CHAPTER 5

PREPARATION & CHARACTERIZATION OF LIPOSOMAL DRY POWDER INHALER

One of the main difficulties in practical application of liposomal products has been the long-term stability of the liposomes. Madden and Co-Workers for the first time addressed this problem (1985) and found that liposomes can be reduced to dry powders if they are dried in the presence of certain sugars. Lyophilization or freeze-drying is often used to stabilize various pharmaceutical products including viral vaccines, protein and peptide formulations, liposome, and small chemical drug formulations (Poste et al, 1983; Ostro et al, 1987). It has been proposed that sugars preserve membrane structure (cryoprotection) by hydrogen bonding to the phospholipid head group and effectively replacing the bound water (Crowe et al, 1973). Evidence in support of this hypothesis has been provided by differential scanning calorimetry and infrared spectroscopic studies (Crowe et al, 1984). Sugars when added to the liposome dispersion form a glassy matrix during freezing. This prevents fusion of the vesicles and provides protection against ice formation (Edwod et al, 1997).

This freeze-dried powder of liposomal formulation was further processed for preparing dry powder inhaler. An alternative approach for pulmonary delivery of liposomes has been reported by Taylor (1990) wherein liposomes containing cromolyn sodium were successfully evaluated for delivery through nebulization, a valid yet less patient compliant approach. A similar approach has also been proposed by Farr et al (1987) where EPC was dissolved in chlorofluorohydrocarbon blends in concentration up to 5% and pressurized. The size of liposome thus formed in situ was found to be depend upon lecithin concentration, blend vapour pressure and adapter orifice diameter making the delivery non-reproducible and thus not suitable for controlled release. The requirement for viable alternatives to ozone depleting metered dose inhalers, coupled with the

opportunity for dehydrating liposomes to powder form, make dry powder aerosol of liposomal drug an attractive choice for modulated inhalation drug delivery. Instead of freeze drying Goldbach et al (1993) have used spray drying of liposomes for pulmonary administration, but found that 65-80 % drug leakage occurred during the spray drying process.

Improving the drug delivery to the lungs from a DPI formulation can be possible by various techniques like smoothing the carrier surface (Ganderton et al, 1992), reducing the particle size of the carrier (French et al, 1993; Steckel et al 1997) and use of ternary powder mix formulation (Staniforth et al, 1996). Addition of micronized lactose to coarse lactose carrier was found to improve the dispersion and deaggregation of salbutamol sulphate and spray dried bovine serum albumin (Zeng et al, 1996; Lucas et al, 1998). Also, techniques like spray drying the drug with phospholipid composites in a suitable range for pulmonary delivery (Kim et al, 2001) or the dissolution of lecithin in chlorofluorohydrocarbon and the formation of liposomes in-situ (Farr et al, 1987) or nebulization of the preformed liposomes (McCallion et al, 1996) can be attempted for liposomal drug delivery to lungs. Recently, many microparticle systems have been reported to be used for pulmonary drug delivery such as oligosaccharide ester derivative (OED), (Davidson et al, 2003) biodegradable ether-anhydride polymer (Fiegel et al, 2004), sodium hyaluronate (Surendrakumar et al, 2003), and poly(lactic-co-glycolic acid) (PLGA) (Sethuraman et al, 2002).

Flow and dispersion characteristic of the developed liposomal DPI formulations are critically important in development of DPI products. These properties are a function of the principal adhesive forces that exist between particles including Van der Waals forces,

electrostatic forces and the surface tension of the adsorbed liquid layer (Hinds et al, 1982). However, prediction of powder rheology based on the potential interplay a number of physicochemical properties is extremely complicated. Instead, flow and dispersion properties are generally characterized using appropriate derived properties including, but not limited to angle of repose, bulk density, compressibility and dustability (Neumann et al, 1967). Thus it is important to identify and control critical parameters, both fundamental and derived to ensure optimum and consistent product performance.

5.1 Lyophilization of liposomes

5.1.1 AMK liposomes

Lyophilization was performed for 48 hours using different cryoprotectants such as maltose, dextrose, trehalose, lactose, and sucrose. Liposomal pellets obtained after centrifuging liposomal dispersions ($3.3 \times 10^6 g$, 2 hours) were suspended in 10 mM Succinate buffer, pH 6.5 containing 1 mM EDTA and containing either lactose or maltose or trehalose or sucrose or dextrose in mass ratio of lipid: sugar (1:2) for AMK33 to AMK42 formulations. PDR of liposomes following dehydration-rehydration cycle were determined. Similarly addition sequence of cryoprotectant (sucrose) (formulations AMK43 to AMK48) and mass ratios of lipid: sucrose (formulations AMK49 to AMK56) was determined for PDR (Table 5.1).

5.1.2 AMB liposomes

Lyophilization was performed for 48 hours using different cryoprotectants such as maltose, dextrose, trehalose, lactose, and sucrose. Liposomal pellets obtained after centrifuging liposomal dispersions ($3.3 \times 10^6 g$, 2 hours) were suspended in 10 mM Tris buffer pH 6.5 containing 1 mM EDTA and containing either lactose or maltose or

trehalose or sucrose or dextrose in mass ratio of lipid: sugar (1:2) for AMB39 to AMB48 formulations. PDR of liposomes following dehydration-rehydration cycle were determined. Similarly addition sequence of cryoprotectant (sucrose) (formulations AMB49 to AMB54) and mass ratios of lipid: sucrose (formulations AMB55 to AMB64) was determined for PDR (Table 5.2).

5.2 Method of preparation of liposomal Dry powder Inhaler formulations

For the preparation of LDPI formulations, during preparation of liposomes by REV technique, amikacin liposomes were prepared by modified REV technique using 10mM Succinate buffer pH 6.5 containing 1 mM EDTA and containing 10% sucrose solution as optimized cryoprotectant such that the mass ratio of lipid: sucrose is 1:4; while for amphotericin B liposomes were prepared by modified REV technique using 10mM Tris buffer pH 6.5 containing 1 mM EDTA and containing 10% sucrose solution as optimized cryoprotectant such that the mass ratio of lipid: sucrose is 1:5. AMK and AMB liposomal drug formulations were extruded through 2μm polycarbonate membrane for 5 cycles and 7 cycles respectively and separated from unentrapped drug as described in chapter 3. The purified liposomal dispersion (1ml) was frozen at –40°C (Remi, India), in 10ml vial and lyophilized for 48 hours as described in previous section (section 5.2).

