CHAPTER 7

IN VITRO DRUG DIFFUSION STUDIES

One of the alarming task facing a researcher in his / her successful development of a viable drug delivery device and experimental assessments of the drug diffusion profile of drugs from the device 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.

7.1 Drug diffusion studies across artificial membrane

7.1.1 Experimental setup

7.1.1.1 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.

7.1.1.2 Design of diffusion cell

For the present study a vertical type of membrane diffusion system was developed (Figure 7.1). 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 magnetic needle (length = 2.5 cm, d = 0.5 cm) and a magnetic stirrer (Remi, India) by the aid of a surrounding water bath. The temperature of the bulk of the solution was maintained at 37 $\pm$ 0.5 ^{o} C. The donor compartment was stirred with a Teflon coated triple blade stirrer (Remi, India) at 50 rpm.

7.1.1.3 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).

7.1.1.4 Selection of diffusion medium

Receptor compartment containing 50 ml of PBS, 1mM EDTA with constant stirring (AMK) simulated highly perfused pulmonary condition (Joshi et al, 1999). AMK is very soluble in PBS, sink condition was maintained with 50 ml PBS, 1mM

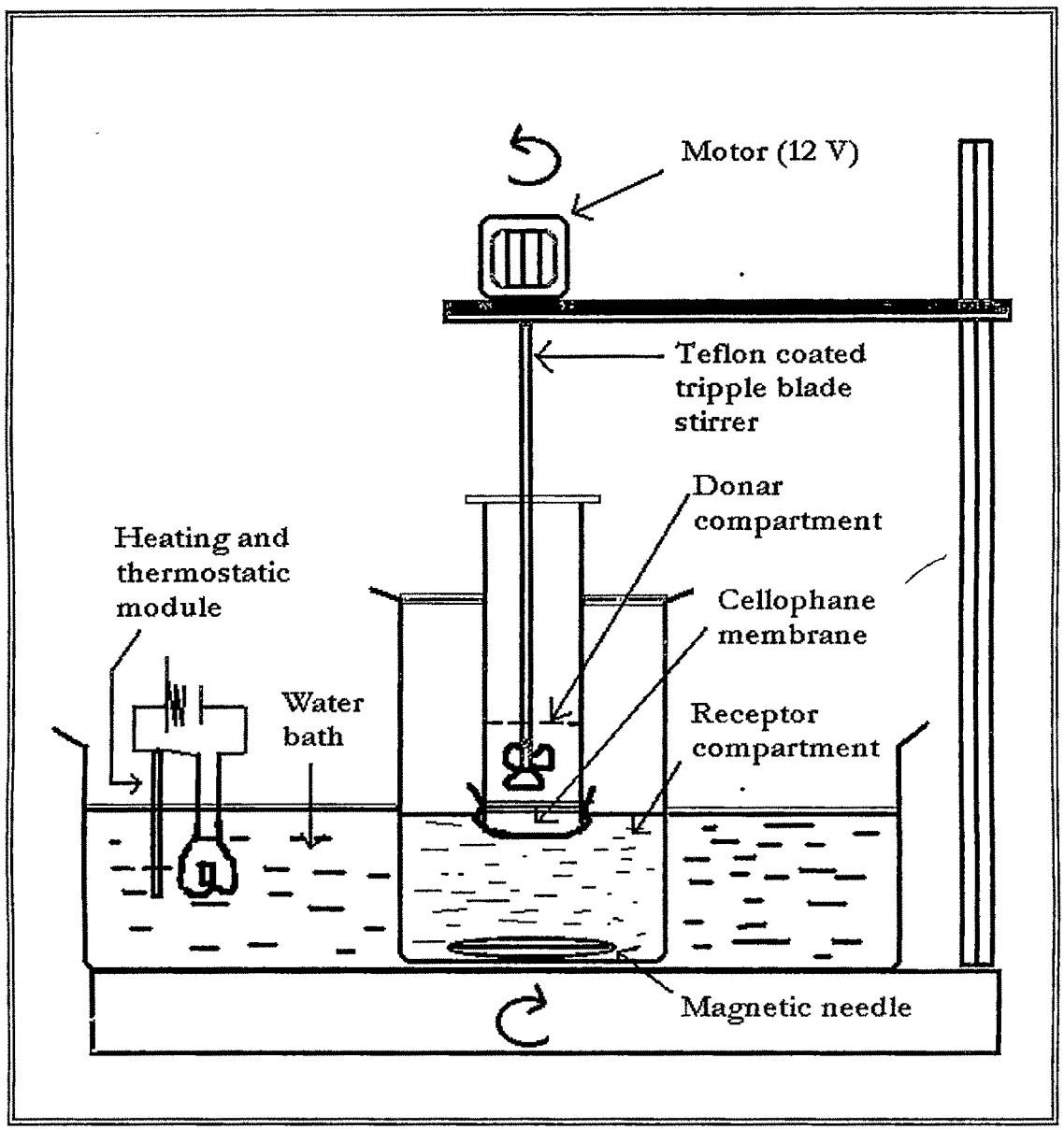

Figure 7.1 Schematic diagram of *in vitro* diffusion cell.

EDTA and zero order flux conditions were maintained. For AMB, being lipophilic drug, diffusion medium used in the receptor compartment was 50ml of mixture of PBS, 1mM EDTA and methanol (6:4) for maintaining sink condition.

7.1.2 Method

Diffusion studies were carried out for plain drugs (AMK and AMB) and LDPI formulations. The study was performed by dissolving AMK in 1 ml of PBS, 1mM EDTA, 10% sucrose solution (such that of original sugar concentration of AMK LDPI formulation) or for AMB a mixture of 2ml of PBS, 1mM EDTA, 10% sucrose solution (such that of original sugar concentration of AMB LDPI formulation) and methanol (6:4). Similarly LDPI formulation of AMK (equivalent to 15 doses; 1000 μg x 15) and LDPI formulation of AMB (equivalent to 10 doses; 250 μg x 10) was dispersed in 1 ml (AMK) and 2ml (AMB) 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 were determined using procedure described in chapter 3, Section 3.3.8 (AMK) and Section 3.4.6 (AMB). All diffusion studies and sample analysis were carried out in triplicate on two consecutive days and mean values along with standard error of mean are recorded in Table 7.1 and shown graphically in figure 7.2 and figure 7.3.

7.2 Statistical analysis

Each batch was prepared 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.

(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.

7.3 Results and discussions

Comparative diffusion study was carried out of plain drug (PD) and its 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) in Fig 7.2 and 7.3. The non-linearity of the graph suggests that the diffusion pattern does not follow zero order kinetics of release. However, the regression coefficients (Table 7.2) of the data of percent drug diffused Vs Root t lies between 0.9492 – 0.9928 indicating that a linear relationship exists between percent drug diffused and Root t suggesting the release obeys Higuchi's diffusion controlled model (Higuchi, 1961).

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

Mean Cumulative Percent Drug Diffused across the membrane (±SEM)*						
Time	**AMIKACIN SULPHATE**			**AMPHOTERICIN B**		
(Hours)	**PDAMK**	**AMK69**	**AMK70**	**PDAMB**	**AMB77**	**AMB78**
01	61.8	12.64	6.95	18.61	3.82	2.21
	(2.78)	(2.03)	(1.93)	(1.83)	(1.56)	(1.20)
02	87.62	18.67	11.62	42.34	12.53	8.61
	(2.61)	(2.29)	(2.22)	(2.10)	(2.32)	(1.86)
03	98.3	27.86	16.32	66.8	16.89	11.56
	(1.83)	(1.86)	(2.15)	(2.19)	(1.64)	(2.29)
04	-----	37.55	23.35	78.62	22.73	17.6
		(2.58)	(2.46)	(2.34)	(1.93)	(2.25)
06		48.97	31.28	98.54	27.5	21.16
		(2.02)	(1.75)	(2.23)	(1.82)	(1.83)
08		55.74	44.18		34.12	27.23
		(1.94)	(2.49)		(1.72)	(2.57)
09		62.27	52.6		41.23	31.88
		(2.31)	(2.06)		(1.55)	(2.05)
10		70.54	61.55		46.71	39.82
		(2.50)	(1.94)		(2.07)	(2.22)
12		84.95	84.53		54.68	50.92
		(1.36)	(2.21)		(2.19)	(2.84)
24					99.59	100.55
					(1.83)	(1.51)

