CHAPTER 7

IN VITRO DIFFUSION STUDIES

7.1 INTRODUCTION

Studies of drug release/diffusion from liposomal systems are directed towards issues that are relevant to the in vivo as well as to the non in vivo arenas (Margalit and Yerushalmi, 1996).

For liposomes in the in vivo arena, the drug release studies are expected to yield data and understanding that will lead to:

a) Minimizing the loss of encapsulated drug on route from the site of administration to the site of drug action.

b) The ability to match the rate of release (once the liposomes arrive at the target) to the requirements of the therapy.

The objectives of drug release studies that concern the non in vivo arena are

a) Physicochemical characterizations of the systems, including liposomes processed into aerosols or reconstituted from freeze dried powders.

b) Various aspects of system optimization such as the selection of liposome type, lipid composition and parameters of shelf life.

c) Criteria for quality assurance.

In order to derive relevant data from such studies, the experimental conditions should be set to fit the specific objectives especially with respect to the extent of liposomes and drug (each, separately) dilutions that the system is anticipated to undergo.

One of the alarming tasks facing a researcher in his / her successful development of a viable drug delivery system and experimental assessments of the drug diffusion profile of drugs from the delivery system is the proper design of an *in vitro* drug diffusion system that permits accurate evaluation and mechanistic analysis of the drug diffusion profiles. Physiological availability of the drug depends on both, the rate of diffusion from the liposomes and permeability through alveolar surface into the lung. The *in vitro* methods are valuable screening procedures for deducing physico-chemical parameters such as fluxes, partition coefficients and diffusion coefficients. Though according to Gemmell and Morison (1957), *in vitro* methods may be of limited predictive value but they are the means of assessing the ability of a vehicle or base to liberate medicament under the conditions of the test. A theoretical

disadvantage of such a technique is that the method does not exactly duplicate the behavior of living tissue in situ, particularly with respect to unpredictable blood supply and metabolism. However, since performing bio-studies on every manufactured batch is impractical and costly, formulators must rely on in-vitro testing to ensure batch-to-batch uniformity and consistency in bioavailability.

At present, *in vitro* test systems have not been developed which can accurately predict the rate of drug diffusion from liposomes *in vivo* (Fielding et al, 1992). Therefore an in vitro diffusion technique is proposed, validated and utilized for drug diffusion studies from optimized LDPI formulations.

7.2 Drug diffusion studies across artificial membrane

7.2.1 Experimental setup

7.2.1.1 Artificial membrane

Dialysis membrane (250-9U, molecular weight cut off: 12000 Dalton; Sigma, Hyderabad, India), 200μm in thickness, pH 5.8 to 8, breaking strength 2.75 kg f / cm and porosity 0.45 μm was used as a artificial membrane for preliminary *In vitro* studies because of simplicity, homogeneity and uniformity. This membrane was pretreated with ethanol (95%) followed by hydration in pH 7.4, phosphate buffer saline (PBS) and 1mM EDTA for 24 hr prior to permeation studies.

7.2.1.2 Design of diffusion cell

For the present study a vertical type of membrane diffusion system was developed. The system consists of a hollow glass tube open at both ends with inner diameter of 18 mm and 6 cm length. The membrane was tied to one end of the tube with a nylon string and this tube acts as a donor compartment. The tube was dipped flush on the surface of a 100 ml beaker containing diffusion medium that is the receptor compartment. The receptor solution was stirred at 100 rpm using a Teflon coated magnetic needle (length = 2.5 cm, d = 0.5 cm) and the surrounding water bath by the aid of a magnetic stirrer (Remi, India). The temperature of the bulk of the solution was maintained at 37 $\pm$ 0.5 $^{\circ}$ C. The donor compartment was stirred with a SS 316 triple blade stirrer (Remi, India) at 50 rpm.

7.2.1.3 Validation of diffusion cell

The hydrodynamic characteristics of the diffusion cell were established using the benzoic acid disc method (Mojaverian et al, 1997; USP 24).

7.2.1.4 Selection of diffusion medium

Receptor compartment containing 50 ml of PBS, with constant stirring simulated highly perfused pulmonary condition (Joshi et al, 1999). AMK is very soluble in PBS, sink condition was maintained with 50 ml PBS and zero order flux conditions were maintained. For RFP, being lipophilic drug, diffusion medium used in the receptor compartment was 50ml of mixture of PBS and methanol (6:4) for maintaining sink condition.