To formulate liposomal DPI formulation, series of experiments were carried out as out lined below:

5.3 AMK LDPI formulation

5.3.1 Effect of carrier addition

The liposomal dispersions containing sucrose as a cryoprotectant were frozen at –40°C and lyophilized for 48 hours. The porous cakes thus formed were sized successively through #120 (125 ± 8.1 μm) and #240 (63 ± 5.3 μm) sieves for AMK57 and AMK58 respectively. Capsules (size "2") were filled with individually weighed powder containing 1000 ± 50 μg of AMK and packed under nitrogen atmosphere in HDPE bottles containing silica bags as dehumactant. Similarly, the sieved lyophilized liposomal powder (through 120# and 240 #) was mixed with lactose carrier (63-90 μm sieved Pharmatose 325M) in varying mass ratios from 1:1 to 1:6. The bottles were stored in a desiccator at refrigeration temperature (2-8°C) till further use. The deposition studies of these formulations were determined using a TSI (Apparatus A, British Pharmacopoeia) after aerosolization of five capsules at 60 L/min via Rotahaler (Cipla, India) as delivery device for AMK59 to AMK66 formulations for fine particle fraction (Table 5.3).

5.3.2 Effect of adding fines

The sieved lyophilized liposomal powders (through 120# and 240 #) were mixed with lactose carrier (63-90 μm Sieved Pharmatose 325M) containing 5% to 15% sieved Sorbolac 400 (#500 – 25μm) in mass ratios of liposome: lactose at 1:5 and these formulations were evaluated using TSI for AMK69 to AMK72 formulations for fine particle fraction (Table 5.3).

5.3.3 Effect of adding sequence of fine

In one set of experiment, the fines (10% sieved Sorbolac 400 – 25μm) were first mixed with lactose carrier (63-90 μm Sieved Pharmatose 325M) forming blend of lactose

and then with sieved lyophilized liposomes (through 120# and 240 #) in a mass ratio of liposome: lactose at 1:5 (Formulation A) for AMK69 and AMK70 respectively. In another set of experiment, the fines (10% sieved Sorbolac 400 – 25μm) were first mixed with sieved lyophilized liposomes (through 120# and 240 #) and then with lactose carrier (63-90 μm Sieved Pharmatose 325M) at same ratio (Formulation B) for AMK69 and AMK70 respectively. These formulations were evaluated using TSI for fine particle fraction (Table 5.3).

5.4 AMB LDPI formulation

5.4.1 Effect of carrier addition

The liposomal dispersions containing sucrose as a cryoprotectant were frozen at – 40°C and lyophilized for 48 hours. The porous cakes thus formed were sized successively through #120 (125 ± 8.1 μm) and #240 (63 ± 5.3 μm) sieves for AMB65 and AMB66 respectively. Capsules (size "2") were filled with individually weighed powder containing 250 ± 7 μg of AMB and packed under nitrogen atmosphere in HDPE bottles containing silica bags as dehumactant. Similarly, the sieved lyophilized liposomal powder (through 120# and 240 #) was mixed with Sorbolac 400 (lactose carrier) in varying mass ratios from 1:2 to 1:8. The bottles were stored in a desiccator at refrigeration temperature (2-8°C) till further use. The deposition studies of these formulations were determined using a TSI (Apparatus A, British Pharmacopoeia) after aerosolization of five capsules at 60 L/min via Rotahaler (Cipla, India) as delivery device for AMB67 to AMB74 formulations for fine particle fraction (Table 5.5).

5.4.2 Effect of adding fines

The sieved lyophilized liposomal powders (through 120# and 240 #) were mixed with Sorbolac 400 (lactose carrier) containing 5% to 15% fines (500# - 25μm sieved Pharmatose 325M) in mass ratios of liposome: lactose at 1:6 and these formulations were evaluated using TSI for AMB75 to AMB80 formulations for fine particle fraction (Table 5.5).

5.4.3 Effect of adding sequence of fine

In one set of experiment, the fines (10% sieved Pharmatose 325M – 25μm) were first mixed with Sorbolac 400 (lactose carrier) forming blend of lactose and then with sieved lyophilized liposomes (through 120# and 240 #) in a mass ratio of liposome: lactose at 1:6 (Formulation A) for AMB77 and AMB78 respectively. In another set of experiment, the fines (10% sieved Pharmatose 325M – 25μm) were first mixed with sieved lyophilized liposomes (through 120# and 240 #) and then with Sorbolac 400 (lactose carrier) at same ratio (Formulation B) for AMB77 and AMB78 respectively. These formulations were evaluated using TSI for fine particle fraction (Table 5.5).

Finally LDPI formulations containing 1000 ± 50 μg of AMK (69 ± 2 mg of AMK69 and AMK70) (Table 5.4) and 250 ± 7 μg of AMB (84.8 ± 2 mg for AMB77 and 71.0 ± 2 mg for AMB78) (Table 5.6) were evaluated using TSI at 30, 60 and 90L/min flow rate

5.5 Residual water content determination

The residual water content of prepared LDPI formulations (1g) was determined by Karl-Fischer titration (Van winden et al, 1997). Commercially available pyridine free reagent was used for the purpose of analysis. The reagent was standardized with known quantity of water (250mg). Before adding sample, 40ml of methanol was added into the titration

vessel and titrated with the reagent to an audiovisual end point to consume any moisture that may be present. The water content determination was carried out five times and results are recorded in Table 5.8.

5.6 Percent drug retained

Percent drug retained is the percentage of drug initially added, determined after lyophilization cycle and in stability samples. 100mg of powder was rehydrated with 1ml of distilled water with gentle, occasional agitation for 30 minutes. The liposomal dispersion thus obtained was separated from the drug leaked during lyophilization cycle by the method described in chapter 3 (Section 3.3.6 for AMK and Section 3.4.4 for AMB). Percent drug retained was further used to calculate the efficiency of lyophilization. The mean percent drug remained entrapped with its standard error of mean, for six determination is shown in Table 5.1 (AMK) and Table 5.2 (AMB).