* Mean of six determinations

PDAMK & PDAMB – Plain drugs, AMK69 & AMB77 – Negatively charged LDPI formulations and AMK70 & AMB78 – Positively charged LDPI formulations

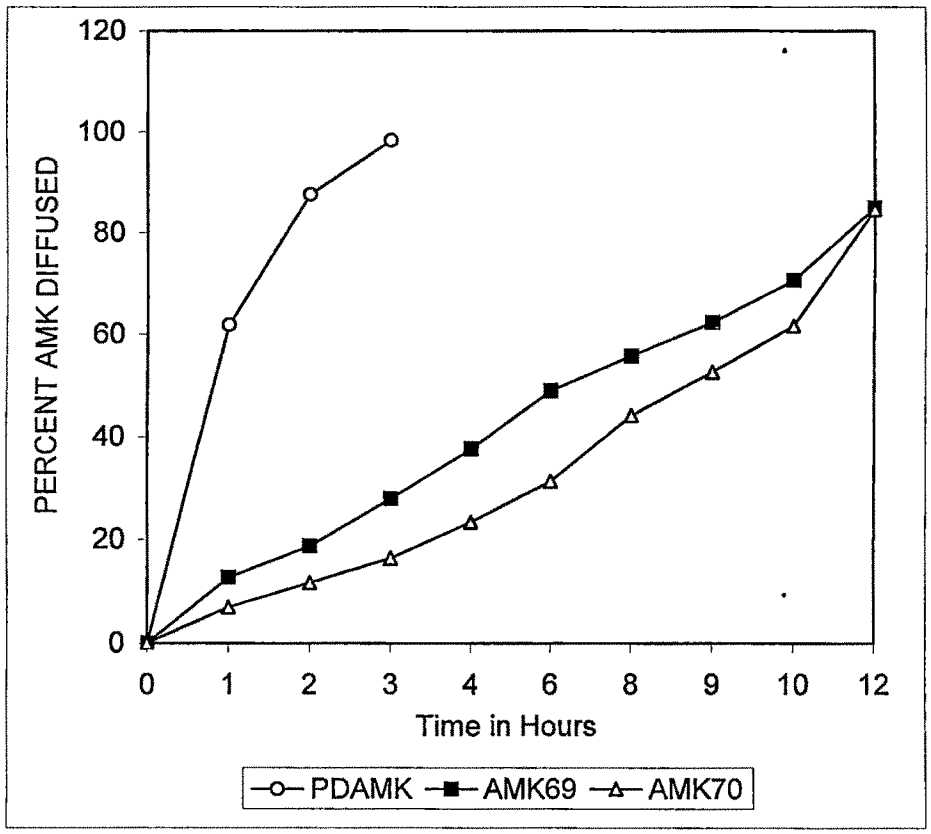

Figure 7.2 Chart illustrating comparative in vitro diffusion of AMK LDPI formulations with plain drug

PDAMK – Plain drugs, AMK69 – Negatively charged LDPI formulation and AMK70 – Positively charged LDPI formulation

