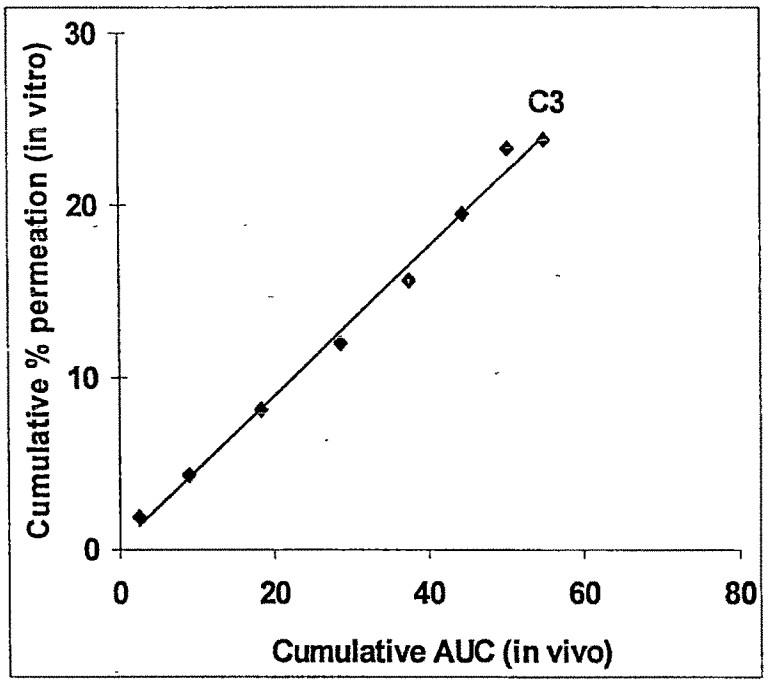


FIGURE 5.5


C3
Cumulative % permeation (in vitro)
30
20
10
0
0
20
40
60
80
Cumulative AUC (in vivo)

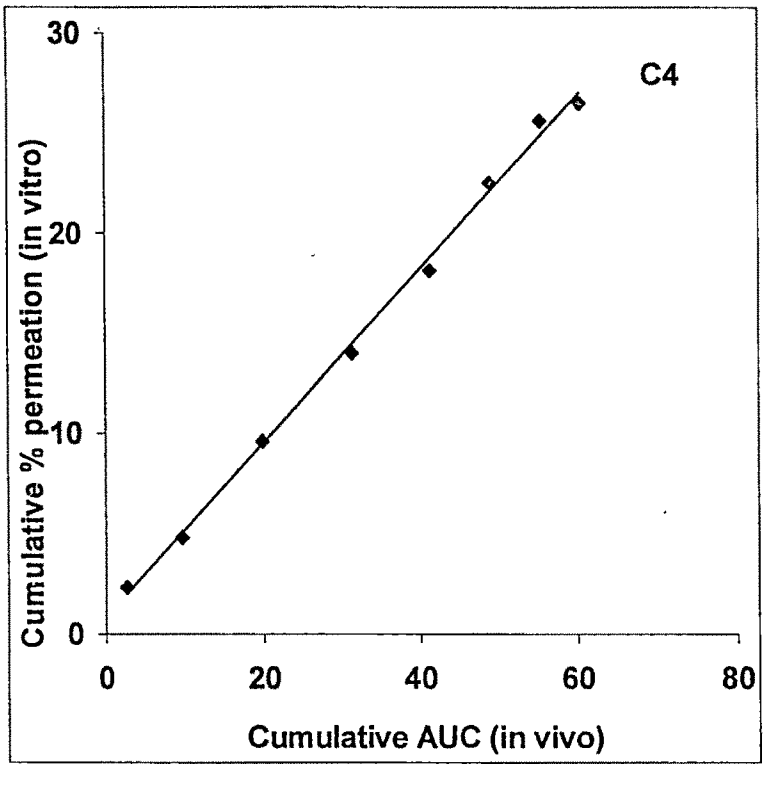
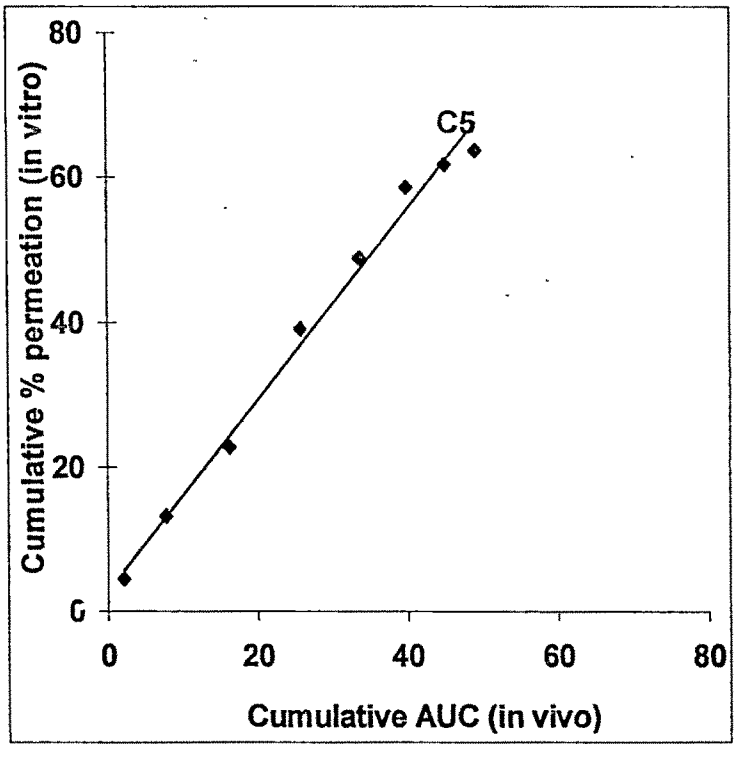

