CHAPTER 5

IN-VITRO DRUG DIFFUSION STUDY

In-vitro Drug Diffusion Study

5.1 INTRODUCTION

The encapsulated drug releases from liposomal systems are useful to establish relevance to the *in-vivo* as well as to the non *in-vivo* arenas (Margalit and Yerushalmi, 1996).

The drug release studies are expected to yield data and gives understanding for prediction of liposomal behavior in the *in-vivo* arena:

a) Minimizing the loss of encapsulated drug on route from the site of administration to the site of drug action i.e. bioavailability.
b) The ability to match the release rate (once the liposomes arrive at the target) to the requirements of the therapy.

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

a) Physicochemical characterizations of the systems, including liposomes lyophilized to form 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 experiments, 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.

The prime factor for successful development of a promising drug delivery system and assessments of the drug release profile of drugs from the delivery system is the proper design and selection of an *in-vitro* drug release system that permits accurate evaluation and mechanistic analysis of the drug release profiles. Physiological availability of the drug depends on the rate of release from the liposomes and permeability through alveolar surface into the lungs. The *in-vitro* methods are valuable and important screening procedures for understanding physico-chemical parameters such as fluxes, partition coefficients and diffusion coefficients etc. Though according to Gemmell and Morison (Gemmell and Morrison, 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 release drug under experimental conditions. The constraint of such

a technique is that the method does not exactly simulate the in situ behavior, especially with respect to unpredictable blood supply and metabolism. However, since performing bio-studies on every manufactured batch is impractical and costlier affair, formulators must rely on *in-vitro* testing to ensure batch-to-batch uniformity and consistency in bioavailability among developed formulations (Mojaverian et al., 1997).

In present scenario, *in-vitro* test systems have not been developed which can accurately predict the rate of drug release from liposomal formulations *in-vivo* (Fielding and Abra, 1992). Therefore an *in-vitro* release technique is proposed, validated and utilized for drug release studies from optimized liposomal formulations.

5.2 EXPERIMENTAL SETUP

5.2.1 Artificial Membrane

Dialysis membrane (250-9U, molecular weight cut off: 12400 Dalton; Sigma, Banglore, India), 200 μm 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.

5.2.2 Activation of Dialysis Membrane

The dialysis membrane tubings were washed in running water for 3-4 hours to remove glycerol followed by treatment of tubing with sodium sulfide solution (0.3% w/v) at 80°C for 1 minute to remove sulfur compounds. Wash with hot water (60°C) for 2 minutes, followed by acidification with a 0.2% (v/v) solution of sulfuric acid, then rinse with hot water to remove the acid. Then the dialysis membranes were dipped overnight in the diffusion medium before dialysis for thorough wetting of the tubings.

5.2.3 Selection of Diffusion Medium

Receptor compartment containing 50 ml of diffusion medium (50 mM Hydroxypropyl-beta-cyclodextrin, 20 mM HEPES, pH 7.4) for RGZ and 50 ml of diffusion medium (100 mM Hydroxypropyl-beta-cyclodextrin, 20 mM HEPES, pH 7.4) for CDS, with constant stirring kept at 37° ± 0.5°C.. This diffusion medium is selected to maintain sink condition (Saarinen-Savolainen et al., 1997).

5.3 METHOD

Diffusion studies were carried out for plain drugs (RGZ, CDS), developed unconjugated liposomal formulations and conjugated liposomal formulations. Known quantity of the plain drug and equivalent amount of the drug-loaded unconjugated and conjugated liposomes were dispersed in 2 ml diffusion medium and were taken into the dialysis bags respectively. Then bags were transferred into 50 ml diffusion medium kept at 37° ± 0.5°C. The dispersion in the dialysis bag was stirred with a magnetic stirrer and the solution outside the bag with an electric stirrer. At fixed time intervals 200 µl samples were withdrawn from outer medium of the bag and replaced with equal volumes of fresh diffusion medium and analyzed by UV spectrophotometric method, as described in chapter no 3, after suitable dilutions with methanol. All diffusion studies and sample analysis were carried out three times and mean values along with standard error of mean are recorded (Nounou et al., 2006; Henriksen et al., 1995).

5.4 DATA AND STATISTICAL ANALYSIS

1) Percentage drug diffused: (Shah et al., 1991; Shah et al., 1993)

$$Percentage\ Drug\ Diffused = \left(\frac{C_r \times V_r}{A}\right) \times 100$$

Where, C_r = conc. of drug in receptor compartment,

V_r = volume of the receptor compartment,

A = amount of drug in donor compartment at zero time.

2) Kinetics of release: The order of drug release was determined by fitting the data in various models utilizing drug release data modeling software DD Solver 1.0 of Microsoft. Percentage drug release was calculated and plotted against the time to accomplish drug release profile (figure 5.1 and 5.2). The *in-vitro* release data obtained were fitted into equations for the zero-order, first- order and higuchi release models. Correlation coefficient values resulting from linear reggression were used to interprete the data (Dash et al., 2010; Enden and Schroeder, 2009; Chien, 1992; Higuchi, 1962; Higuchi, 1961; Koizumi et al., 1975).

