CHAPTER 8

IN VIVO INSTILLATION STUDIES

Aerosol therapy is an effective means of delivering relatively small doses of an active ingredient (for localized action) directly to the respiratory system. Localization f 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 so giving a 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).

8.1 Intratracheal Instillation (Gonzalez-Roth et al, 1996; 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. Five rats of either sex were used in each group, at every time interval. With five sampling points and eight formulations to compare a total of approximately 200 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. Solutions containing 500µg (AMK), 250µg (AMB) of non-encapsulated drug (PD) or liposome-encapsulated drug prepared by rehydration (for 30 min) of DPI with 250µl of distilled water was slowly instilled over a 1min period followed by 50µl normal saline. Animals to be sacrificed at 3, 6 and 9 hours after

administration had cannula secured with sutures and the access cannula excised to leave a 1 cm protrusion.

8.2 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 spectrofluorometric method for amikacin (Chapter 3, Section 3.3.9) and spectrophotometer for Amphotericin B (Chapter 3, Section 3.4.7). 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 (Chapter 3, Section 3.3.9).

8.3 Statistical Analysis

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.

The various pharmacokinetic parameters calculated for comparing are defined as below:

C_{max} Maximum concentration of drug attained in lung during the study.

The drug concentration in the lung is the drug estimated in lung homogenate (LH).

T_{max} The time point at which maximum drug concentration is attained in lung homogenate (i.e. the time interval of Cmax).

AUC^{24h}_{0} The area under the curve of drug concentration in lung homogenate Vs time, over the period of study (24 hrs).

$T_{1/2}$ Pulmonary half-life of drug is calculated by:

1. Calculating the sum of the values of drug concentration in BAL and LH at individual sampling points.
2. Regressing the calculated sum over the entire duration of study.
3. Deriving the time point at which the sum of drug level is 50% compared to instilled quantity (i.e. deriving the median of the regression line).

LH Lung homogenate

BAL Broncho alveolar lavage

8.4 Results and discussion

The in vivo evaluation was carried out by the estimation of the percent drug in BAL and LH after the administration of rehydrated LDPI formulations and of plain drug (PDAMK and PDAMB). The dose of 500 µg (AMK) or 250µg (AMB) was intra-tracheally instilled. Amount of drug present in the LH was considered as the drug absorbed and available for the pharmacological response and the amount of drug present in the BAL

was considered as drug not absorbed into the lung tissue but still retained in the bronchial spaces (in liposomally encapsulated form). Later 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 summarized in Table 8.1 (AMK) and Table 8.2 (AMB) and shown in Figure 8.1 (AMK) and Figure 8.2 (AMB). From the drug concentration in lung-time plot pharmacokinetic parameters were calculated and are recorded in Table 8.3.

After instillation of liposomal drug, 40-60% of the drug was recovered in BAL during the first 6 hours, which decrease by 12 to 24 hours. Lung tissue recovery of drug, after administration of liposomes, increased with time (Figure 8.1 and Figure 8.2). The total drug balance between the percent drug diffused and percent drug still present in liposomes (estimated in BAL) was not 100 %. It was assumed that the amount of drug that could not be accounted might have either metabolized or systemically absorbed or both.