C4
Cumulative % permeation (in vitro)
30
20
10
0
0
20
40
60
80
Cumulative AUC (in vivo)

FIGURE 5.5


80
60
40
20
0
Cumulative % permeation (in vitro)
C5
0
20
40
60
80
Cumulative AUC (in vivo)

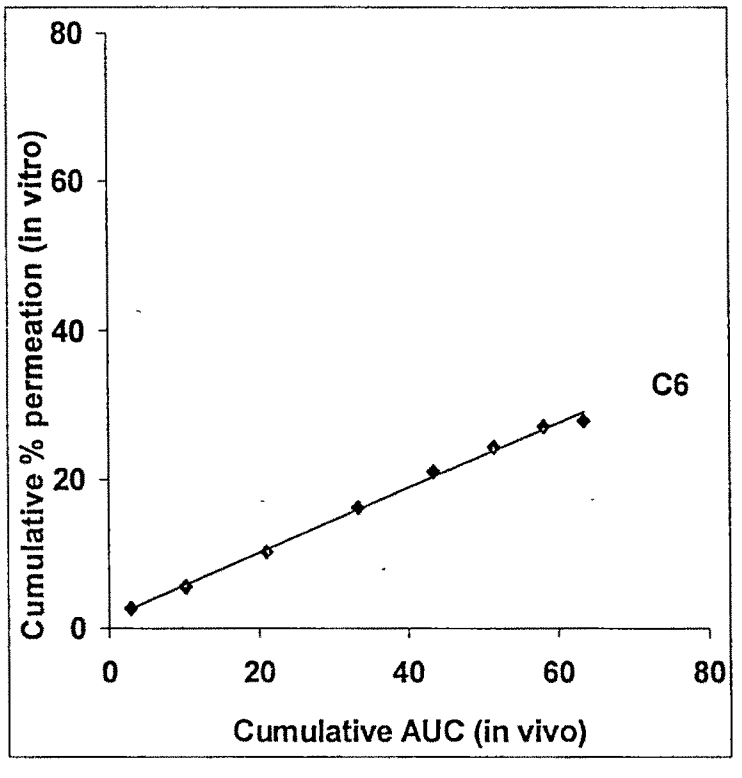
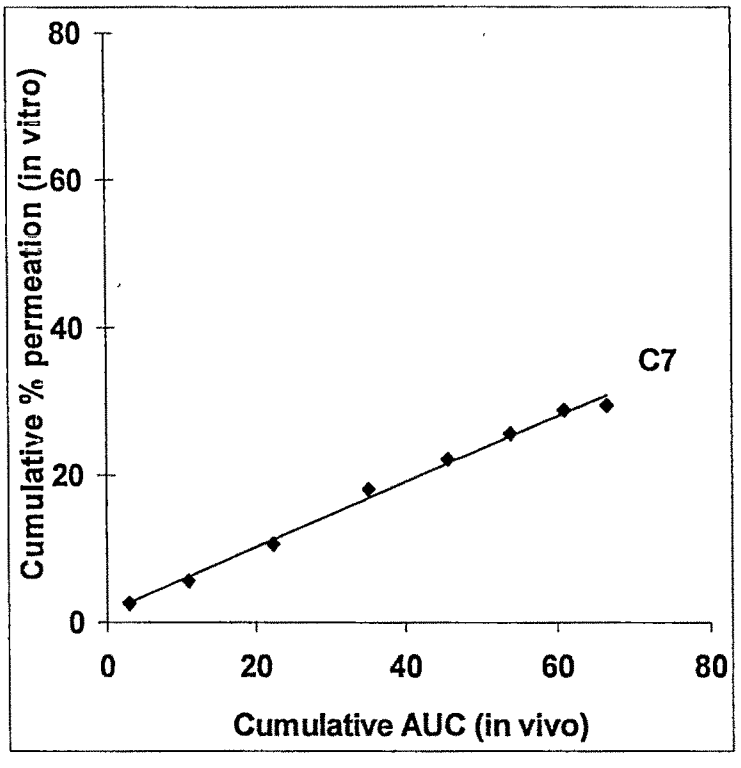

