CHAPTER 8

IN VIVO STUDIES

8.1 INTRODUCTION

Aerosol therapy is an effective means of delivering relatively small doses of an active ingredient (for localized action) directly to the respiratory system. Localization of drug maximizes the therapeutic effect while minimizing unwanted systemic activity or toxicity. The use of liposomes in pulmonary delivery was first investigated as a potential treatment for respiratory distress syndrome (Ivy et al, 1976). However, subsequent studies have indicated that liposomes have an inherent capacity to act as a drug carrier system for localized pulmonary drug therapy (McCullough et al, 1979; Juliano et al, 1980; Woolfrey et al, 1988).

Most investigation of liposomal drug delivery has relied on parental route of administration to achieve targeted delivery to the lung, however the direct administration of liposomes into the airways has the advantage of circumventing systemic dilution and removal by other tissues and organs (Shek et al, 1990). In order to compare preparations on an equivalent basis during animal studies, it was necessary to ensure that in each case 100 percent of the total dose was delivered directly to the lung (Rebuck et al, 1984). The lung tissue very well takes liposomes, after intratracheal administration givien as bolus dose of the preparations at the bifurcation of the trachea will meet this requirement (Shek et al, 1990).

In vitro diffusion studies are dependent on the instrument's hydrodynamic condition and the diffusion medium. It cannot predict physiological variables such as phagocytosis and mucociliary clearance. The in vivo parameters that help in assessing the rate and extent of absorption, AUC, C_{max} and T_{max} may not be sufficient to evaluate the pharmacokinetic performance, particularly the diffusion rate of controlled release liposomal formulations. However, when in vivo and in vitro data are combined it would add another useful dimension for the evaluation of a product's performance (Mojaverian et al, 1997).

8.2 INTRATRACHEAL INSTILLATION

The intratracheal instillation of INH & RFP LDPI formulations were carried out by well adapted method (Gonzalez-Roth et al, 1996 and Brown et al, 1983). Albino rats were selected for study because of the ease in their availability, handling and sampling. The study was carried out in accordance with the guidelines for the care and use of laboratory animals as adopted and promulgated by the animal ethics

committee. The rats were procured from Deep Biolabls, Ahmedabad (India). Rats selected for the study were weighing between 200-240 g and were housed in individual plastic cages in a constant temperature environment. Three rats of either sex were used in each group, at every time interval. With five sampling points and four formulations to compare a total of approximately 60 rats were used for the entire study. Animals were allowed free access to water and rat feed but were food fasted overnight prior to each experiment.

Intra peritoneal administration of pentobarbitone sodium (40mg/kg) was used to anesthetize rats. The trachea was exposed by blunt dissection of the sternohyoideus muscle and a small midline incision was made over the trachea. A small hole was made in trachea between the fifth and the sixth tracheal rings using as 20-guage needle. The trachea was cannulated with a PE 200 tubing (5 to 7cm) with the tip positioned approximately at the tracheal bifurcation. PE50 (10 to 15cm) tubing connected with a glass Hamilton syringe (waters, India) was inserted into the cannula and advanced to the bifurcation of the trachea. Dose equivalent to 10mg/ml of drug in the form of solutions/dispersions containing INH and RFP of non-encapsulated drug (PD) or liposome-encapsulated drug prepared by rehydration of LDPI with 250µl of distilled water was slowly instilled over a 1min period followed by 50µl normal saline. Animals to be sacrificed at 8, 12 and 24 hours after administration, had cannula secured with sutures and excised to leave a 1 cm protrusion to the access cannula. At the end of each time point (2, 4, 8, 12 and 24 hr) biological samples were collected and the animals are sacrificed.