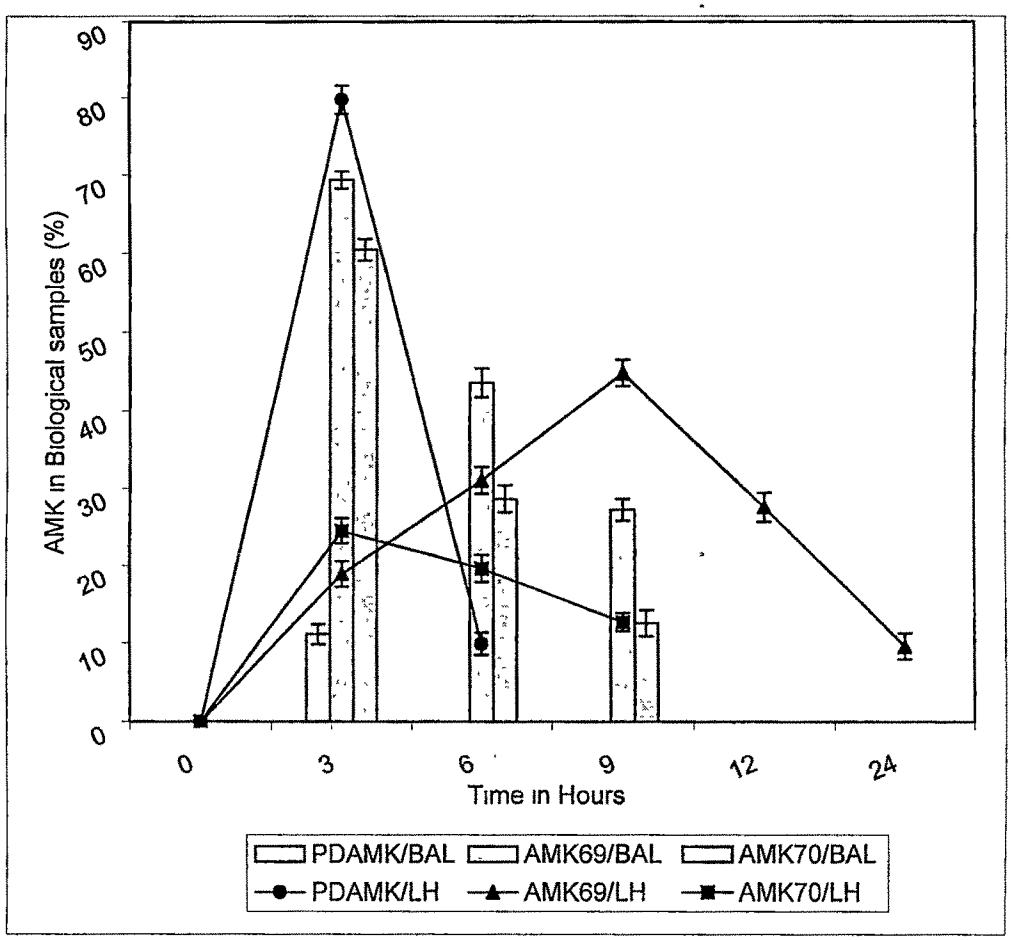
When the concentration-time profiles were examined upto 12-24 hours post-instillation there was a rank order decrease in Cmax from plain drug to the formulation containing negative and positive charge in their composition i.e. PDAMK>AMK69>AMK70 and PDAMB>AMB77>AMB78. Accordingly Tmax of all four formulations were found to be more than their respective plain drugs. Similarly, there was an increase in AUC $^{24h}_{0}$ for liposomal formulations compared to plain drug, the percent increase in the AUC $^{24h}_{0}$ for negatively charged liposomal formulations were more compared to positively charged liposomal formulation suggesting that negatively charged liposomal formulations sustains more in vivo diffusion of drugs (i.e. drug absorbed). The presence of more proportion of

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

Time (Hrs)	AMK IN BIOLOGICAL SAMPLES (%) Mean* ± (SEM)					
	BRONCHO ALVEOLAR LAVAGE			LUNG HOMOGENATE		
	PDAMK	AMK69	AMK70	PDAMK	AMK69	AMK70
03	11.08 ± 1.26	69.34 ± 1.12	60.42 ± 1.36	79.68 ± 1.88	18.89 ± 1.66	26.48 ± 1.61
06	-----	43.57 ± 1.84	28.61 ± 1.75	9.85 ± 1.42	31.02 ± 1.73	19.59 ± 1.76
09	-----	27.20 ± 1.39	12.49 ± 1.68	-----	44.81 ± 1.67	12.62 ± 1.15
12		14.84 ± 1.97	-----	-----	27.54 ± 1.86	05.84 ± 1.24
24		-----			9.56 ± 1.64	-----