5.7 Characterization of liposomal DPI formulations

5.7.1 Angle of repose

The pile of powder was carefully built up by dropping the powder material through a funnel tip till from height of 2cm (Carr et al, 1965). The angle of repose was calculated by inverting tangentially the ratio of height and radius of the formed pile (Table 5.8).

5.7.2 Compressibility index

The compressibility index was determined by tapping the formulation for 500 taps to reach plateau condition.

5.7.3 Particle size determination

The vesicle size of rehydrated liposomes was determined by laser diffraction technique using Mastersizer (Malvern Instruments ltd. UK) operating at a beam length of 2.40 mm

and range of lens at 300 mm. The LDPI formulations were rehydrated with equivalent proportion of distilled water for 30 minutes and obtained dispersion was centrifuged to remove lactose and subjected to particle size determination. The liposomal dispersions were concentrated before analysis so further diluted with the hydrating medium to a factor of 10,000. The mean liposomal size with their respective size range is summarized in Table 5.8 for AMK and AMB.

5.7.4 Photomicrography

All the batches of the LDPI formulations were hydrated and viewed under Olympus (BX40F4, Japan) with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity. Representative photomicrographs showing liposomal vesicles before lyophilization (figure 5.1) and liposomal vesicles after lyophilization (figure 5.2) for AMB 77 formulation.

5.7.5 SEM photomicrographs

Scanning electron microscopy (Philips XL30 ESEM, The Netherlands) of the representative LDPI formulation was carried out and photomicrographs of final formulations (AMK69, AMK70, AMB77 and AMB78) are shown in figure 5.3 – 5.6.

5.7.6 Fine particle fraction

The twin stage impinger (TSI) (Apparatus A, British Pharmacopoeia) was used to obtain the FPF values. The device is presented schematically in figure 5.7.

For AMK LDPI formulations, the volume of capturing solvent (water) in the upper (stage 1) and lower (stage2) were 7 and 30ml respectively in TSI (B.P. Apparatus A) (1993) while for AMB LDPI formulations, the volume of capturing solvent (methanol) in the upper (stage 1) and lower (stage2) were 7 and 30ml respectively in TSI. Rotahaler (Cipla,

India) was used as delivery device at flow rate of 30 ± 2 L/min, 60 ± 2 L/min and 90 ± 2 L/min for 5 s for 5 capsules. Fluidization of the formulation was achieved with aid of rotary vacuum pump (Model F16, Bharat, Banglore, India). The pump was previously set with a flow control valve to generate a relevant (19-21) air flow rate of 30, 60 & 90 L/min measured with Flow meter (Copley Instruments, UK). A trap of sulfuric acid was placed between the impinger and the vacuum pump to protect it from the vaporizing solvent. The inhaler body, capsule shells, mouthpiece, stage 1 and stage 2 were washed five times with water (for AMK) or methanol (for AMB) and analyzed to measure the amount of drug retained as described in chapter 3 (Section 3.3.6 for AMK and Section 3.4.4 for AMB). The fine particle dose (FPD) was denoted as the quantity (μg) of the particles per capsule that deposited in the lower stage of the TSI after aerosolization at 30 L/min, 60 L/min and 90 L/min. Each capsule contained a powder mass of 69 ± 2 mg equivalent to nominal dose of 1000 ± 50 μg AMK (AMK69 and AMK70) and 250 ± 7 μg of AMB (84.8 ± 2 mg for AMB77 and 71.0 ± 2 mg for AMB78). The recovered dose (RD) was taken as the total quantity of drug recovered per capsule after each actuation, while emitted dose (ED) was that emitted from the inhaler device. Percent emission was calculated as the percentage of emitted dose to total dose. Fine particle fraction (FPF) was the ratio of FPD to RD, while dispersibility was the percentage of FPD to ED (Table 5.4 for AMK and Table 5.6 for AMB formulations). As a control, a marketed preparation (Asthalin Rotacaps, Cipla Ltd., India) containing salbutamol sulphate powder was used and the FPF determined at 30 L/min, 60 L/min and 90 L/min flow rate using Rotahaler (Cipla, India) as delivery device (Table 5.4, 5.6).

5.8 Statistical analysis

Each batch was prepared six times and data from all experiments are expressed as mean ± SEM unless specified. Process variables were studied by comparing PDE of two batches having all other variables same. PDE was expressed as the percentage of the drug initially added. Similarly, the PDR is relative to the drug initially entrapped after lyophilization. Efficiency of lyophization was determined from PDR by the following formula :

$$\text{Percentage efficiency of lyophilization} = \frac{\text{Percentage of drug remained entrapped after lyophilization}}{\text{Percentage drug entrapped before Lyophilization}} \times 100$$

The compressibility index was calculated by the following formulae:

$$\frac{100\,(V_0 - V_f)}{V_0}$$

Where V_0 is the initial volume of the weighed sample &

V_f is the final volume of the sample after 500 taps

Effective index is the geometric mean of the total emitted dose and FPF, represented by the equation (Hino et al, 1998):

$$EI = \sqrt{(100 - DF) \times FPF}$$

Where, DF is the device fraction.

Significant differences were calculated by ANOVA and mutual differences were detected with Students t-test and differences at $P<0.05$ were considered as significant.

5.9 Results and discussion

Liposomes were prepared by REV method in the presence of cryoprotectant and formed dispersion was frozen at –40°C for 24 hours. In the initial experiments for selection of cryoprotectant and the phase of its addition, the liposomal dispersion was ultra centrifuged at 5.33 x 10^6 x g and the formed pellet of liposomes was re-suspended into the hydrating medium with cryoprotectant. Freezing followed by drying for 48 hours. In the process of freeze-drying the formulation variables were optimized to obtain dry powder of liposome with maximum efficiency of lyophilization. Liposomes were frozen slowly in deep freezer, rather than quickly submerging the samples in boiling nitrogen, to achieve higher retention of the entrapped drug (Edwoud et el, 1997).