8.3 BIOLOGICAL SAMPLING

Broncho alveolar lavage (BAL) was performed on anaesthetized and recannulated (as necessary) animals with 12ml PBS, pre-warmed to 37°C. For performing the lavage the Hamilton syringe connected to the PE50 tubing was replaced with a 3-way stopcock attached with two 20ml syringes. The tubing was reinserted through the cannula and advanced till the tracheal bifurcation. Fluid (PBS) was slowly injected into the lung via one syringe and then BAL withdrawn by gentle aspiration via the other (Shek et al, 1990). This BAL yielded between 7 to 11 ml liquid, which was centrifuged at 4.38 x 103 x g for 5 min. the supernatant was mixed with 10% Triton – x-100 in a ration of 9:1 respectively to dissolve the liposomes (Tabak et al, 1994), if required with the aid of gentle warming. It was then extracted and assayed by HPLC

method for INH (Chapter 3, Section 3.4.3.8) and RFP (Chapter 3, Section 3.4.4.8). The lungs and the portions of tracheal below the instillation site were excised and homogenized (LH) in 10ml PBS containing 1% Triton-X-100 and the diffused drug was analyzed. Serum sampling was done by direct cardiac puncturing.

8.4 TOXICITY STUDIES

The levels of SGOT, SGPT and ALP were measured before and on 24 hour serum samples.

8.5 DATA AND STATISTICAL ANALYSIS

For drug targeting or drug delivery in general it is important to be able to quantitatively assess the site-targeting effectiveness (Bodor and Buchwald, 2003). The various pharmacokinetic assessment parameters calculated for comparison are defined as below:

AUC^{24h}_{0} The area under the curve of drug concentration in lung homogenate and Broncho alveolar lavage (LH+BAL) or blood Vs time, over the period of study (24 hrs).

LH Lung homogenate

BAL Broncho alveolar lavage

Site targeting index (STI) = $\frac{AUC_{target}}{AUC_{blood}}$

Site-exposure enhancement factor (SEF) = $\frac{AUC_{target}^{delivery\ system}}{AUC_{target}^{drug\ alone}}$

Targeting enhancement factor (TEF) = $\frac{STI^{delivery\ system}}{STI^{drug\ alone}}$

Each testing was carried out three times and data from all experiments are expressed as mean ± SEM unless specified. The statistical analysis of the data was carried out using ANOVA and unpaired student's t-test. Differences greater than $p<0.05$ were considered significant.

8.6 RESULTS AND DISCUSSION

The in vivo evaluation was carried out by the estimation of the drug concentrations in BAL, LH and serum after intra-tracheal instillation of INH, RFP (INHPD; RFPPD) alone and re-hydrated LDPI formulations (INH70; INH71; RFP70 & RFP71). The dose of 1000 μg (INH) or 500μg (RFP) was intra-tracheally instilled. Amount of drug present in serum was considered as drug adsorbed, amount of drug in LH as the drug released from liposomes and available for the systemic absorption and the amount of drug present in the BAL as unreleased drug present within the liposome. Drug in BAL represents a reservoir of drug that eventually would be absorbed by the lung tissue. Mean lung drug concentration-time data following each individual treatment are recorded in Table 8.1 (INH) and Table 8.2 (RFP) and shown in Figures 8.1 (INH) and 8.2 (RFP). From the drug concentration in lung-time plot, pharmacokinetic parameters were calculated and are recorded in Table 8.3.

After plain and liposomal drug instillations, the drug concentrations recovered in BAL was observed to decrease; while the LH and serum drug levels increased with time (Figure 8.1 and Figure 8.2) for the studied time duration (24 hours). The drug mass balance between the amount of drug diffused and drug still present in liposomes (estimated in BAL) was not confirmed. It was assumed that the amount of drug that could not be accounted might have either metabolized or distributed or both.

When the concentration-time profiles were examined upto 12-24 h post-instillation, higher Tmax for liposome formulations (INH70, INH71, RFP70 and RFP71) was observed than of plain drug. There was an increase in AUC 24h0 for liposomal formulations compared to plain drug. The higher Tmax and AUC 24h0 observed for liposomal formulations confirm prolonged drug retention in lung than plain drug assuring a better therapeutic activity. The in vivo concentration-time profile showed the kinetics of LDPI formulations was altered by liposomal encapsulation depending on composition of bilayers. In another set of data analysis, the STI, SET and TEF were calculated and recorded in Table 8.3. The STI defined as the ratio between the area under the concentration-time curves (AUC) for the drug concentration at the targeted site (Lung) and at systemic site (Serum). STI were observed to be higher for LDPI formulations (INH70- 1.08, INH71-1.96, RFP70-1.73 and RFP71-1 54) than for plain drugs (INHPD-0.19 and RFPPD-0.94. STI gives an accurate measure on how effectively the drug is actually delivered to intended site of action. Hence, the free drug was rapidly absorbed from the lung to systemic circulation; while the liposomal