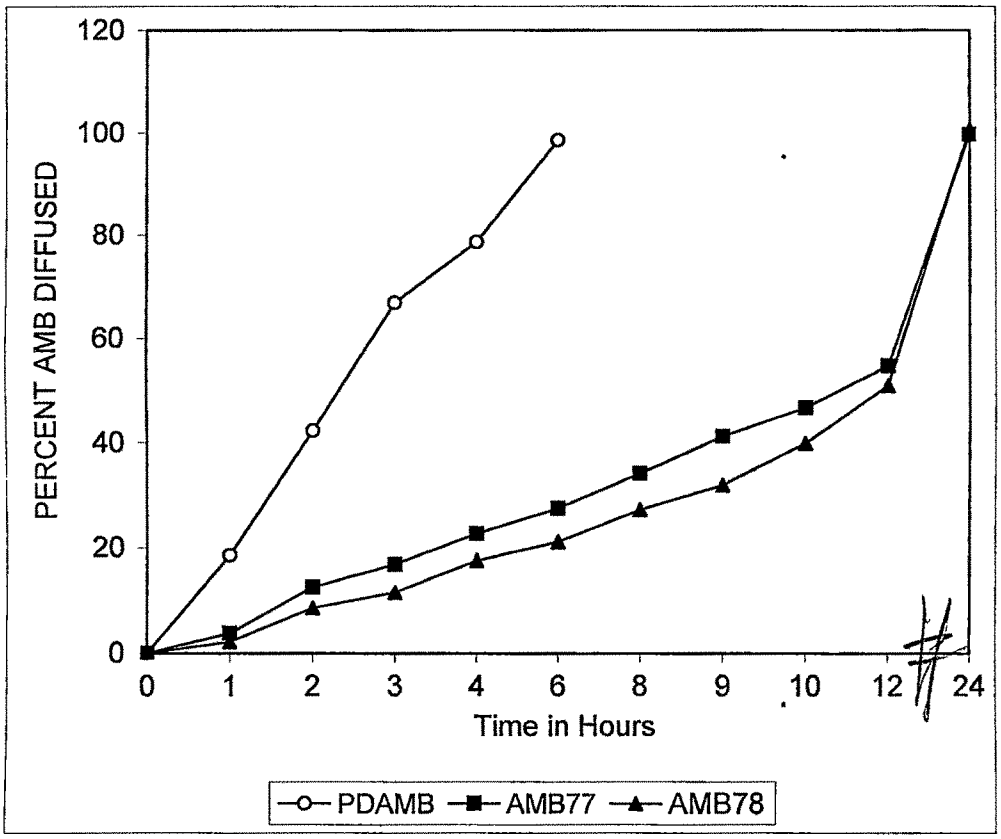

Figure 7.3 Chart illustrating comparative in vitro diffusion of AMB LDPI formulations with plain drug

PDAMB – Plain drugs, AMB77 – Negatively charged LDPI formulation and AMB78 – Positively charged LDPI formulation

The Mean flux values of plain drug (PD) and its LDPI formulations (AMK69, AMK70, AMB77 and AMB78) were calculated and recorded Table 7.2 and shown graphically in Figure 7.4. The diffusion coefficient of PD and its LDPI formulations were also calculated and recorded in Table 7.3 and shown graphically in Figure 7.5. The mean flux values of PD is found to be four to five times higher than those of LDPI formulations underscore sustained drug diffusion from liposomally encapsulated drug formulations. Similarly, the diffusion coefficient of the PD is 7 to 8 times larger than that of LDPI formulations, confirming a sustained diffusion following liposomal encapsulation of these drugs.

On comparing the flux of both positively and negatively charged liposomal DPI formulations, negatively charged liposomal DPI formulations found to diffuse less drug than positively charged formulations (AMK69 and AMB 77). It may be due to ionic interaction of these 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 enter into the sampling port and not regulating the drug diffusion to the receptor compartment. This further validates the applicability of the designed diffusion cell for the evaluation of liposomal formulations.

