CHAPTER 6

STABILITY STUDIES

As industrially produced drug or antigen containing liposomes will reach the patient or person to be vaccinated only after a prolonged time, the liposome dispersion should not change its characteristics or lose the associated drug or antigen during storage or transport. In general, a shelf life of atleast one year is minimum prerequisite in the pharmaceutical industry. Attention has been focused on two processes affecting the quality and therefore acceptability of liposomes (Talsma et al, 1993). First, the encapsulated drugs can leak from the vesicles into the extra liposomal compartment. Second, liposomes can aggregate and/or fuse, forming larger particles. Both these processes change the disposition of the drug in vivo and thereby presumably affect the therapeutic index of the drug involved. Other physical parameters may also change drugs storage like hydrolysis of phospholipid causes the formation of fatty acids and lysophopholipids (Grit et al, 1993). Though under dehydrated storage, there is least possibility of the formulation to encounter hydrolytic degradation. Another aspect to consider is liposome oxidation (Frokjaer et al, 1984). Oxidation of unsaturated phospholipids and cholesterol may be initiated mainly by the action of light and heavy metals. We have tried to inhibit peroxidation of lipids within membranes by addition of metal chelators such as EDTA, protection from light, oxygen free atmosphere and the addition of α - tocopherol and low temperature storage (Mowri et al, 1984).

Stability is generally considered as chemical stability of drug substance in a dosage form, however, the performance of a drug when given as a liposomal dry powder inhaler system is not only dependent upon the content of the drug substance, but also reproducible in vivo performance of the formulations. Drugs under study were considered chemically stable so the study can be focused on monitoring the drug leakage from

liposomes. The stability protocol was designed as per ICH guidelines (Singh et al, 1999) for countries falling under zone III (hot, dry) and zone IV (very hot, humid).

6.1 Method

Comparative stability studies were carried out of the potential LDPI formulations at freezer (-10°C ± 2°C), refrigerated (5°C ± 2°C), controlled room temperature (30°C ± 2°C, 60 ± 5 % RH) and accelerated (40°C±2°C, 75±5% RH) conditions up to one year. LDPI formulations containing 250 µg AMB and 1000 µg AMK were filled into gelatin capsule shells (Size "2"). The empty shell weight for size "2" capsule was 68 ± 2 mg. These capsules were packed in HDPE bottles under nitrogen cover and the bottle was sealed with PVC coated aluminum foil. The bottles also contained silica bags as dehumactant and were resealed with flush of nitrogen after each sampling. Thirty sets of 10 capsules from a batch were filled in the HDPE bottles for each condition. The study was done with three batches of same composition.

During sampling, one bottle containing 10 capsules was withdrawn at definite time interval, rehydrated with distilled water for 30 minutes. The quantity of water used for rehydration was equivalent to give HSPC concentration of the formed dispersion proportionate to initial (i.e. before lyophilization). The obtained dispersion was centrifuged at 4.38 x 10^3 g for 3 minutes to sediment lactose. For AMK, the supernatant was subjected to separation of leaked drug by dialysis followed by analysis of drug content (Chapter 3, Section 3.3.7). For AMB, the leaked drug got separated along with lactose and so pellet was directly analyzed for the drug content (Chapter 3, Section 3.4.5). The result calculated in terms of percent drug retention and recorded in Tables 6.1 (AMK) and Table 6.2 (AMB) and shown in figures 6.1 and 6.2 (for AMK) and 6.3 and

6.4 (for AMB). The PDR is the percentage of drug initially added, determined after lyophilization. The liposomal dispersion was also evaluated for change in mean liposomal size and size distribution by laser diffraction; the results are recorded in Table 6.5. The LDPI formulations were also examined visually for the evidence of cacking and discoloration.

6.2 Statistical Analysis

Three batches of each formulations and each batch was evaluated three times, data of nine experiments are expressed as Mean ± SEM. The data were compared using ANOVA and students t-test and difference larger than the value at $p<0.05$ were considered significant.

The differences in liposomal size prior to and after storage at controlled room temperature and refrigerated conditions after 3 and 6 months respectively were compared using students paired t-test at $p = 0.05$. The analysis was carried out among the storage condition taking particle size values of all the formulations as component of one group for a specific storage condition and compared it with the control, which included the initial values.