encapsulated drug remained in the lung for a prolonged period of time as reported by Juliano et al, 1980. Similarly, the higher SEF values of LDPI formulations compared to plain drug corroborated the effectiveness of the liposomal drug delivery. A good delivery system not only increases exposure of the active agent at the target site, but also decreases the corresponding systemic exposure. TEF, a measure of relative improvement in the STI, produced by administration of the delivery system compared with administration of the plain drug was evaluated and recorded in Table – 8.3. TEF is the most rigorous measure and used in quantifying the targeting produced by a delivery system. It compares not only concentrations, but also concentrations along a time period and it compares actual, active drug concentration, both at target and systemic sites. Higher TEF values were obtained for LDPI formulations than plain drug confirms that the liposomal drug delivery to lung not only increases exposure of the active agent at the target site, but also decreases the systemic exposure. Toxicities induced by free and liposomal drugs were performed by monitoring the levels of SFOT, SGPT, and ALP on control and on treated animals after 24 h and results is recorder in table – 8.4. From the results, it is evident that the encapsulation of INH and RFP in liposomes significantly reduced ($p>0.05$) the levels of SFOT, SGPT, and ALP compared to those observed for plain drugs due to reduction in systemic exposure.

Different portions of broncho-pulmonary tree possess different characteristics; it is possible that drug diffusion from liposomal DPI formulation is affected by its distribution within the lung and later altered by mucociliary transport and other mechanisms. Animal studies reported till date has utilized instillation of liquid formulations in order to obtain accurate dosimetry. Such results depend upon the spreading of the instilled dose within the lung. The distribution and absorption of inhaled aerosols in the lungs and airways are different from those of instilled liquid (Brown et al, 1993; Brain et al, 1976) and it is possible that diffusion kinetics of aerosol formulations in humans may differ considerably from diffusion kinetics of the instilled formulations in animals. Additionally, the size and aerodynamic properties of human airways may result in a significantly different distribution and rehydration of aerosolized liposomes to rodent animals, which may affect observed diffusion kinetics, duration, onset and intensity of effect.

Findings of these studies conclusively demonstrated superiority of LDPI formulations over plain drug by exhibiting maintenance of effective drug concentrations in the lung tissues for prolonged time, slow clearance from the lung and reduced toxicities.

Table 8.1: Drug level in biological samples following instillation of INH plain drug and liposomal DPI

Time (Hrs)	INH IN BIOLOGICAL SAMPLES (%) Mean* ± (SEM)								
	BRONCHO ALVEOLAR LAVAGE (μ/ml)*			LUNG HOMOGENATE (μ/ml)*			SERUM (μ/ml)*		
	INHPD	INH70	INH71	INHPD	INH70	INH71	INHPD	INH70	INH71
02	15.07 ± 5.22	66.12 ± 4.27	67.04 ± 3.97	65.60 ± 8 13	29.63 ± 8.72	42.77 ± 9.02	79.81 ± 5 46	12.23 ± 7.49	6.17 ± 8.89
04	3.48 ± 3.65	48.81 ± 4.03	53.77 ± 4.79	29.71± 9.03	37.60 ± 8 10	65.30 ± 10.6	54 64 ± 6.51	20.51 ± 8.77	12.34 ± 8.26
08	ND	31.00 ± 5 33	40.41 ± 4.34	ND	80 43 ± 10.8	74 70 ± 9.52	33.06 ± 10.5	30.59 ± 6.51	26.74 ± 6.59
12	ND	20.70 ± 6.64	28.74 ± 3.90	ND	45.87 ± 7.70	94.53 ± 8.08	17.63 ± 9.17	47.54 ± 9.03	35.30 ± 7.66
24	ND	9.37 ± 5.81	18 74 ± 5.57	ND	31.87 ± 12.1	58.60 ± 7 68	12.36 ± 8.26	30.64 ± 8.21	32.39 ± 9.46

* Mean ± SEM (n = 3)

INHPD – Plain drugs, INH70 – Neutral LDPI formulations and INH71 – Negatively charged LDPI formulations, ND – Concentration not detectable.