7.2.2 Method

Diffusion studies were carried out for plain drugs (INH and RFP) and LDPI formulations. The study was performed by dissolving INH in 1 ml of PBS, or for RFP a mixture of 2ml of PBS, and methanol (6:4). Similarly LDPI formulation of INH (equivalent to 10 doses; 1000 µg x 10) and LDPI formulation of RFP (equivalent to 10 doses; 500 µg x 10) was dispersed in 1 ml (INH) and 2ml (RFP) of distilled water. Formulations to be compared were separately transferred to the donor compartment and stirred at 50 rpm while the receptor compartment was stirred at 100 rpm. 1ml of the sample was withdrawn from the receptor compartment at definite time intervals and equivalent amount of fresh medium was replaced to the receptor compartment. The estimation of drug in the samples was determined using procedure described in chapter 3, Section 3.4.3.7 (INH) and Section 3.4.4.7 (RFP). All diffusion studies and sample analysis were carried out six times and mean values along with standard error of mean are recorded in Table 7.1.

7.3 In vitro alveolar macrophages uptake studies:

Advances in the cell culture technology have provided an additional tool for the study of cell/membrane uptake, absorption, transport and clearance of therapeutic agents (Wilson et al, 1990). Alveolar phagocytic cells, through uptake and release of antibiotics, play a key role in delivery of drugs to the lung (Carlier et al, 1987).

7.3.1 Method

Before isolation of the cells, the thorax was opened, and the blood was removed by cardiac puncture. The lungs were removed from the thoracic cavity en bloc and lavaged with 10 ml of chilled PBS. This procedure was repeated four times. The lung lavages were pooled per animal and centrifuged (100 *g*, 10 min, 4°C). The cellular pellet, i.e., alveolar macrophages, was suspended in solution containing 140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 2.0 mM

$CaCl_2$, and 1.3 mM $MgSO_4$ to a concentration of 2×10^6 cells/ml and stored on ice until further use. The average yield was 5×10^6 alveolar macrophages/rat. A total of 3×10^5 cells were incubated with various concentrations of liposomes (1 mg of lipids/ml) at 37°C (final vol. 500 µl) in a shaking water bath. After 1 h, the incubation was terminated by addition of 2 ml of ice-cold PBS. The cell suspension was centrifuged at 100 *g* for 10 min at 4°C. The supernatant was removed, and the cells were suspended in 2 ml of ice-cold PBS and centrifuged again. This wash procedure was repeated twice. Finally, the pellet was re-suspended in 200 µl of cold PBS, and cells and free liposomes were counted using a Hemacytometer chamber. The viability of cells after liposome uptake was also examined by trypan blue exclusion.

7.4 Data and statistical analysis

(a) Percent Drug Diffused (Shah et al, 1993)

The percent drug diffused was determined by the formula

$$\% \text{ Drug diffused} = \frac{C_r . V_r}{Cd \ Vd} \times 100$$

Where,

C_r = Conc. of drug in receptor compartment.

V_r = Volume of the receptor compartment.

Cd = Conc. of drug in donor compartment.

Vd = Volume of donor compartment.

(b) Kinetics of Release

The order of drug release was determined by performing regressions over the mean values of percent drug diffusion Vs T and percent drug diffusion Vs Root t.

(c) Mean steady state flux.

The flux across the membrane was calculated using the following formula:

$$J = V \ (dc/dt)$$ (Chien et al, 1993; Vincent et al, 1993)

Where, J = flux of the drug across the membrane.

V = Volume of receptor compartment.

(dc/dt) = Rate of change of concentration.

Mean steady state flux is the mean of individual flux values at all sampling points.

(d) Diffusion coefficient

The diffusion coefficient of the drug at every sampling point was calculated using the following equation:

$$R = 200 \sqrt{\frac{DT}{\pi h^2}} \quad \text{(Higuchi, 1962)}$$

Where,

R = Percent drug diffused
h = thickness of the membrane (0.02 cm)
t = time (sec)
D = diffusion coefficient (cm^2/ sec)

The diffusion coefficient used for the discussion is the mean of the value (D) obtained at each sampling point.

Each test was conducted six times and data from all experiments are expressed, as mean ± SEM. The data were compared using ANOVA and Student's t-test and difference at $p < 0.05$ were considered significant.