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

Batch No.	Regression coefficient (r)
AMK69	0.9928
AMK70	0.9768
AMB77	0.9492
AMB78	0.9272

low

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)
PDAMK	81.92	1.36 E-08
AMK69	19.07	3.16 E-09
AMK70	17.40	1.97 E-09
PDAMB	7.38	8.57 E-09
AMB77	2.08	1.45 E-09
AMB78	1.88	1.08 E-09

PDAMK & PDAMB – Plain drugs, AMK69 & AMB77 – Negatively charged LDPI formulations and AMK70 & AMB78 – Positively charged LDPI formulations

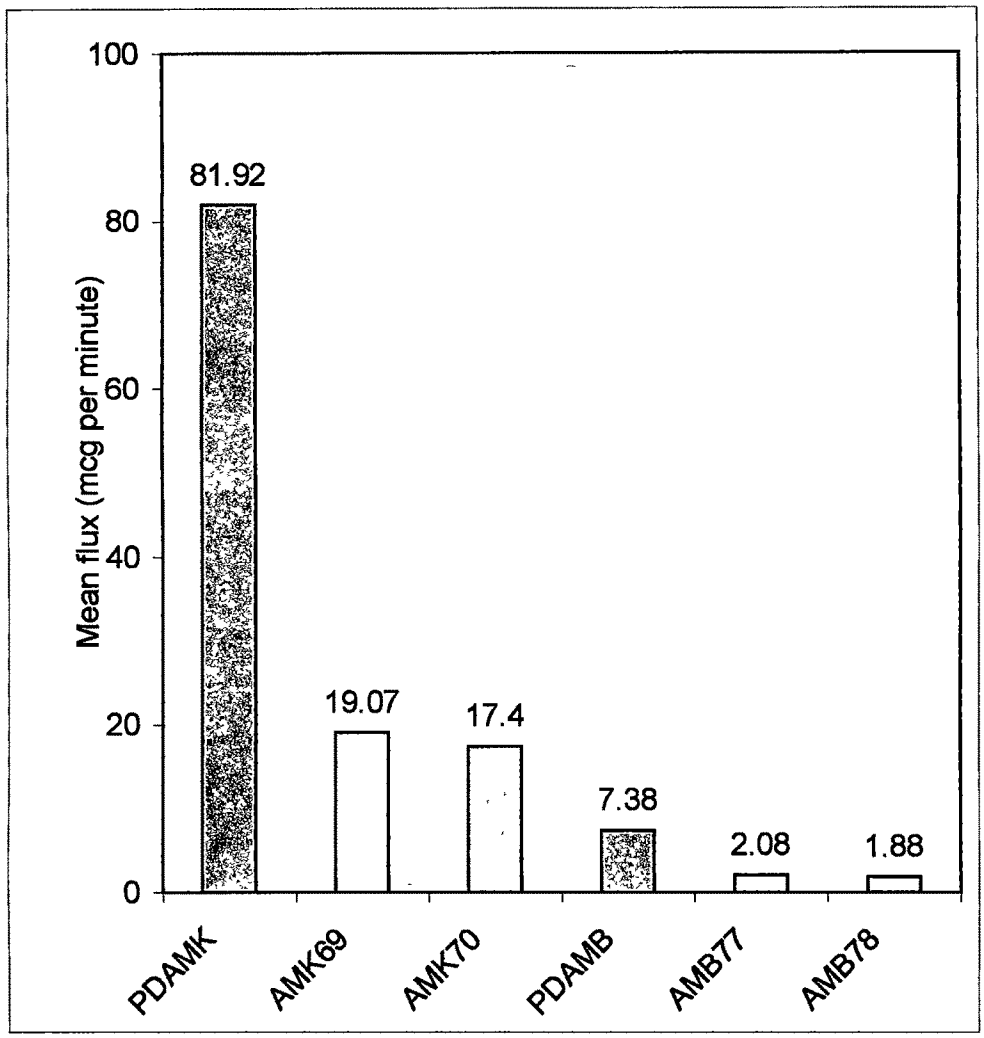

Figure 7.4 Comparative mean flux of plain drug and LDPI formulations across artificial membrane

PDAMK & PDAMB – Plain drugs, AMK69 & AMB77 – Negatively charged LDPI formulations and AMK70 & AMB78 – Positively charged LDPI formulations