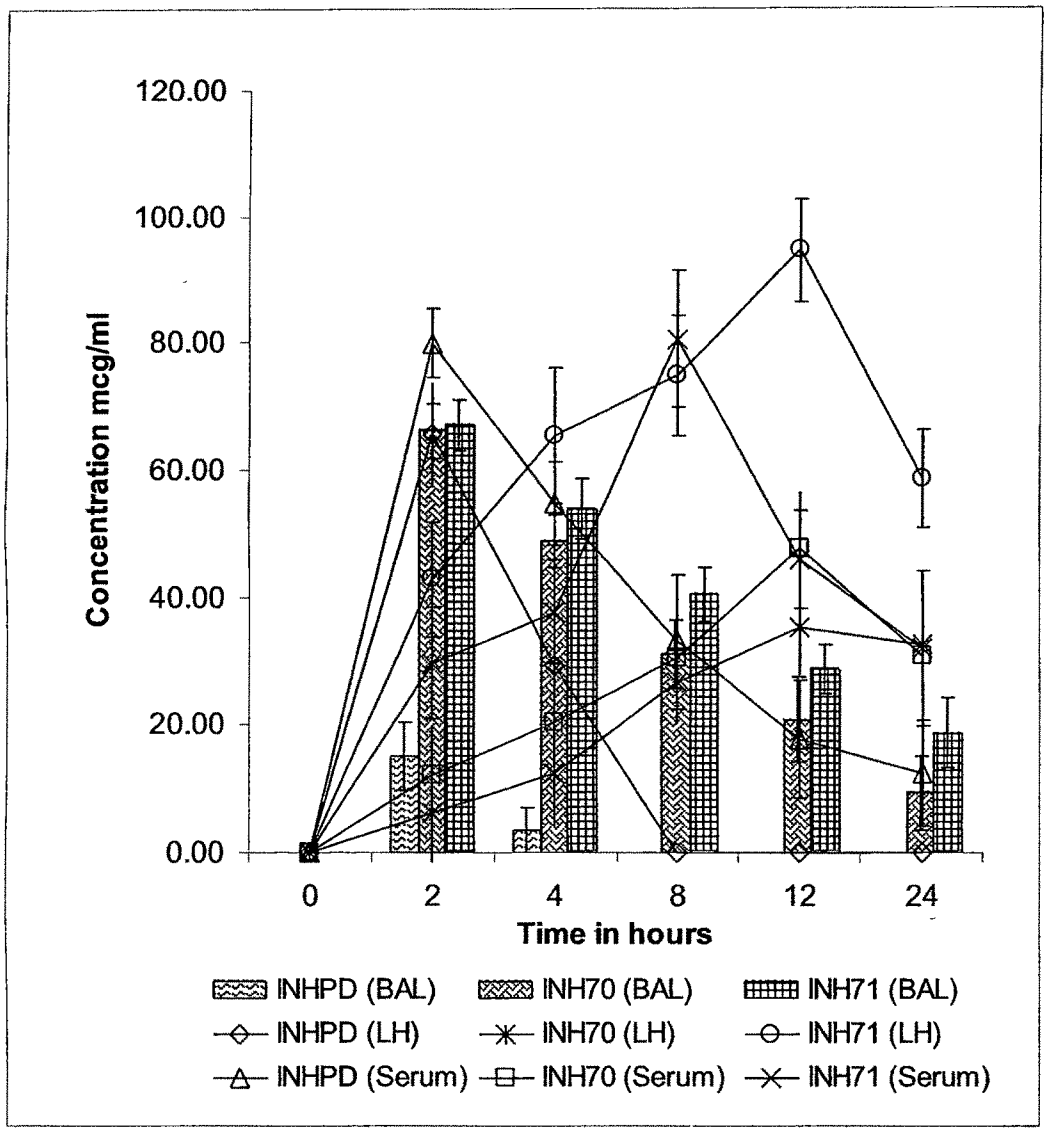

Figure 8.1: Drug level in biological samples following instillation of INH plain drug and liposomal DPI; INHPD – Plain drugs, INH70 – Neutral LDPI formulations and INH71 – Negatively charged LDPI formulations, BAL – Bronchoalveolar Lavage and LH - Lung Homogenate