5.9.1 Influence of formulation parameters

5.9.1.1 Selection of cryoprotectant

Liposomal dispersion of composition AMK31, AMK32, AMB37 and AMB38 obtained with the optimized variable (Chapter 4) were centrifuged in 20ml polypropylene tubes at 5.33 x 10^6 x g to pellet the liposomes. The liposomal pellet obtained after centrifuging liposomal dispersion was suspended in 10mM Succinate buffer pH 6.5 containing 1 mM EDTA (for AMK)/ 10mM Tris buffer pH 6.5 containing 1 mM EDTA (for AMB) containing either lactose or maltose or trehalose or sucrose or dextrose in mass ratio of lipid: sugar (1:2). PDR of liposomes following dehydration-rehydration cycle was determined. The results are shown in Table 5.1 (AMK) and 5.2 (AMB) reveal that Trehalose resulted in highest percent drug retained (PDR) (AMK 35, 69.38 %; AMK36 70.86 %; AMB41, 62.44 % and AMB42 58.38 %) as compared to sucrose (AMK41, 62.29 %; AMK42 64.02 %; AMB47, 60.33 % and AMB48, 56.04 %), lactose (AMK39,

41.84 %; AMK40, 42.91; AMB45, 48.57 % and AMB46, 45.59 %), dextrose (AMK37, 40.23 %; AMK38, 39.44 %; AMB43, 38.80 % and AMB44, 35.74 %) and maltose (AMK33 45.62 %; AMK34 43.84; AMB39, 45.82 % and AMB40 42.20 %). This is in good agreement with the work of Madden and Co-workers (1985) who examined the effectiveness of number of sugars in maintaining structural and functional properties of microsomal membranes at low mean liposomal size and found trehalose to be the most effective one. However, at higher concentrations, sucrose was found to be equally effective for liposomes with large mean size. Also, sucrose is easily available at low cost compared to trehalose and hence sucrose was selected as optimized cryoprotectant. Different sugars have markedly different effect on stability. The apparent difference between the ability of these sugars to preserve dry liposomes may be related to fundamental difference in their mode of interaction with the bilayer. Non-reducing disaccharides are the most effective at protecting against drug leakage during freeze-drying. Leopold and Vertucci (1987) have suggested that sugars that tend to crystallize more readily may not be as effective and so lactose and maltose despite of being disaccharides are not effective compared to sucrose.

Table 5.1 Selection and optimization of cryoprotectant for efficient lyophilization of AMK liposomes.

Batch No.	Variable studied	PDE Mean ± SEM	PDR Mean ± SEM	Percentage efficiency of lyophilization
SELECTION OF CRYOPROTECTANT (1:2, LIPOSOME: SUGAR)				
AMK33	Maltose	96.71 ± 1.97	45.62 ± 2.48	47.49 ± 2.05
AMK34	Maltose	98.54 ± 1.40	43.84 ± 1.88	44.46 ± 1.95
AMK35	**Trehalose**	**96.71 ± 1.97**	**69.38 ± 1.14**	**71.70 ± 1.57**
AMK36	**Trehalose**	**98.54 ± 1.40**	**70.86 ± 1.83**	**71.93 ± 1.82**
AMK37	Dextrose	96.71 ± 1.97	40.23 ± 2.40	41.56 ± 2.01
AMK38	Dextrose	98.54 ± 1.40	39.44 ± 1.22	40.08 ± 2.18
AMK39	Lactose	96.71 ± 1.97	41.84 ± 1.67	43.29 ± 1.86
AMK40	Lactose	98.54 ± 1.40	42.91 ± 2.14	43.50 ± 1.95
AMK41	Sucrose	96.71 ± 1.97	62.29 ± 2.47	64.43 ± 1.76
AMK42	Sucrose	98.54 ± 1.40	64.02 ± 2.27	64.91 ± 1.80
PHASE OF CRYOPROTECTANT ADDITION (SUCROSE)				
AMK43	External	96.71 ± 1.97	62.28 ± 2.48	64.35 ± 2.16
AMK44	External	98.54 ± 1.40	64.22 ± 2.31	65.20 ± 2.22
AMK45	Internal *	97.12 ± 1.97	54.64 ± 2.73	56.18 ± 2.57
AMK46	Internal *	98.60 ± 1.40	61.80 ± 1.48	62.57 ± 1.59
AMK47	**Both**	**97.12 ± 1.79**	**74.22 ± 2.42**	**76.45 ± 2.31**
AMK48	**Both**	**98.60 ± 1.58**	**77.83 ± 2.50**	**78.90 ± 2.24**
MASS RATIO OF SUCROSE (LIPID: SUCROSE)				
AMK49	1 : 2	97.12 ± 1.79	74.22 ± 2.44	76.45 ± 2.31
AMK50	1 : 2	98.60 ± 1.58	77.83 ± 2.50	78.90 ± 2.24
AMK51	**1 : 4**	**98.16 ± 1.67**	**97.59 ± 2.20**	**99.37 ± 1.86**
AMK52	**1 : 4**	**99.20 ± 1.82**	**98.50 ± 1.88**	**99.24 ± 1.54**
AMK53	1 : 6	98.49 ± 1.54	97.23 ± 2.54	98.75 ± 2.02
AMK54	1 : 6	99.64 ± 1.88	98.10 ± 1.72	98.42 ± 2.42
AMK55	1 : 8	98.76 ± 1.73	95.31 ± 1.47	96.66 ± 1.55
AMK56	1 : 8	99.72 ± 1.50	97.24 ± 2.20	97.48 ± 2.03

* The lipid: sucrose ratio of 1:2 was not maintained.

Table 5.2 Selection and optimization of cryoprotectant for efficient lyophilization of AMB liposomes.