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

Figure 8.1 Drug level in biological samples following instillation of liposomal DPI of AMK and plain drug



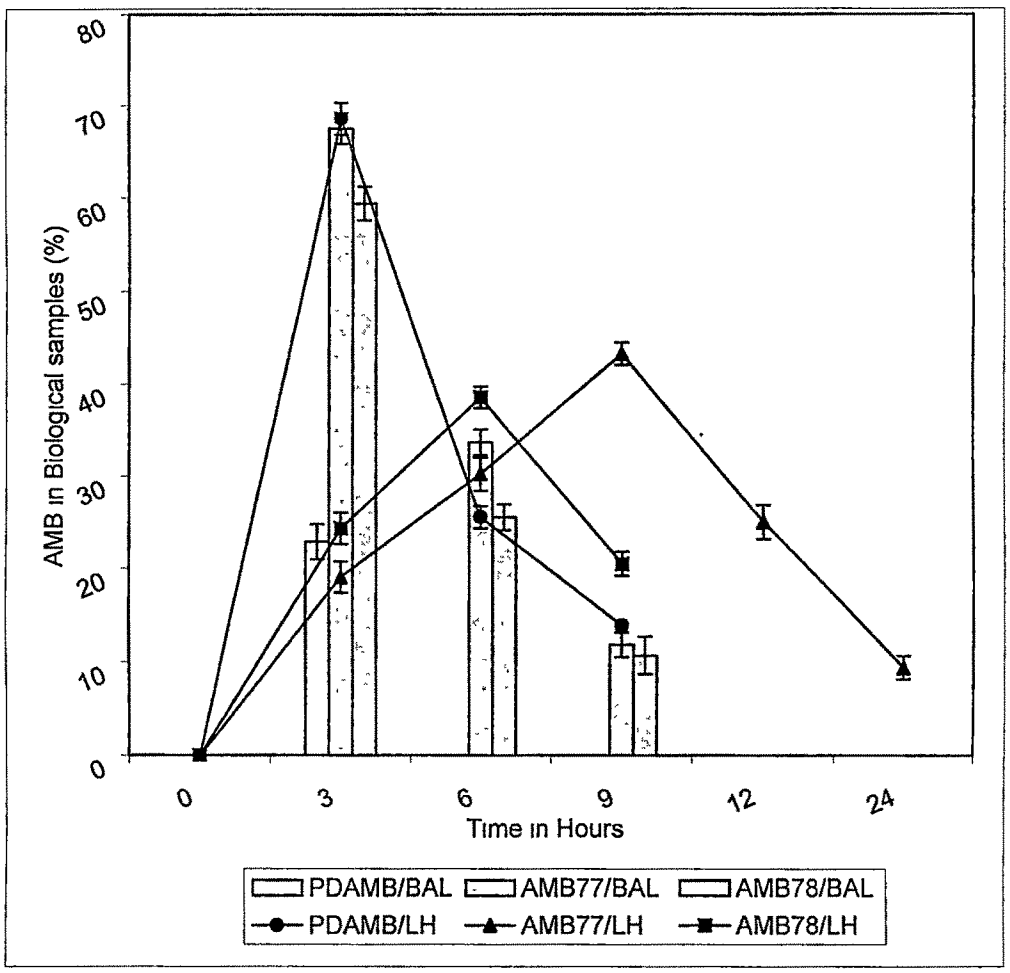
PDAMK – Plain drug, AMK69 – Negatively charged LDPI formulation, AMK70 – Positively charged LDPI formulation, BAL – Bronchoalveolar Lavage and LH - Lung Homogenate

Table 8.2 Drug level in biological samples following instillation of liposomal DPI of AMB and plain drug formulation