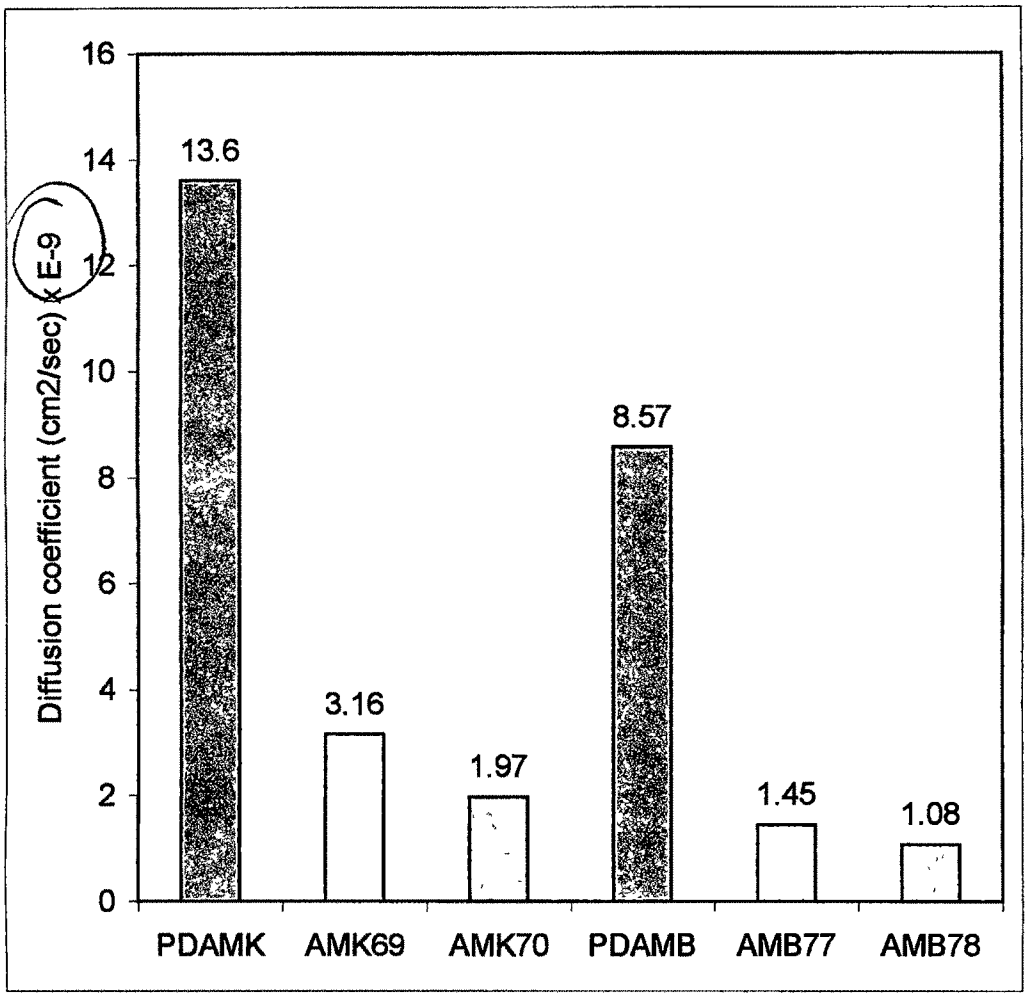

Figure 7.5 Comparative diffusion coefficient of plain drug and LDPI formulations across artificial membrane

PDAMK & PDAMB – Plain drugs, AMK69 & AMB77 – Negatively charged LDPI formulations and AMK70 & AMB78 – Positively charged LDPI formulations

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 formulation 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. Increasing the quantity of CHOL in the liposomal membrane (AMB78) reduces the permeability of the membrane providing a more sustained diffusion. Hence by altering the composition of the liposomal membrane (use of charge and PC: CHOL ratio), 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.

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