Batch No.	Variable studied	PDE Mean ± SEM	PDR Mean ± SEM	Percentage efficiency of lyophilization
SELECTION OF CRYOPROTECTANT (1:2, LIPOSOME: SUGAR)				
AMB39	Maltose	95.84±1.50	45.82 ±1.86	47.78 ± 2.02
AMB40	Maltose	87.92±1.33	42.20 ±2.48	47.85 ± 2.31
AMB41	**Trehalose**	**95.84±1.50**	**62.44 ± 1.90**	**65.14 ± 1.84**
AMB42	**Trehalose**	**87.92±1.33**	**58.38 ± 2.04**	**66.42 ± 2.00**
AMB43	Dextrose	95.84±1.50	38.80 ± 2.16	40.43 ± 1.86
AMB44	Dextrose	87.92±1.33	35.74 ± 2.32	40.66 ± 2.01
AMB45	Lactose	95.84±1.50	48.57 ± 2.20	50.69 ± 2.16
AMB46	Lactose	87.92±1.33	45.59 ± 2.04	51.81 ± 1.94
AMB47	Sucrose	95.84±1.50	60.33 ± 2.29	62.88 ± 2.09
AMB48	Sucrose	87.92±1.33	56.04 ±2.10	63.79 ± 1.80
PHASE OF CRYOPROTECTANT ADDITION (SUCROSE)				
AMB49	External	95.84±1.50	60.27 ± 2.32	62.86 ± 1.95
AMB50	External	87.92±1.33	56.03 ± 2.08	63.71 ± 1.75
AMB51	Internal *	96.40±1.85	48.56 ± 2.55	50.29 ± 2.31
AMB52	Internal *	88.62±1.47	42.45 ± 1.93	47.85 ± 2.08
AMB53	**Both**	**96.40±1.85**	**70.34 ± 2.12**	**73.01 ± 1.99**
AMB54	**Both**	**88.62±1.47**	**66.21 ± 2.17**	**74.67 ± 2.04**
MASS RATIO OF SUCROSE (LIPID: SUCROSE)				
AMB55	1 : 2	96.40±1.85	70.34 ± 2.11	73.01 ± 1.99
AMB56	1 : 2	88.62±1.47	66.21 ± 2.24	74.67 ± 2.04
AMB57	1 : 4	96.68±1.98	83.40 ± 1.86	86.15 ± 2.14
AMB58	1 : 4	92.60±1.79	80.16 ± 2.07	86.62 ± 1.83
AMB59	**1 : 5**	**98.50±1.56**	**96.58 ± 1.81**	**98.12 ± 1.55**
AMB60	**1 : 5**	**96.22±1.87**	**94.73 ± 2.43**	**98.48 ± 2.05**
AMB61	1 : 6	98.89±1.32	96.42 ± 2.00	97.55 ± 1.81
AMB62	1 : 6	96.62±2.05	95.10 ±1.86	98.36 ± 2.06
AMB63	1 : 8	98.30±2.16	96.03 ± 1.57	97.61 ± 1.94
AMB64	1 : 8	95.92±1.77	94.55 ± 2.33	98.47 ± 1.52

* The lipid: sucrose ratio of 1:2 was not maintained.

5.9.1.2 Phase of cryoprotectant addition

In the experiments (AMK43, AMK44, AMB49 and AMB50) sucrose was present only in the external phase during freeze-drying. To study the effect of its presence in the internal aqueous phase, AMK liposomes were prepared using 10mM Succinate buffer pH 6.5 containing 1 mM EDTA and containing 10% sucrose solution as optimized cryoprotectant such that the mass ratio of lipid: sucrose is 1:2; while for amphotericin B liposomes were prepared by modified REV technique using 10mM Tris buffer pH 6.5 containing 1 mM EDTA and containing 10% sucrose solution as optimized cryoprotectant such that the mass ratio of lipid: sucrose is 1:2. The formed liposomal dispersion after separation of unentrapped drug was centrifuged at $5.33 \times 10^6 \times g$ to form pellet of liposomes. The pellet was re-suspended into the hydrating medium without sucrose for both drugs (AMK45, AMK46, AMB51 and AMB52). In a similar set of experiment, where the effect of presence of sucrose in both the phases was investigated and the pellet obtained after centrifugation was again re-suspended in the same composition of hydrating medium (lipid: sucrose ratio to be 1:2) (AMK47, AMK48, AMB53 and AMB54). When sucrose was present only on the outside of the vesicles, less lyophilization efficiency was achieved i.e. AMK43 (64.35 %), AMK44 (65.20 %), AMB 49 (62.86 %) and AMB50 (63.71 %). Similarly, when sucrose was present only on inside of the liposomes, AMK45 (56.18 %), AMK46 (62.57 %), AMB51 (50.29%) and AMB52 (47.85 %) lyophilization efficiency was obtained. In comparison to these two both set of experiments, when sucrose was present on both side of bilayer; AMK47 (76.45 %), AMK48 (78.90%), AMB53 (73.01%) and AMB54 (74.67 %) lyophilization efficiency was achieved (Table 5.1 and 5.2). Damage to the liposomes due to freezing may be

related to osmotic dehydration. The presence of osmogen (sucrose) depresses the freezing point, which ultimately results in the difference of freezing point among the solution of either phase when sucrose was present only at one side. As a result, dehydration of liposomes occurs when extra liposomal water freezes prior to the liposome contents and vice versa. Thus sucrose should be present on both sides of the bilayer to preserve its structure in the dry state (Crowe et al, 1986, 1987). It can be concluded that during freeze-drying process of liposomes, liposomes constrict and get coated on the optimum surface of crystallized sugar. Hydration of polar head groups with hydroxyl group of sucrose leads to stabilization of liposomes. If the sucrose concentration is less than optimum, the crystallized sugar does not provide adequate surface for the adherence of constricted bilayer leading to drug leakage. Hence, it may be concluded that the bulk concentration of sugar required as cryoprotectant depends upon the type of sugar selected and saturation of the polar head groups of the bilayer by drug or other formulation components. The lamellarity and size of liposomes are expected to change these requirements. The above conclusion is based on the assumption that the liposomal vesicles when lyophilized in presence of sucrose retain their contents without disrupting the bilayer structures.

5.9.1.3 Mass ratio of lipid: sucrose

The samples were sequentially diluted with sucrose solution so as to obtain mass ratio as shown in Table 5.1 and 5.2 for AMK and AMB respectively. From the results, it is evident that mass ratio of lipid: sucrose is important parameter for further stabilization of liposomal drugs. A mass ratio of 1:4 for AMK and mass ratio of 1:5 for AMB was found to be optimum with high lyophilization efficiency (AMK51, 99.37 %; AMK52 99.24 %;

AMB59 98.12 % and AMB60, 98.48 %). Further increase in the lipid to sucrose mass ratio did not show any improvement in percentage efficiency of lyophilization for both drugs. It may be due to sufficient dilution of liposome in the sugar solution is required to have the polar head groups to be saturated with sugar molecules and to protect the liposomes from the deleterious effect of icing sugar (Crowe and Crowe, 1988). Thus it can be concluded that optimal lipid to sucrose mass ratio is required to retain drug in the liposomes during freeze-drying.