3) Steady state flux:

$$Steady\ state\ flux\ (J) = V_r \times \left(\frac{dc}{dt}\right)$$

Where, V_r = volume of receptor compartment and

(dc/dt) = rate of change of concentration,

5.5 RESULTS AND DISCUSSIONS

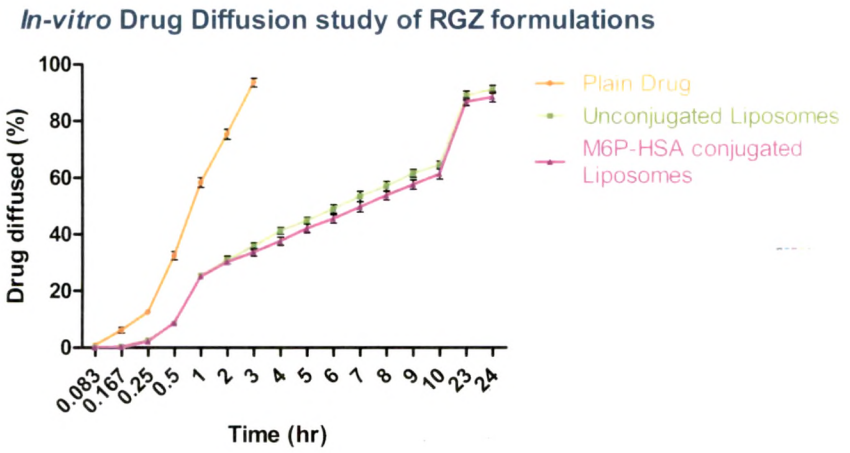
Comparative diffusion studies were was carried out of plain drugs, unconjugated and M6P-HSA conjugated liposomal formulations using diffusion membrane for a period of 24 hours. The results of these studies are recorded in Table 5.1, 5.2 and 5.3.

Table 5.1 Comparative *in-vitro* drug diffusion of plain RGZ and RGZ liposomal formulations

Time (hrs.)	Mean Cumulative Percent Drug Diffused across the membrane (mean ± SEM)*		
	RGZ formulations		
	Plain RGZ	Unconjugated Liposomes	M6P-HAS conjugated Liposomes
0.083	0.79 ± 0.031	0.03 ± 0.003	0.02 ± 0.003
0.167	6.10 ±1.016	0.28 ± 0.018	0.23 ± 0.010
0.25	12.57 ± 0.604	2.65 ± 0.211	2.11 ± 0.199
0.5	32.44 ± 1.445	8.43 ± 0.652	8.70 ± 0.545
1	58.30 ± 1.735	25.48 ± 0.655	25.05 ± 0.750
2	75.36 ± 1.788	30.88 ± 1.430	30.10 ± 0.862
3	93.59 ± 1.576	35.70 ± 1.259	33.60 ± 1.320
4	--	41.24 ± 1.180	37.55 ± 1.402
5	--	44.89 ± 1.162	42.08 ± 1.532
6	--	48.95 ± 1.480	45.45 ± 1.526
7	--	53.37 ± 1.809	49.67 ± 1.758
8	--	57.017 ± 1.663	53.67 ± 1.540
9	--	61.53 ± 1.435	57.57 ± 1.678
10	--	64.50 ± 1.366	61.30 ± 1.846
23	--	88.96 ± 1.589	86.75 ± 1.286
24	--	91.22 ± 1.439	88.48 ± 1.793

* n=3

Figure 5.1 Comparative *in-vitro* drug diffusion profiles of plain RGZ and RGZ liposomal formulations



n = 3 (± SEM)

P<0.001: (Plain drug and unconjugated liposomes; plain drug and conjugated liposomes)

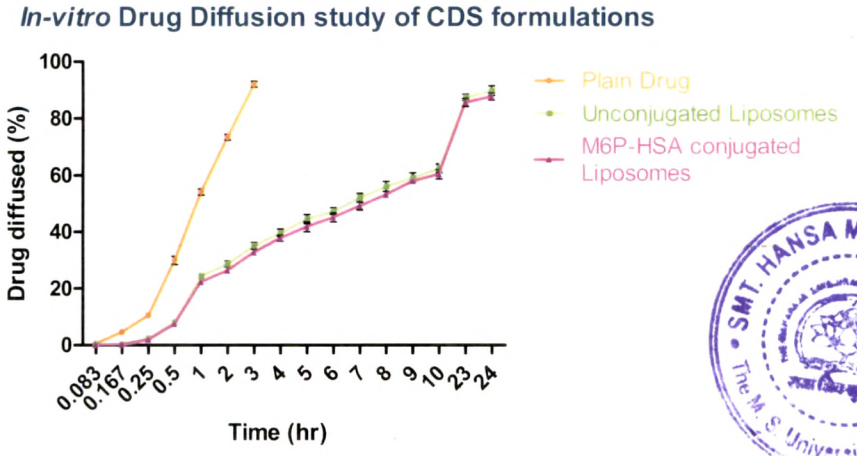
P > 0.05: (Unconjugated liposomes and conjugated liposomes)