Table 8.2: Drug level in biological samples following instillation of RFP plain drug and liposomal DPI

Time (Hrs)	INH IN BIOLOGICAL SAMPLES (%) Mean* ± (SEM)								
	BRONCHO ALVEOLAR LAVAGE (μ/ml)*			LUNG HOMOGENATE (μ/ml)*			SERUM (μ/ml)*		
	RFPPD	RFP70	RFP71	RFPPD	RFP70	RFP71	RFPPD	RFP70	RFP71
02	31.73 ± 2.98	63.96 ± 3.84	68.72 ± 3.53	54.90 ± 7.72	40.47 ± 10.5	28.53 ± 8.52	68.91 ± 6.01	8.90 ± 6.04	4.63 ± 5.50
04	20.19 ± 4.37	52.28 ± 4.45	57.60 ± 4.37	87.73 ± 9.70	60.83 ± 9.28	48.83 ± 10.5	61.77 ± 7.47	26.31 ± 6.20	17.83 ± 6.06
08	10.12 ± 3.54	36.84 ± 3.22	42.78 ± 3.89	107.07 ± 7.08	75.50 ± 7.34	65.13 ± 9.64	47.54 ± 9.21	46.30 ± 7.31	40.41 ± 7.49
12	5.27 ± 3.84	26.78 ± 4.09	36.93 ± 2.89	60.80 ± 10.68	91.27 ± 8.42	80.50 ± 6.92	34.54 ± 7.54	37.83 ± 6.44	46.30 ± 6.67
24	ND	18.47 ± 4.45	24.70 ± 3.82	48.93 ± 9.62	74.47 ± 10.3	73.93 ± 10.2	26.09 ± 6.69	23.06 ± 6.64	34.46 ± 7.91

* Mean ± SEM (n = 3)

RFPPD – Plain drugs, RFP70 – Neutral LDPI formulations and RFP71 – Negatively charged LDPI formulations, ND – Concentration not detectable.