5.9.2 Optimization of LDPI formulation

5.9.2.1 AMK LDPI formulation development

To formulate AMK LDPI formulation, series of experiments were conducted as described in the method section of this chapter (Section 5.3.1). Lyophilized liposomes when formulated as LDPI formulation without using any carrier molecule resulted in low FPF value. This observation describes the importance of addition of lactose carrier in formulating the LDPI formulation. Pharmatose 325M was sieved to get 63-90 μm size range fraction as a carrier to formulate LDPI formulations. The lyophilized liposomes were mixed with sieved Pharmatose 325M (63-90 μm) in range of liposome: lactose mass ratio from 1:1 to 1:6 and its effect on FPF were studied. The data revealed the optimum liposome: lactose mass ratio of 1:5 (Table 5.3). Optimum concentration of carrier is required to achieve detachment of liposomal drug from carrier molecule. Carrier concentration is less or more than optimum resulted in to low FPF or no further increase in FPF. Further, effect of adding fines (sieved Sorbolac-400 through 500#) in 5%, 10% & 15% proportion with carrier (63-90μm) keeping final liposome: lactose mass ratio of 1: 5.

Table 5.3 Optimization of AMK LDPI formulation

Batch No.	Variable studied	FPF * (Mean ± SEM)
Effect of liposome: lactose ratio		
AMK57	1: 0	7.5 ± 2.1
AMK58	1: 0	6.8 ± 2.3
AMK59	1: 1	9.8 ± 2.4
AMK60	1: 1	8.3 ± 2.5
AMK61	1: 3	11.6 ± 2.0
AMK62	1: 3	10.3 ± 1.5
AMK63	**1: 5**	**14.5 ± 1.6**
AMK64	**1: 5**	**12.9 ± 1.8**
AMK65	1: 6	14.8 ± 2.2
AMK66	1: 6	13.5 ± 2.4
Effect of sieved lactose (liposome: lactose ratio was 1:5)		
AMK67	5 %	19.4 ± 2.2
AMK68	5 %	24.6 ± 2.4
AMK69	**10 %**	**25.9 ± 1.8**
AMK70	**10 %**	**29.2 ± 2.1**
AMK71	15 %	22.1 ± 1.5
AMK72	15 %	25.2 ± 2.0

* n = 6 (±SEM) at 60l min^{-1}

Table 5.4 Comparative characterization of potential batches of AMK LDPI formulations

Parameters	AMK69						AMK70					
	30L/min		**60L/min**		90L/min		30L/min		**60L/min**		90L/min	
	Mean	***SD***	Mean	***SD***	Mean	***SD***	Mean	***SD***	Mean	***SD***	Mean	***SD***
FPD (μg)	213.6	6.9	256.4	8.2	292.1	6.5	244.8	5.8	294.9	7.6	345.4	6.4
FPF (%)	21.8	2.2	25.9	1.8	29.5	2.6	24.6	2.4	29.2	2.1	34.2	2.0
Dispersibil-ity (%)	24.7	1.8	29.1	1.6	33.0	1.9	29.4	1.5	34.6	1.4	40.3	1.7
Effective Index	43.8	1.6	41.9	1.5	51.4	1.7	45.4	1.8	49.7	1.8	53.9	1.9
Control: Asthalin (Cipla Ltd., India): Delivery device: Rotahaler (Cipla Ltd., India) **FPF = 27.1 ± 2.0, EI = 48.6 ± 1.7 at 60L/min**												

At 10% level of fines, high-energy adhesion sites (HA) of lactose may bind strongly to the liposomal drug particles and low-energy adhesion sites (LA) may allow the formation of more reversible bonds with liposomal drug. This results in efficient detachment of liposomal drug from the carrier as observed with plain DPI formulations (Staniforth et al, 1996). Hence, 10% sieved Pharmatose 325M added to AMK LDPI formulation occupies HA sites leaving LA sites for attachment of liposomal drug and thus resulted in higher FPF. This observation was also confirmed by observing the effect of adding sequence of fines to the liposomal formulation. Blending the fines (10% sieved Sorbolac 400) with carrier (63-90 μm) resulted in higher FPF with interestingly different device fraction (Table 5.4). Liposomal drug powder adheres to carrier particles as seen in scanning electron microscopy photographs of AMK LDPI formulations (Figure 5.3 and 5.4). The FPD (μg), FPF (%), Dispersibility (%) and Emission (%) at 30, 60 & 90 L/min flow rate using Rotahaler (Cipla, India) as dispersing device are shown in Table 5.4.

5.9.2.2 AMB LDPI formulation development

Similarly to formulate AMB LDPI formulation, series of experiments were conducted as described in the method section of this chapter (Section 5.3.2). Lyophilized liposomes when formulated as LDPI formulation without using any carrier molecule resulted in low FPF value. This observation describes the importance of addition of lactose carrier in formulating the LDPI formulation. Sorbolac 400 was used as a carrier to formulate LDPI formulations. The lyophilized liposomes were mixed with Sorbolac 400 in range of liposome: lactose mass ratio from 1:2 to 1:8 and their effects on FPF were studied. The data revealed the optimum liposome: lactose mass ratio of 1:6 (Table 5.5).