6.3 Results and discussion

The physical stability of liposomes may be one of the largest obstacles in formulation commercially viable product (Fildes et al, 1981). Liposomes should be stable for 1-2 years preferably at room temperature to be pharmaceutically accepable with preservation of entrapped drug during this time. The drug leakage studies were carried out as per ICH guidelines (Singh et al, 1999) for countries falling under zone III (hot, dry) and zone IV (very hot, humid).

The percentage of drug remained entrapped for LDPI formulations of AMK after one year of storage at freezer condition was 84.27 % (AMK69) and 82.69 % (AMK70), at refrigerated storage condition it was 79.31 % (AMK69) and 77.57 % (AMK70), at controlled room temperature storage it was 68.30 % (AMK69) and 67.28 % (AMK70). While at six months accelerated storage it was 49.61 % (AMK69) and 45.73 % (AMK70). Thus there was decrease in percent drug retained entrapped with the increase in the temperature of storage. No major difference was observed due to effect of charge on stability for AMK LDPI formulations.

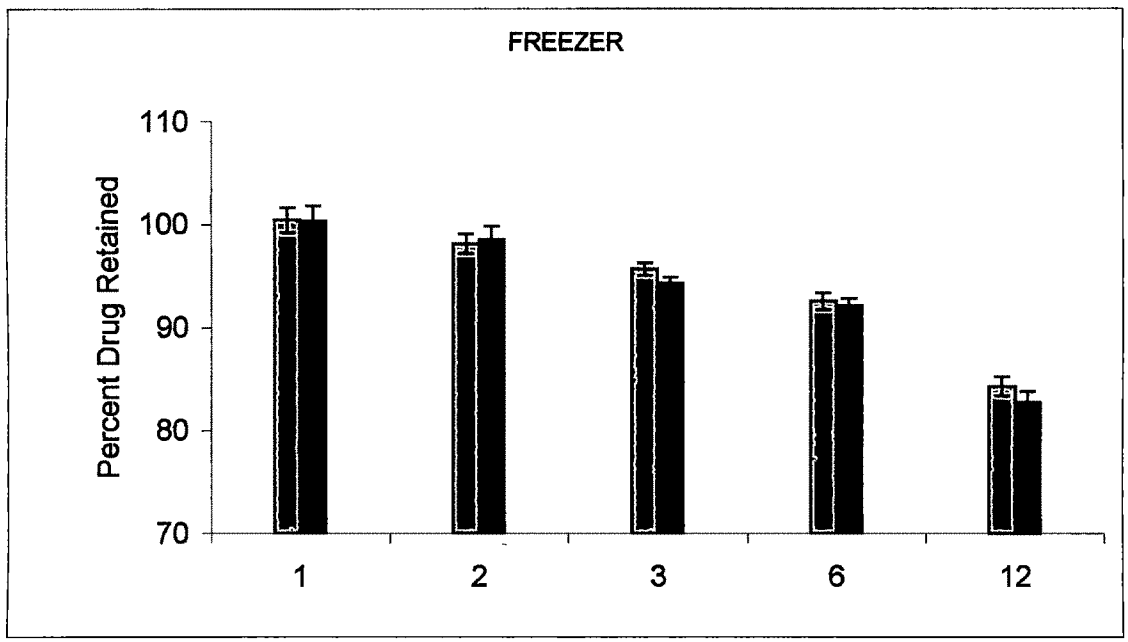
Similarly, percent drug retained for AMB LDPI formulations after one year of storage at freezer condition was 93.17 % (AMB77) and 9358 % (AMB78), at refrigerated storage condition it was 91.24 % (AMB77) and 90.76 (AMB78), at controlled room temperature storage it was 79.48 % (AMB77) and 76.16 % (AMB78). While at six months accelerated storage condition it was 73.49 % (AMB77) and 65.17 % (AMB78). Thus there was decrease in percent drug retained entrapped with the increase in the temperature of storage. No major difference was observed due to effect of charge on stability for AMB LDPI formulations at freezer and refrigerated storage but at controlled room temperature and accelerated storage conditions after 6 months slight difference was observed. It may be due to higher proportion of CHOL being present in AMB78 (positively charged LDPI formulation). At accelerated storage i.e. near Tg (phase transition temperature), decrease in the PDR for LDPI formulations (particularly for AMB78) was observed due to increased fluidity of bilayer resulting to drug leakage. Higher PDR of AMB77 at controlled room and accelerated storage may be due to presence of higher proportion of amphiphilic PC leading to re-encapsulation of the leaked drug by the liposomes.