80
60
40
20
0
Cumulative % permeation (in vitro)
C6
0
20
40
60
80
Cumulative AUC (in vivo)

FIGURE 5.5


Cumulative % permeation (in vitro)
80
60
40
20
0
C7
0
20
40
60
80
Cumulative AUC (in vivo)

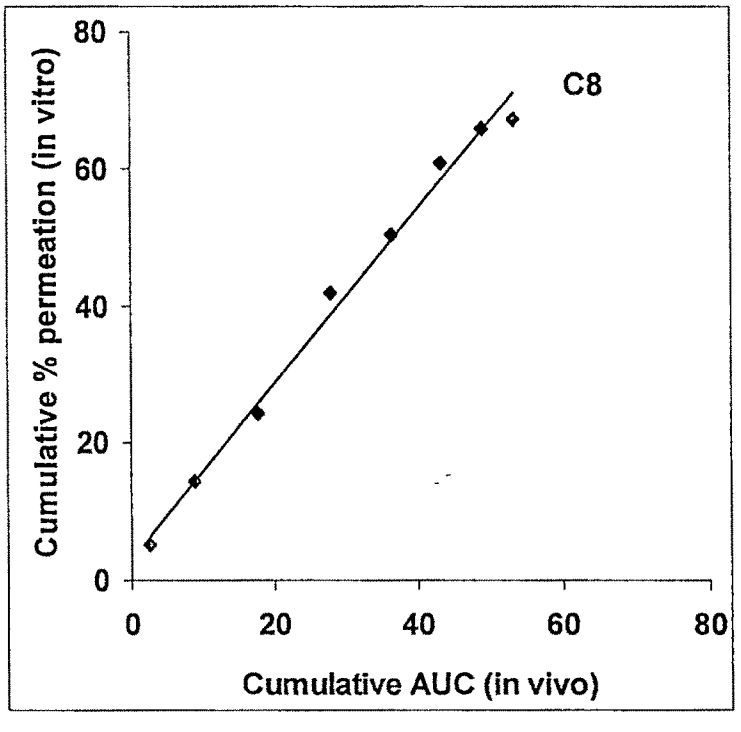

Cumulative % permeation (in vitro)
80
60
40
20
0
C8
0
20
40
60
80
Cumulative AUC (in vivo)

FIGURE 5.5

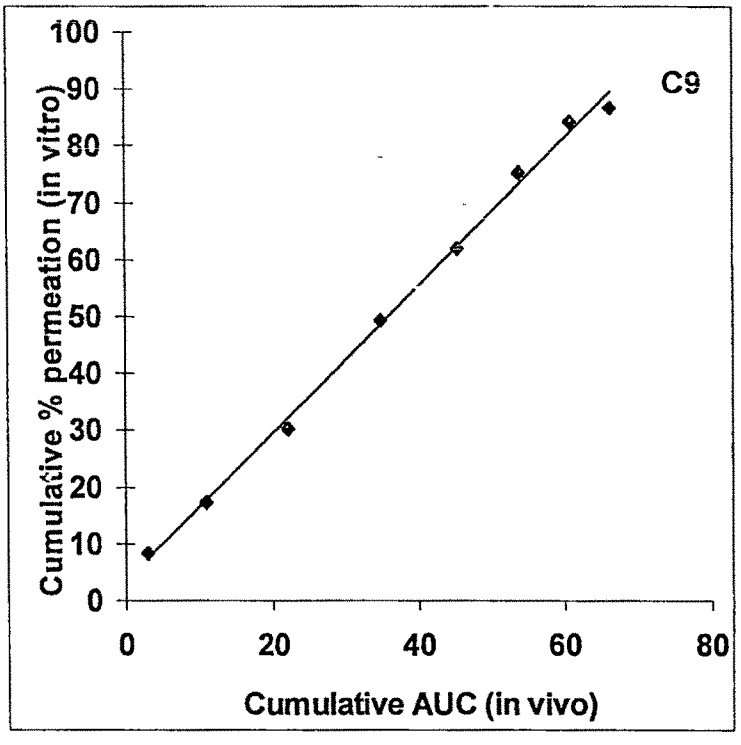

C9
Cumulative % permeation (in vitro)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
Cumulative AUC (in vivo)

FIGURE 5.5

5.10 REFERENCES

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