Table 5.5 Optimization of AMB LDPI formulation

Batch No.	Variable studied	FPF * (Mean ± SEM)
Effect of liposome: lactose ratio		
AMB65	1: 0	4.5 ± 2.0
AMB66	1: 0	2.5 ± 1.4
AMB67	1: 2	12.3 ± 2.2
AMB68	1: 2	8.5 ± 2.4
AMB69	1: 4	15.1 ± 3.0
AMB70	1: 4	11.6 ± 2.2
AMB71	**1: 6**	**17.5 ± 2.4**
AMB72	**1: 6**	**13.2 ± 3.1**
AMB73	1: 8	16.4 ± 2.7
AMB74	1: 8	11.9 ± 2.8
Effect of sieved lactose (liposome: lactose ratio was 1:5)		
AMB75	5 %	19.2 ± 2.6
AMB76	5 %	14.9 ± 2.5
AMB77	**10 %**	**25.3 ± 1.8**
AMB78	**10 %**	**19.6 ± 1.5**
AMB79	15 %	20.1 ± 1.9
AMB80	15 %	14.6 ± 2.3

* n = 6 (±SEM) at 60 L/min

Table 5.6 Comparative characterization of potential batches of AMB LDPI formulations

Parameters	AMB77						AMB78					
	30L/min		60L/min		90L/min		30L/min		60L/min		90L/min	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FPD (μg)	56.6	1.8	64.5	1.5	73.9	2.0	40.7	1.7	47.0	1.4	56.1	1.8
FPF (%)	23.1	1.5	25.3	1.8	28.4	2.1	17.3	2.2	19.6	1.5	22.9	1.9
Dispersibil-ity (%)	26.1	1.5	28.4	2.2	31.8	2.1	20.8	2.4	23.4	1.8	27.1	1.3
Effective Index	45.2	1.2	47.5	1.9	50.4	1.4	37.9	2.0	40.5	1.6	44.0	1.7
Control: Asthalin (Cipla Ltd., India): Delivery device: Rotahaler (Cipla Ltd., India) **FPF = 27.1 ± 2.0, EI = 48.6 ± 1.7 at 60L/min**												

Optimum concentration of carrier is required to achieve detachment of liposomal drug from carrier molecule. Carrier concentration is less or more than optimum resulted in to low FPF or no further increase in FPF. Further, effect of adding fines (sieved Pharmatose 325M through 500#) in 5%, 10% & 15% proportion with carrier (Sorbolac 400) keeping final liposome: lactose mass ratio of 1: 6. At 10% level of fines, high-energy adhesion sites (HA) of lactose may bind strongly to the fines and low-energy adhesion sites (LA) may allow the formation of more reversible bonds with liposomal drug. This results in efficient detachment of liposomal drug from the carrier as observed with plain DPI formulations (Staniforth et al, 1996). Hence, 10% sieved Pharmatose 325M added to AMB LDPI formulation occupies HA sites leaving LA sites for attachment of liposomal drug and thus resulted in higher FPF. This observation was also confirmed by observing the effect of adding sequence of fines to the liposomal formulation. Blending the fines (10% sieved Pharmatose 325M) with carrier (Sorbolac 400) resulted in higher FPF with interestingly different device fraction (Table 5.6). Liposomal drug powder adheres to carrier particles as seen in scanning electron microscopy photographs of AMB LDPI formulations (Figure 5.5 and 5.6). The FPD (μg), FPF (%), Dispersibility (%) and Emission (%) at 30, 60 & 90 L/min flow rate using Rotahaler (Cipla, India) as dispersing device are shown in Table 5.6.

The batches namely AMK69, AMK70, AMB77 and AMB78 were selected for the further characterization studies. The formulation components of these potential batches are shown in detail in Table 5.7

Table 5.7 Essential formulation components of the LDPI batches selected for the further studies.

Batch No.	Drug (μg)	HSPC (mg)	α-Tocopherol (μg)	CHOL (mg)	SPG-3 (μg)	SA (μg)	Sucrose (mg)	Lactose (mg)	Total (mg)
AMK69	1000	2.122	21.22	0.525	107	N.A.	8.548	57.2	68.6
AMK70	1000	2.122	21.22	0.525	N.A.	344.6	8.548	57.2	68.6
AMB77	250	1.890	18.9	0.359	118	N.A.	8.234	73.64	84.76
AMB78	250	1.479	14.79	0.662	N.A.	454	6.762	61.28	70.8

5.9.3 Characterization of LDPI formulations

The residual water content of the prepared LDPI formulations (1g) was determined by Karl-Fischer Titration (Van Winden et al, 1997). The formulations had residual water content (<2 %), which confirms its low aggregation tendency (Table 5.8).

Evaluation and control of flow and dispersion (deaggregation) characteristics of the formulation are of critical importance in the development of DPI products. Inter-particle forces that influence flow and dispersion properties are particularly dominant in micronize or microcrystalline powders required for inhalation therapy (< 5μm) (Gonda, 1992; Hickey, 1996). It has been demonstrated that powder adhesion, mediated in part by Van der Waal forces, is directly related to particles < 10 μm (Hickey et al 1996). Predictions of powder rheology based on the possible relationship of a number of physicochemical properties are extremely complicated. Hence, flow and dispersion properties like angle of repose, dispersibility index, compressibility index, moisture content and FPF are characterized and controlled (Table 5.8).

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Nelson (1955) was the first to use angle of repose measurements to determine the flow properties of pharmaceutical materials. The angle of repose for the formulations fall in the range of 27.1 (AMK69) to 29.7 (AMB78) degrees, where as Carr (1965) concluded that an angle of repose of 25 degrees as optimum with cent percent points awarded in the point score evaluation proposed by him.

Table 5.8 Characterization of LDPI formulations

Variable studied	Amikacin sulphate		Amphotericin B	
	AMK69	AMK70	AMB77	AMB78
Mean size (liposomes)	2.05 ± 0.25	1.93 ± 0.32	1.86 ±0.23	2.03 ± 0.34
Residual water content (%)	1.6 ± 0.8	1.9 ± 0.9	1.4 ± 1.0	1.5 ± 0.7
Angle of Repose	27.1 ± 0.4	28.7 ± 0.5	28.3 ± 0.6	29.7 ± 0.4
Compressibility index	23.8 ± 2.4	21.9 ± 2.0	23.5 ± 1.8	22.3 ± 2.4
FPD (µg)	256.4 ± 8.2	294.9 ± 7.6	64.5 ± 1.5	47.0 ± 1.4
FPF (%)	25.9 ± 1.8	29.2 ± 2.1	25.3 ± 1.8	19.6 ± 1.5
Emission (%)	88.9 ± 2.0	84.4 ± 1.7	89.1 ± 1.6	83.7 ± 1.9
Dispersibility	29.1 ± 1.6	34.6 ± 1.4	28.4 ±1.0	23.4 ± 0.6
Effective Index	41.9 ± 1.5	46.1 ± 1.8	47.5 ± 1.9	40.5 ± 1.6
Control: Ashthalin Capsules (Cipla Ltd., India): FPF = 27.1 ± 2.0, EI = 48.6 ± 1.7 n = 6 at 60 l min^{-1}.				