Time (Hrs)	AMB IN BIOLOGICAL SAMPLES (%) Mean* ± (SEM)					
	BRONCHO ALVEOLAR LAVAGE			LUNG HOMOGENATE		
	PDAMB	AMB77	AMB78	PDAMB	AMB77	AMB78
03	22.82 ± 1.90	67.50 ± 1.65	59.37 ± 1.80	68.54 ± 1.73	19.05 ± 1.65	24.27 ± 1.68
06	----	33.67 ± 1.43	25.46 ± 1.41	25.49 ± 1.22	30.20 ± 1.84	38.55 ± 1.18
09	-----	11.79 ± 1.31	10.66 ± 1.99	13.88 ± 1.71	43.29 ± 1.22	20.46 ± 1.31
12		-----	-----	-----	24.94 ± 1.85	12.07 ± 1.10
24					9.39 ± 1.24	-----

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

Figure 8.2 Drug level in biological samples following instillation of liposomal DPI of AMB and plain drug formulation



PDAMB – Plain drug, AMB77 – Negatively charged LDPI formulation, AMB78 – Positively charged LDPI formulation, BAL – Bronchoalveolar Lavage and LH - Lung Homogenate

CHOL and positive charge in AMB78 has resulted in least AUC compared to negatively charged formulation (AMB77). Similar type of observation was also observed for AMK LDPI formulations. Cholesterol is know to protect liposomes from in vivo destabilization (Abra et al, 1990), but up to an optimum concentration after which it contributes to the destabilization of the liposomes in vivo as revealed from AUC $^{24h}_{0}$ for AMB78 with 1:1 HSPC: CHOL ratio. The destabilization caused by the inclusion of cholesterol is more for hydrophobic drug like AMB. Thus the kinetics of LDPI formulations in lung is found to be dependent on drug's physicochemical property and on the composition of liposomes. The inclusion of CHOL is must for the physical stability of liposomes however the biological stability its level to be optimum and a further increase leads to a relatively rapid diffusion of the medicament.

In another set of statistical analysis, the pulmonary half-life ($T_{1/2}$) was calculated and recorded in Table 8.3. It is relatively new approach to study drug concentration and is proposed because C_{max} and T_{max} were not calculated by integrating the values over the entire time period of study. The integration was not done due to the constraint of having different set of animals used at each sampling point, which makes the integration a lesser valid approach. When the $T_{1/2}$ values were examined, there was increase in $T_{1/2}$ values from plain drug to negatively charged liposomal DPI formulations while for positively charged liposomal DPI formulations it was similar or slight more than plain drug. Thus 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 (Juliano et al, 1980) particularly negatively charged LDPI formulations (AMK69 and AMB78). It also confirms that inclusion of cholesterol in the liposomal membrane decreases the membrane permeability.

Table 8.3 Mean Pharmacokinetic parameters of liposomal formulation comparative to plain drug

Formulation	AUC $^{24h}_{0}$ (µg-h/ ml)	C_{max} (µg)	T_{max} (hr)	$T_{1/2}$ (hr)
Amikacin Sulphate				
PDAMK	1342.95	398.4	3	4.3
AMK69	2740.35	224.05	9	14.8
AMK70	924.15	132.4	3	8.5
Amphotericin B				
PDAMB	757.275	171.35	3	5.2
AMB77	1302.525	108.225	9	15
AMB78	669.8625	96.375	6	9.2

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

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. Severe P.aeuroginosa or fungal infection in patients may also alter the distribution and product performance compared to normal humans.

Findings of these studies give only comparative performance of plain drug and liposomal drug without any predictive value on performance in humans. Though the drug concentrations maintained in the lung tissues (LH) and less rapid clearance from the lung perfusate (BAL) demonstrate the superiority of LDPI formulations compared to plain drugs. Maintenance of effective drug concentrations in the lung tissues (LH) for prolonged time and less rapid clearance from the lung perfusate (BAL) demonstrates the superiority of negatively charged LDPI formulations over plain drug solutions. The inclusion of negative charge in the liposomal formulations increases the pulmonary half-life of liposomally encapsulated drugs i.e. for AMK LDPI formulations, 4.3 hrs to 14.8 hrs and for AMB LDPI formulations, 5.2 hrs to 15 hrs.

References

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