Table 6.1 Drug leakage profiles of LDPI formulations of AMK

	PERCENT DRUG RETAINED (%) Mean ± (SEM)*				
TIME IN MONTHS	**1**	**2**	**3**	**6**	**12**
Batch No.	**FREEZER CONDITION**				
AMK 69	100.36	98.10	95.64	92.53	84.27
	(1.23)	(0.95)	(0.57)	(0.82)	(0.94)
AMK 70	100.24	98.52	94.27	92.07	82.69
	(1.51)	(1.30)	(0.61)	(0.73)	(1.11)
	REFRIGERATED CONDITION				
AMK 69	98.60	96.95	93.48	91.34	79.31
	(1.40)	(0.92)	(1.02)	(1.26)	(0.92)
AMK 70	98.27	95.83	94.15	91.23	77.57
	(1.67)	(1.15)	(0.69)	(1.08)	(1.32)
	CONTROLLED ROOM TEMPERATURE CONDITION				
AMK 69	96.47	93.20	90.82	86.25	68.30
	(0.76)	(0.64)	(0.57)	(0.73)	(1.49)
AMK 70	95.81	93.62	91.54	85.34	67.28
	(1.24)	(1.04)	(0.49)	(1.10)	(1.20)
	ACCELERATED CONDITION				
AMK 69	92.41	78.42	66.26	49.61	33.87
	(1.62)	(0.68)	(1.67)	(1.96)	(2.51)
AMK 70	92.60	76.30	65.92	45.73	30.47
	(1.91)	(1.25)	(1.16)	(1.62)	(2.20)

* Mean of nine determinations

Figure 6.1 Drug leakage profile under freezer and refrigerator storage of LDPI formulations of AMK



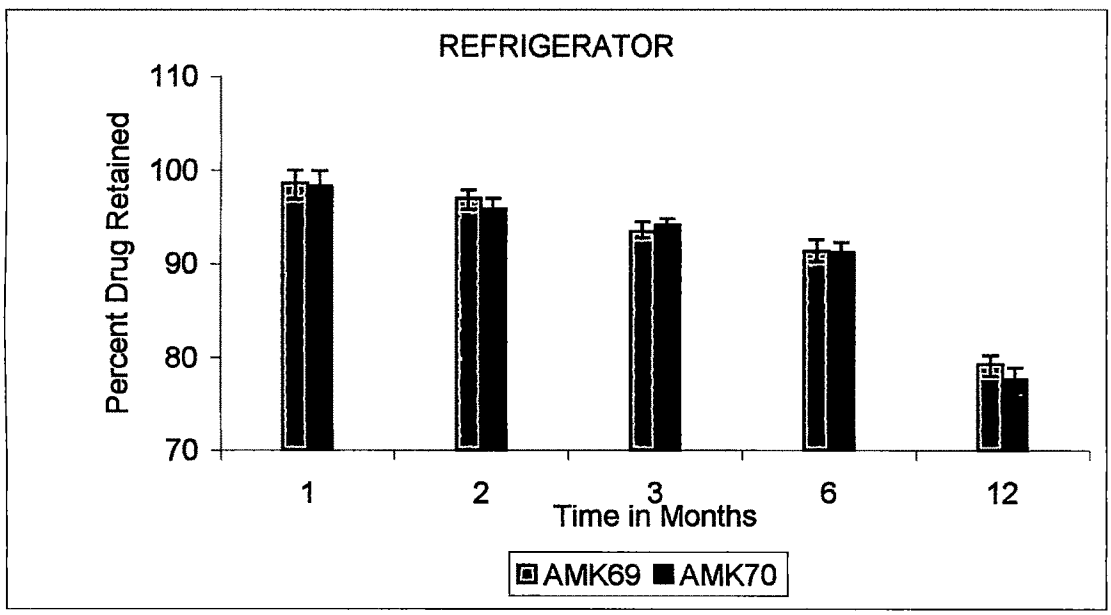


Figure 6.2 Drug leakage profile under controlled room temperature and accelerated storage of LDPI formulations of AMK