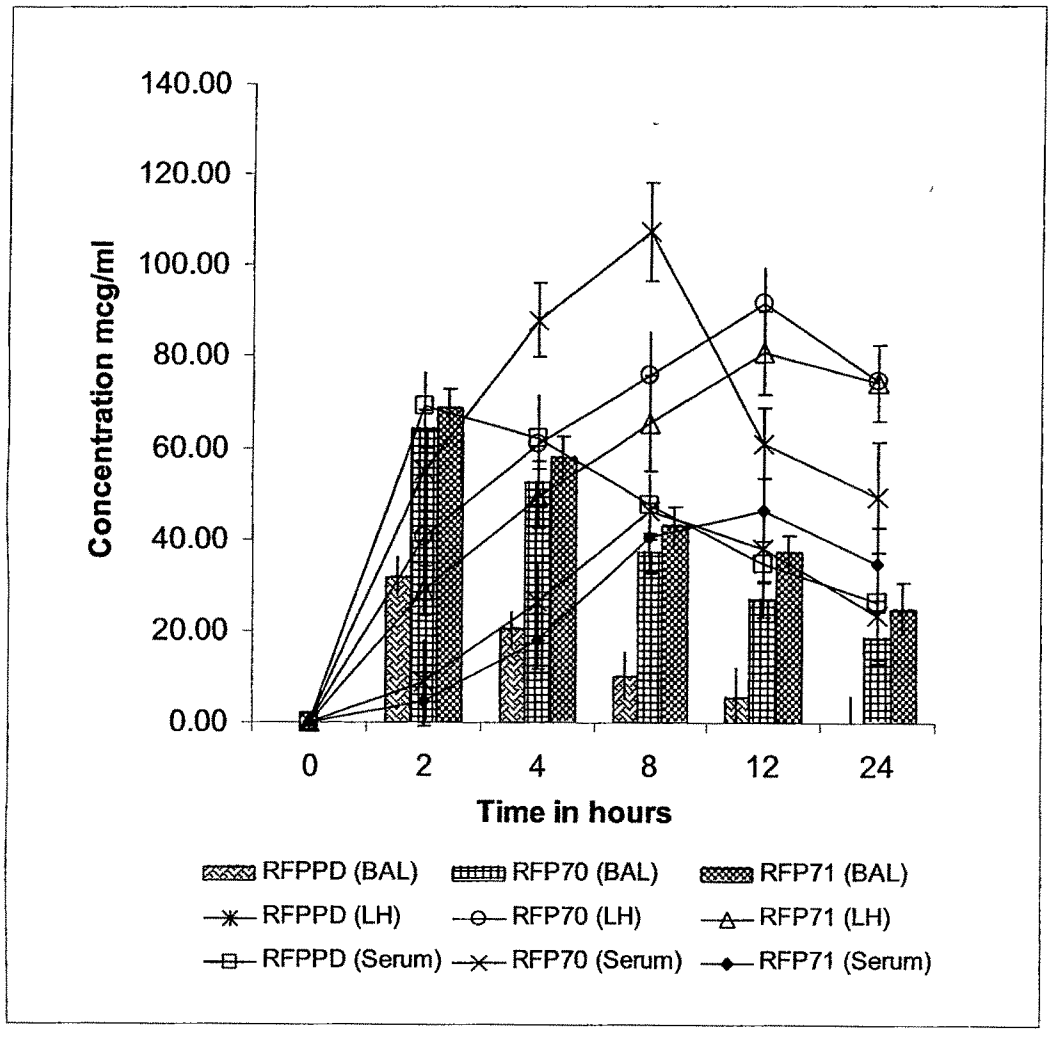

Figure 8.2 Drug level in biological samples following instillation of RFP plain drug and liposomal DPI; RFPPD – Plain drugs, RFP70 – Neutral LDPI formulations and RFP71 – Negatively charged LDPI formulations, BAL – Bronchoalveolar Lavage and LH - Lung Homogenate

Table 8.3: Comparative pharmacokinetic assessment parameters of LDPI formulations to plain drug

Formulation	AUC $^{24h}_{0}$ (µg . h/ml)		STI	SEF	TEF
	Lung	Serum			
Isoniazid					
INHPD	129.629	670.957	0.19	-	-
INH70	838.196	772.543	1.08	6.47	9.59
INH71	1243.75	633.057	1.96	5.62	10.17
Rifampicin					
RFPPD	893.954	946.171	0.94	-	-
RFP70	1249.78	722.914	1.73	1.39	1.39
RFP71	1238.37	801.543	1.54	1.83	1.64

INHPD & RFPPD – Plain drugs; INH70 & RFP70 – Neutral LDPI formulations and INH71 & RFP71 – Negatively charged LDPI formulations

TABLE 8.4: Comparative serum levels of SGOT, SGPT and ALP

Group	SGOT (U/ml)	SGPT (U/ml)	ALP (U/ml)
Control	72.80 ± 3.80	46.08 ± 4.21	67.7 ± 4.67
Free INH	117.24 ± 4.36	86.47 ± 3.67	103.41 ± 5.42
Liposomal INH70	68.25 ± 4.24	48.24 ± 4.51	74.23 ± 4.57
Liposomal INH71	66.24 ± 5.32	42.48 ± 3.64	68.12 ± 5.24
Free RFP	123.24 ± 4.54	92.41 ± 5.42	118.24 ± 5.42
Liposomal RFP70	86.24 ± 4.21	51.04 ± 4.61	78.24 ± 4.64
Liposomal RGP71	79.54 ± 5.61	54.67 ± 3.56	82.42 ± 3.56

8.7 REFERENCES

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