7.5 Results and discussions

Comparative diffusion studies were was carried out of plain drugs (INHPD & RFPPD and LDPI formulations using self designed and validated diffusion cell for a period of 12 to 24 hours. The results of these studies are recorded in Table 7.1. Cumulative percent drug diffusion was plotted against time (t) and shown in Figure 7.2 and 7.3. The non-linearity of the graph suggests that the diffusion pattern does not follow zero order kinetics of release. The regression coefficients (0.9422 – 0.9873) of the data of percent drug diffused Vs Root t (Table 7.2) suggest that a linear relationship exists between percent drug diffused and Root t confirming the release obeys Higuchi's diffusion controlled model (Higuchi, 1961).

Mean flux values of plain drug (PD) and its LDPI formulations (INH70, INH71, RFP70 and RFP71) were calculated and recorded Table 7.3. The diffusion coefficient of PD and its LDPI formulations were also calculated and recorded in Table 7.3. The mean flux values of PD is found to be two times higher than those of LDPI formulations underscore sustained drug diffusion from liposomally encapsulated drug formulations. Similarly, the diffusion coefficient of the PD is four times higher than that of LDPI formulations, confirming a sustained diffusion following liposomal encapsulation of these drugs.

On comparing the flux of both neutral (INH70 – 87.37 µg/min) and negatively charged (INH71 – 74.40 µg/min) liposomal DPI formulations, negatively charged

liposomal DPI formulations found to diffuse slower than neutral formulations. It may be due to ionic interaction of drugs with charge present in liposomal membranes and thus retarding the drug diffusion. The diffusion coefficient is governed by the concentration of free drug in the donor compartment and depends on the rate of drug diffusion from liposomes.

There are two rate-controlling barriers influencing the drug diffusion to the receptor compartment, one is the liposomal membrane and the other is the artificial membrane. The diffusion coefficient and flux of liposomal drugs are found to be dependent upon the composition and presence of charge. Hence, we can conclude that the liposomal membrane controls the drug diffusion and not the artificial membrane. The artificial membrane acts only as physical barrier preventing the liposomes to diffuse into the donor compartment and not regulating the drug diffusion to the receptor compartment.

In the in vitro alveolar macrophage uptake studies liposomes demonstrated an apparent maximal uptake of 73 ± 8.7% at 20 μg/ml. No significant differences were observed in the uptake of different types of liposomes size and charge. The photomicrograph of alveolar macrophage before and after liposomal uptake is shown in figure 7.3. This suggests that liposomes with different concentrations demonstrated the uptake of liposomes by alveolar macrophages, which indicates a major role of the alveolar macrophages in the uptake of lipid carriers.

Hence, liposomal encapsulation, composition of liposomal membrane and charge are expected to help in retaining the drug within the lung. All these observations lead us to the conclusion that liposomal DPI formulations delivery has a greater potential for sustained diffusion of drug. Drug diffusion from LDPI formulations obeys Higuchi's diffusion controlled model and the diffusion rate is close to first order kinetics. The diffusion rate depends upon the physicochemical property, concentration of drug within the liposomes and the composition of the liposomal membrane. Hence by altering the composition of the liposomal membrane, different loading dose followed by maintenance dose can be achieved. This model of diffusion study may be used to assess the desired diffusion pattern by modulating the composition of the bilayer membrane and evaluation the formulation's in vitro before going for in vivo.

Hence the LDPI formulations studied for in vitro diffusion was further subjected to in vivo studies and availability of drug to lungs was studies with that of the plain drug.

Table 7.1: Comparative in vitro drug diffusion of Optimized LDPI formulations.

Mean Cumulative Percent Drug Diffused across the membrane (±SEM)*						
Time	**ISONIAZID**			**RIFAMPICIN**		
(Hours)	**INHPD**	**INH70**	**INH71**	**RFPPD**	**RFP70**	**RFP71**
01	77.52	33.54	26.34	28.31	13.43	7.41
	(3.41)	(2.68)	(2.76)	(2.83)	(3.22)	(2.46)
02	97.8	47.67	34.38	44.31	26.49	10.46
	(3.23)	(2.52)	(2.74)	(3.10)	(2.64)	(3.29)
03	-----	54.77	42.17	67.68	32.83	21.42
		(3.24)	(2.39)	(2.56)	(2.95)	(2.55)
04		64.28	54.62	76.64	47.45	27.46
		(2.61)	(2.36)	(3.34)	(2.32)	(2.83)
06		77.54	64.57	97.64	54.72	32.43
		(2.60)	(1.94)	(3.25)	(2.52)	(2.87)
08		94.35	83.23		61.26	38.87
		(3.26)	(2.71)		(2.55)	(2.55)
09			95.24		66.74	47.62
			(3.42)		(2.27)	(3.22)
10					74.66	57.22
					(2.59)	(3.42)
12					84.49	75.56
					(3.83)	(2.56)
24					98.59	97.55
					(3.83)	(3.51)

* Mean of six determinations

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

Table 7.2: Regression coefficient (r) of the line of percent drug released Vs square root of time.