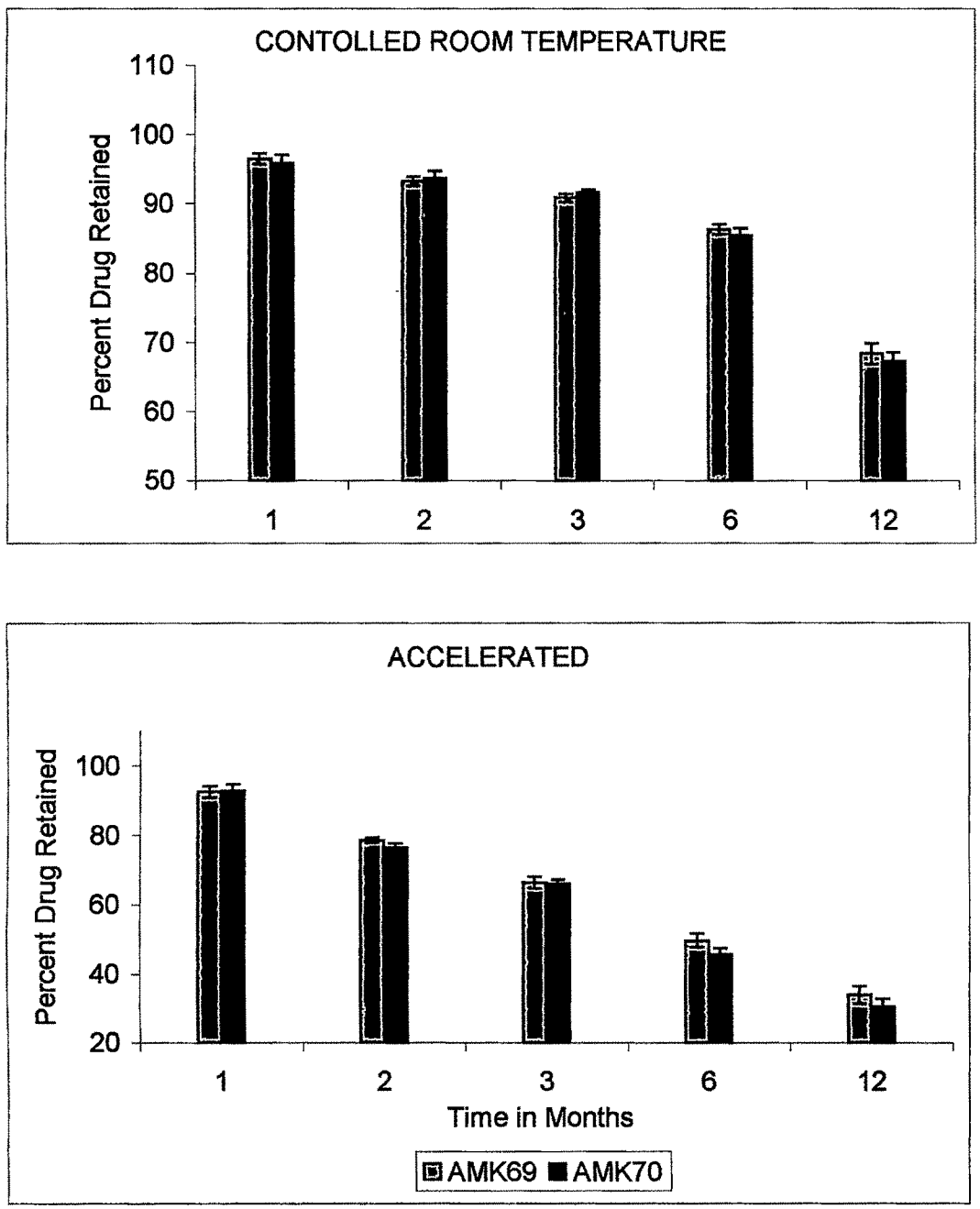


Table 6.2 Drug leakage profiles of LDPI formulations of AMB

	PERCENT DRUG RETAINED (%) Mean ± (SEM)*				
TIME IN MONTHS	**1**	**2**	**3**	**6**	**12**
Batch No.	**FREEZER CONDITION**				
AMB 77	99.21	97.92	96.24	94.8	93.17
	(0.67)	(1.15)	(1.52)	(0.94)	(0.86)
AMB 78	99.64	97.49	96.73	95.16	93.58
	(1.13)	(1.57)	(0.88)	(0.73)	(1.18)
	REFRIGERATED CONDITION				
AMB 77	98.52	97.16	95.33	94.27	91.24
	(0.95)	(1.18)	(1.64)	(0.58)	(1.02)
AMB 78	98.94	97.30	95.17	93.44	90.76
	(1.15)	(0.98)	(0.56)	(1.34)	(0.61)
	CONTROLLED ROOM TEMPERATURE CONDITION				
AMB 77	97.24	95.43	93.27	90.50	79.48
	(1.26)	(1.58)	(1.85)	(2.10)	(1.96)
AMB 78	98.17	96.75	92.81	88.36	76.16
	(1.10)	(1.42)	(1.70)	(1.97)	(1.64)
	ACCELERATED CONDITION				
AMB 77	94.25	91.47	83.43	73.49	66.67
	(1.28)	(1.63)	(1.67)	(1.36)	2.14
AMB 78	95.71	91.52	75.90	65.17	60.72
	(1.64)	(1.27)	(1.35)	(1.82)	(2.02)

* Mean of nine determinations

Figure 6.3 Drug leakage profile under freezer and refrigerator storage of LDPI formulations of AMB