Table 5.2 Comparative *in-vitro* drug diffusion of plain CDS and CDS liposomal formulations

Time (hrs.)	Mean Cumulative Percent Drug Diffused across the membrane (mean ± SEM)*		
	CDS formulations		
	Plain CDS	Unconjugated Liposomes	M6P-HAS conjugated Liposomes
0.083	0.52 ± 0.020	0.02 ± 0.003	0.02 ± 0.003
0.167	4.62 ± 0.477	0.17 ± 0.009	0.14 ± 0.009
0.25	10.56 ± 0.383	2.36 ± 0.264	1.91 ± 0.149
0.5	29.92 ± 1.455	8.10 ± 0.532	7.37 ± 0.551
1	54.16 ± 1.091	24.38 ± 0.753	22.29 ± 0.866
2	73.43 ± 0.993	28.69 ± 1.037	26.29 ± 0.788
3	92.04 ± 1.019	35.09 ± 1.229	32.69 ± 0.909
4	--	39.75 ± 1.305	37.75 ± 1.009
5	--	44.55 ± 1.558	41.75 ± 1.671
6	--	47.28 ± 1.140	45.09 ± 1.515
7	--	52.09 ± 1.547	49.14 ± 1.393
8	--	56.06 ± 1.788	53.14 ± 0.607
9	--	59.16 ± 1.692	57.96 ± 0.841
10		62.44 ± 1.512	60.35 ± 1.601
23	--	87.43 ± 1.086	85.62 ± 1.406
24	--	89.82 ± 1.675	87.72 ± 1.210

n=3

Figure 5.2 Comparative *in-vitro* drug diffusion profiles of plain CDS and CDS liposomal formulations



n = 3 (± SEM)

P<0.001: (Plain drug and unconjugated liposomes; plain drug and conjugated liposomes)

P > 0.05: (Unconjugated liposomes and conjugated liposomes)

Table 5.3 Drug diffusion model, Regression coefficient (r^2) and mean steady state flux of different formulations

	Name of the formulation	Drug diffusion model	Regression coefficient (r^2)	Mean steady state flux
RGZ and liposomal formulations	**Plain RGZ**	First order model	0.9833	37.78
	Unconjugated Liposomes	Higuchi model	0.9768	6.89
	M6P-HAS conjugated Liposomes	Higuchi model	0.9796	6.48
CDS and liposomal formulations	**Plain CDS**	First order model	0.9827	30.66
	Unconjugated Liposomes	Higuchi model	0.9789	6.58
	M6P-HAS conjugated Liposomes	Higuchi model	0.9818	6.08

The order of drug release was determined by fitting the data in various models utilizing drug release data modeling software DD Solver 1.0 of Microsoft. Percentage drug diffused was calculated and plotted against the time to accomplish drug release profile. Cumulative percent drug diffusion was plotted against time (t) and shown in Figure 5.1 and 5.2. The *in-vitro* release data obtained were fitted into equations for the zero-order, first- order and higuchi release models. Correlation coefficient values, resulting from linear regression, were used to interpret the data. The non-linearity of the graph for unconjugated and conjugated liposomal formulations suggests that the diffusion pattern does not follow zero order kinetics of release. Highest regression coefficient value for the first order model was found for plain drugs [RGZ (0.9833); CDS (0.9827)] and for the higuchi model for both the unconjugated [RGZ (0.9768); CDS (0.9789)] and M6P-HSA conjugatd liposomes [RGZ (0.9796); CDS (0.9818)], indicating diffusion to be the predominant mechanism of drug release in both the cases of liposomes. In case of RGZ, Mean steady state flux were 37.78, 6.89 and 6.48 for plain drug, unconjugated liposomes and M6P-HSA conjugatd liposomes respectively. In case of CDS, mean steady state flux were 30.66, 6.58 and 6.08 for plain drug, unconjugated liposomes and M6P-HSA conjugatd liposomes respectively.

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 percentage drug diffusion of liposomal drugs is found to be dependent upon the composition of formulation. 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.

5.6 CONCLUSION

Hence, liposomal encapsulation, composition of liposomal membrane and charge are expected to help in retaining the drug within the liposomes. All these observations lead us to the conclusion that liposomal drug delivery has a greater potential for sustained diffusion of drug. Drug diffusion from liposomal 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 *in-vitro* evaluation of the formulations before going for *in-vivo* studies.

5.7 REFERENCES

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