Batch No.	Regression coefficient (r^2)
INH70	0.9485
INH71	0.9716
RFP70	0.9873
RFP71	0.9422

Table 7.3: Mean flux and Diffusion coefficient values of optimized liposomal formulations across artificial membrane

BATCH NO.	MEAN FLUX (µg/ min)	DIFFUSION COEFFICIENT (cm^2/ sec)
INHPD	169.00	2.04E-03
INH70	87.37	8.09E-04
INH71	74.40	6.61 E-04
RFPPD	72.22	1.28 E-03
RFP70	33.38	5.96 E-04
RFP71	33.13	4.10 E-04

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

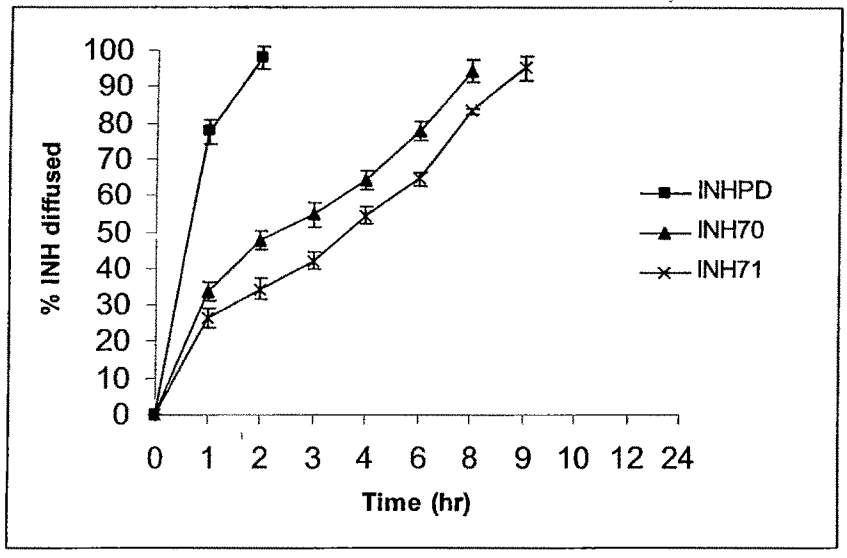

Figure 7.1: In vitro diffusion profile of INH plain drug and LDPI formulations.
INHPD – Plain drugs, INH70 – Neutral LDPI formulations and INH71 – Negatively charged LDPI formulations

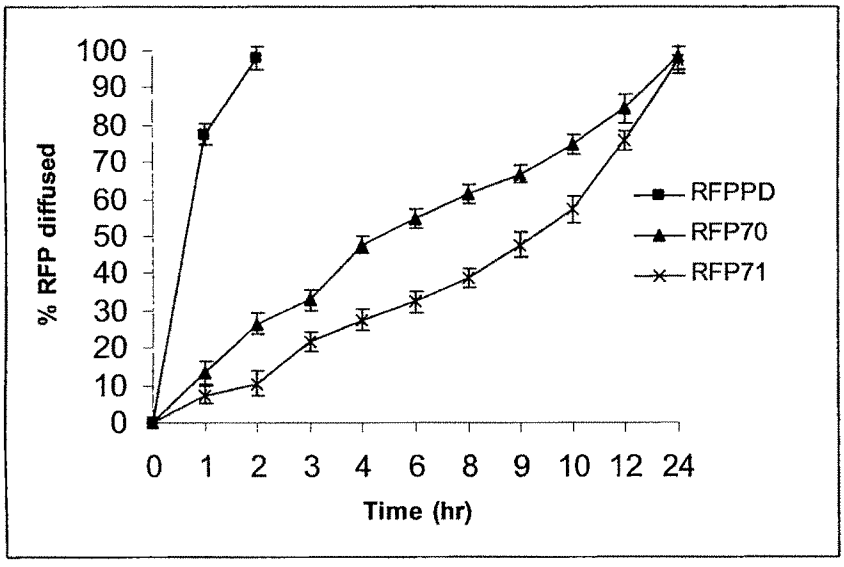

Figure 7.2: In vitro diffusion profile of RFP plain drug and LDPI formulations.
RFPPD – Plain drugs, RFP70 – Neutral LDPI formulations and RFP71 – Negatively charged LDPI formulations