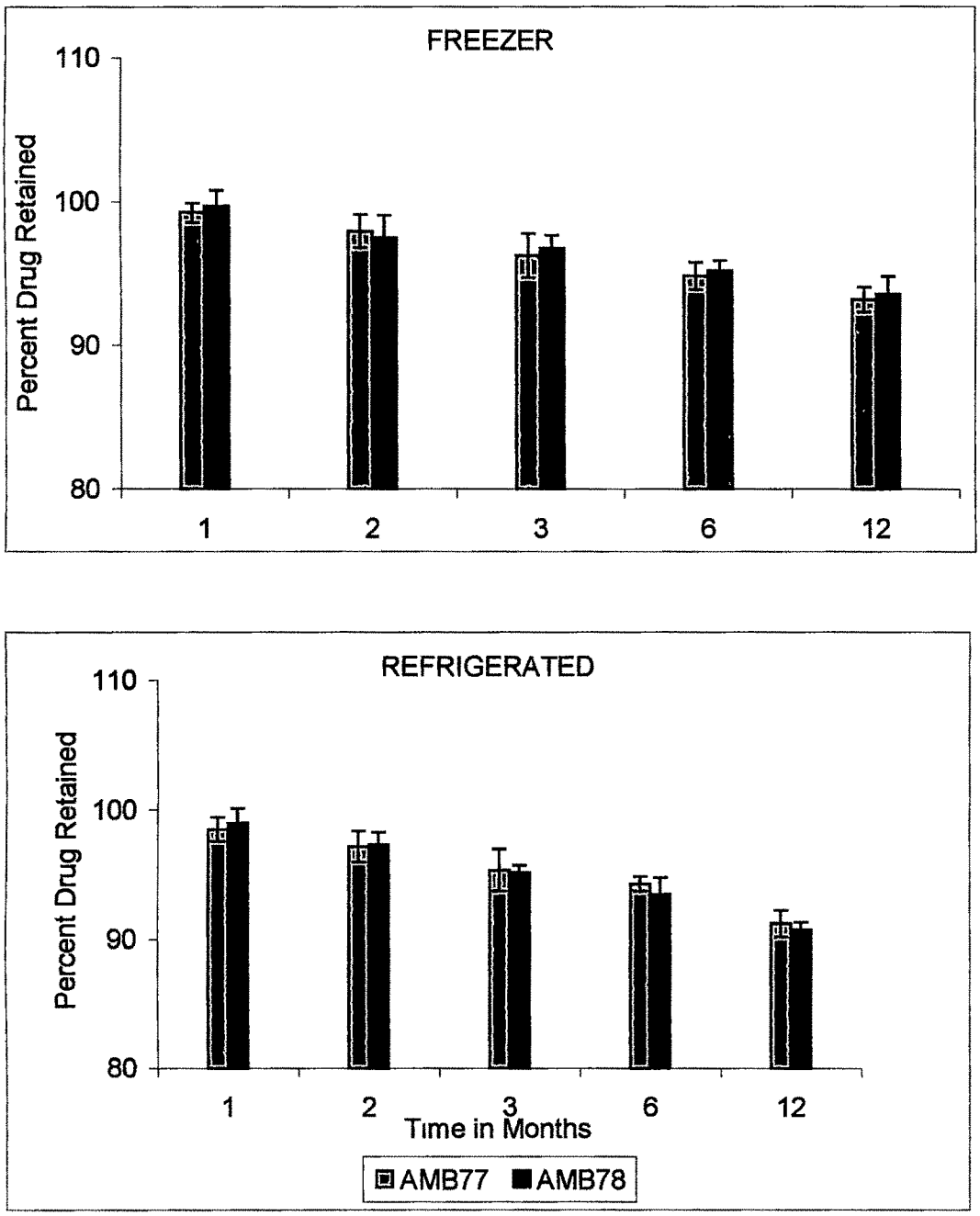
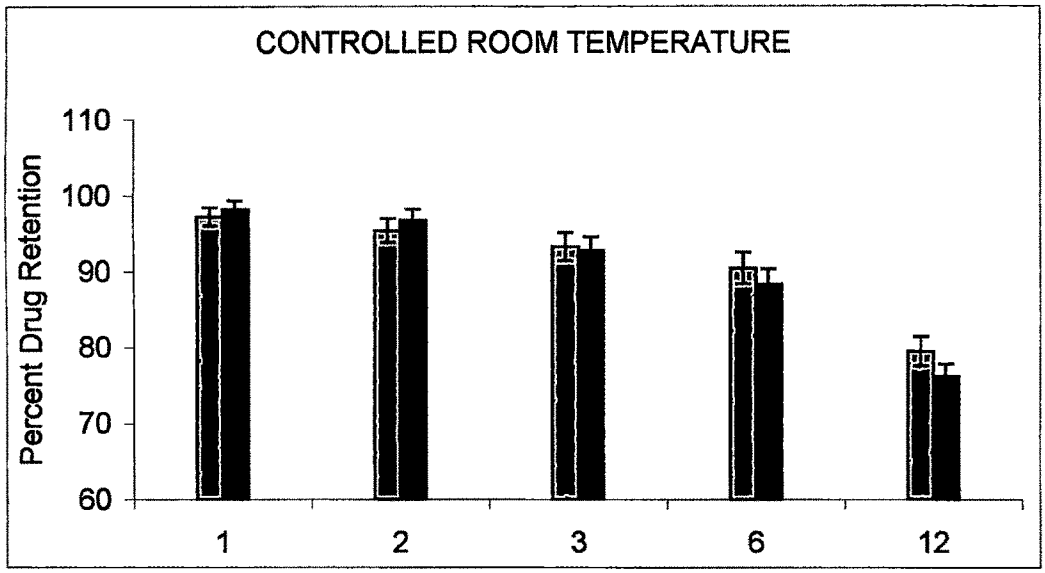
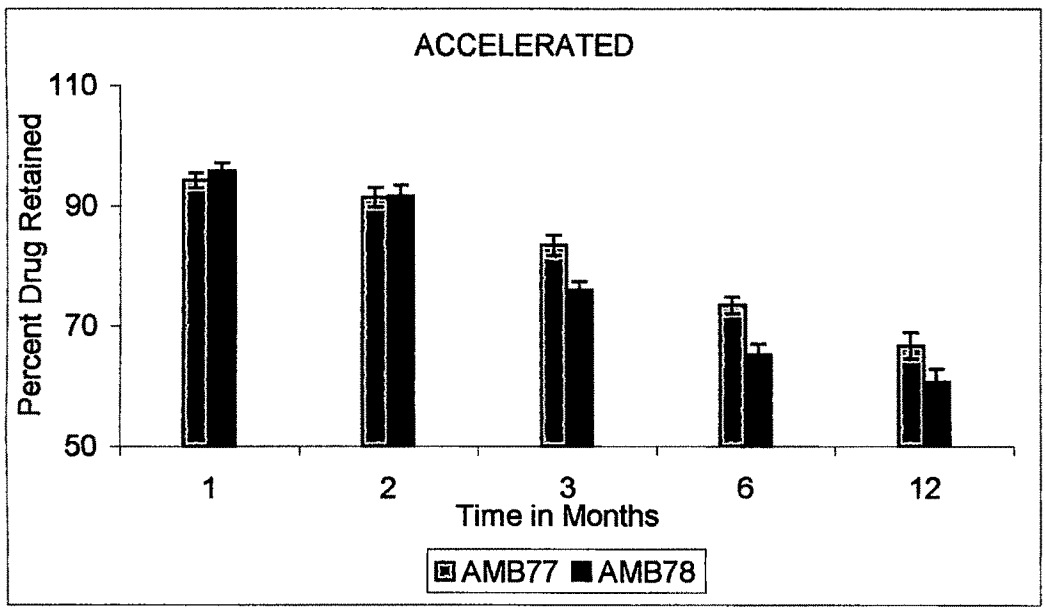