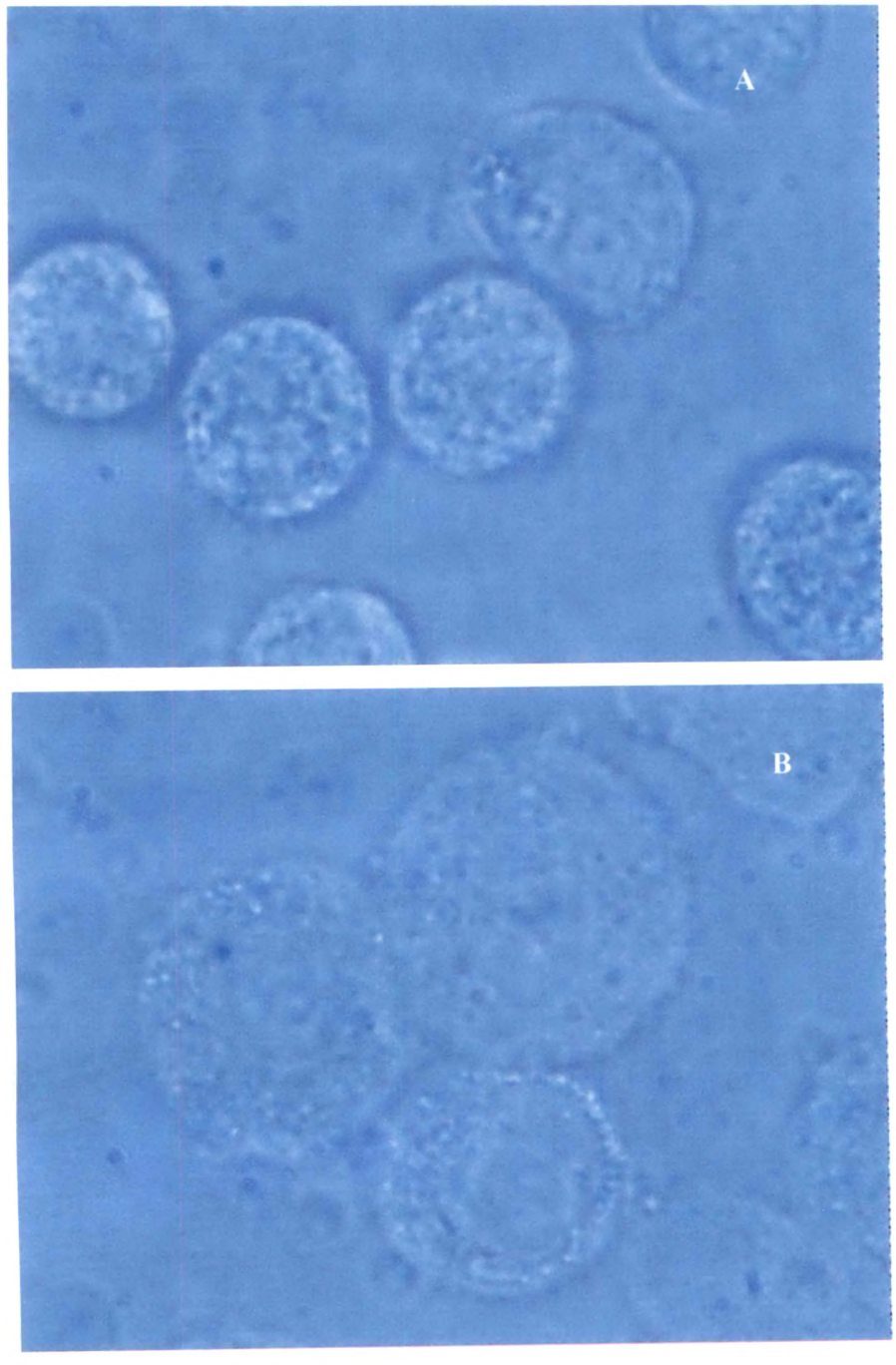

Figure 7.3: Photomicrograph of alveolar macrophage (A) before and (B) after liposomal uptake

7.6 References

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