Figure 6.4 Drug leakage profile under controlled room temperature and accelerated storage of LDPI formulations of AMB





T90 (it refers to the time period when the drug preserved entrapped is 90 %) for accelerated storage condition it was between 1.5 months to 2.5 months (Figure 6.2 and 6.4), for controlled room temperature storage condition it was between 3.5 months to 6 months (Figure 6.2 and 6.4) and at refrigerated & freezer storage condition it was 9 months for AMK LDPI formulation and 12 months for AMB LDPI formulations (Figure 6.1 and 6.3).

After 9 months of storage of AMK LDPI formulations & 12 months storage for AMB LDPI formulations did not show significant ($p<0.05$) PDR at freezer and refrigerated conditions. It may be due to presence of CHOL in the formulation increasing the rigidity of membrane thereby increasing PDR below phase transition temperature of the bilayers for both drugs. PDR data of same formulations, when stored at controlled room temperature and accelerated storage, show significant decrease in PDR for AMB78 formulation compared to AMB77 LDPI formulation (66.67 % v/s 60.72%). At accelerated storage conditions, the fluidity of bilayer increases leading to leakage of drug from the bilayers. For AMB 77 LDPI formulation (negatively charged LDPI formulation), presence of more proportion of amphiphilic PC (70% v/s 50%) leads to re-encapsulation of leaked drug in to the bilayers; while there was less proportion of PC in AMB78 LDPI formulation (50% v/s 70%) and more proportion of CHOL (50% v/s 30%) leading to more fluid bilayer and hence more drug leakage from AMB78 LDPI formulation.

Table 6.3 Mean liposomal size range of LDPI formulations at different storage time and conditions

Batch No.	Initial Mean size (μm)	Controlled room temperature (3 months) Mean size (μm)	Refrigerator (6 months) Mean size (μm)	Refrigerator (12 months) Mean size (μm)
AMK69	2.01	2.02	2.01	2.09
AMK70	1.94	1.96	1.95	2.03
AMB77	1.84	1.84	1.84	1.86
AMB78	2.04	2.05	2.05	2.07

Thus a shelf life of 9 months for AMK LDPI formulations and 12 months for AMB LDPI formulations can be assigned. The increase in the shelf life with increase in the lipophilicity of drug is indicative of better potential for the delivery of hydrophobic drug entities by this delivery system. The increase in liposome size upon rehydration was determined from change in particle size for all the batches prior to and after storage at controlled room temperature (for 3 months) and refrigerated storage (for 6 months and 12 months) (Table 6.3). The differences were compared using Student's paired t-test at 95 % confidence level. $P = 0.0917$ ($P>0.05$) for controlled room temperature storage, $P = 0.1816$ for refrigerated storage (6 months) and $P = 0.1256$ ($P>0.05$) for refrigerated storage (12 months for AMB LDPI formulations) was obtained. For AMK LDPI formulations, refrigerated storage at 12 months yielded $P = 0.0374$ ($P<0.05$) which reveals that there is significant change in liposome size after 12 months of refrigerated storage condition. It can be concluded that change in liposomal mean size is non significant before and after storage up to 3 months controlled room temperature storage, 6 months refrigerated storage (for both formulations) and 12 months refrigerated storage for AMB LDPI formulation. The slight increase in the liposome size may be due to aggregation on storage that is of insignificant level.

The formulations were also observed for caking and discoloration of LDPI formulations. Marginal caking or discoloration was observed for batches stored under accelerated storage conditions after 3 months. These type of observations were less visible at controlled room temperature storage, not visible at refrigerated and freezer storage of both LDPI formulations.

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