The flowability and floodability expressed by angle of repose (27.1 to 29.7°), compressibility index (21.9 - 23.8) falls under category of good and floodable, in the point score evaluation expressed by Carr (1965). This suggests that there is no significant interparticulate interactions among the LDPI formulations and assure optimal dispersion in stream of air upon inhalation.

The liposomal size of rehydrated liposomes was determined by laser diffraction spectroscopy using Mastersizer 2000 (Malvern Instruments Ltd. UK) operating at a beam length of 2.40 mm and range of lens at 300 mm. There was no significant change in the liposome size by lyophilization (Table 5.9). Thus it could be concluded that after freeze drying according to this protocol provided sufficient cryoprotection to the liposomes.

All the batches of LDPI formulations were rehydrated and viewed under Olympus (BX 40F4, Japan) with attachment of polarizing lens to study their shape and lamellarity. Representative photomicrographs showing liposomal vesicles before lyophilization (figure 5.1) and liposomal vesicles after lyophilization (figure 5.2) for AMB 77 formulation. The prepared liposomes were found to be multilamellar and identified by Maltese crosses in liposomal photomicrographs.

Table 5.9 Particle size range of LDPI formulations

Batch No.	Post extrusion Mean size (Size range) (μm)	Post rehydration Mean size (Size range) (μm)
Amikacin Sulphate		
AMK69	1.92 [0.50 – 28]	2.01 [0.50 – 28]
AMK70	1.88 [0.50 – 26]	1.94 [0.50 – 26]
Amphotericin B		
AMB77	1.76 [0.50 – 32]	1.84 [0.50 – 32]
AMB78	1.97 [0.50 – 35]	2.04 [0.50 – 35]

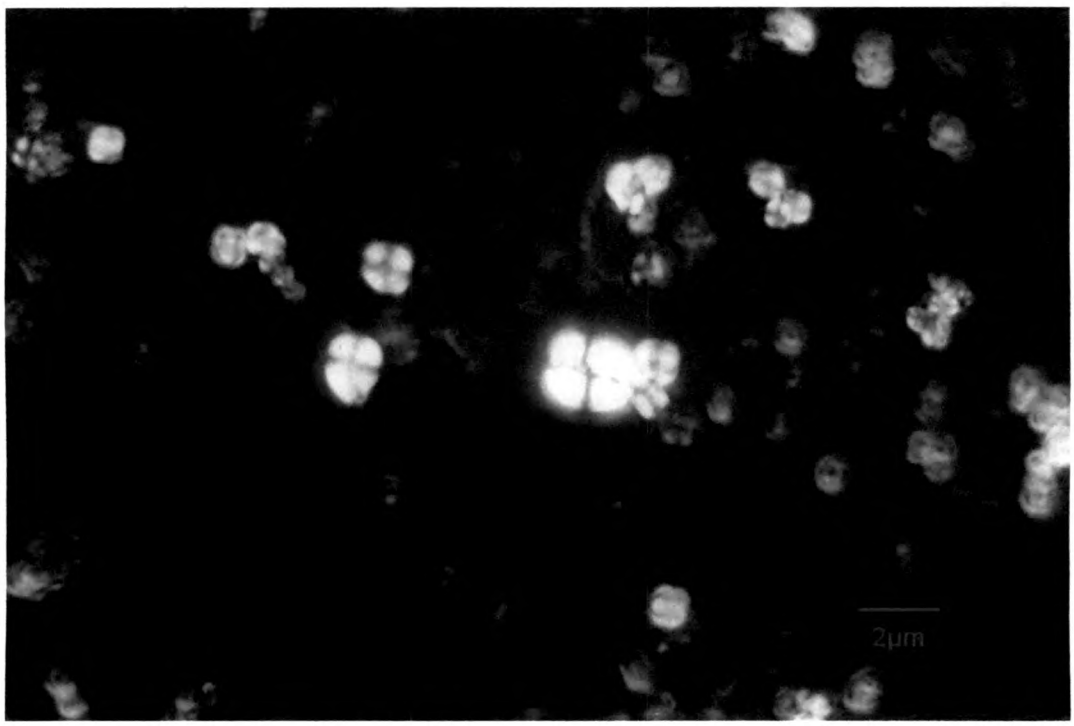

Figure 5.1 AMB liposomes before lyophilization under polarized light

Figure 5.2 AMB liposomes after lyophilization under polarized light

All four batches of LDPI formulations (AMK69, AMK70, AMB77 and AMB78) were viewed by Scanning Electron Microscopy (Philips XL30 ESEM, The Netherlands) and shown in figure 5.3, 5.4, 5.5 and 5.6 respectively. From SEM photographs, Liposomal drug powder adheres to carrier particles can be clearly differentiated. Thus it can be concluded that the liposomal powder detaches from carrier molecules upon inhalation.

In DPI formulations, certain particles may be aggregated to form free flowing spheres, reducing the surface free energy of the micronized powder. The aerosol performance of the agglomerated system is dictated predominantly by the interparticulate forces acting between the particles. Optimal inhalation performance requires that the dispersive forces, generated within the device upon patient's inspiration, exceed the strength of the interparticulate forces acting between the particles. The in-vitro aerosol behavior of the developed LDPI formulations was investigated in terms of respirable fraction or Fine particle fraction (FPF). The twin stage impinger (Apparatus A) (Figure 5.7) official in British Pharmacopoeia (1993) was used to obtain FPF values as reported in Table 5.8. Apparatus was set in-house as per BP specifications; FPF value of marketed preparation (Asthalin - Salbutamol sulphate DPI formulation, Cipla Ltd, India) was used as control. The data derived from these devices reflect the fraction of drug likely to deposited in the lungs upon inhalation. The particles passing to the lower impingement chamber (Stage 2) are respirable and considered as FPF.

Ideal LDPI formulation should provide small device fraction (effective emission from the device) and large FPF when inhaled. The FPF values (Table 5.8) for the LDPI formulation falls in the range of 19.6% (AMB78) to 29.2 (AMK70), which is comparable with that of the marketed control value (27.1%).

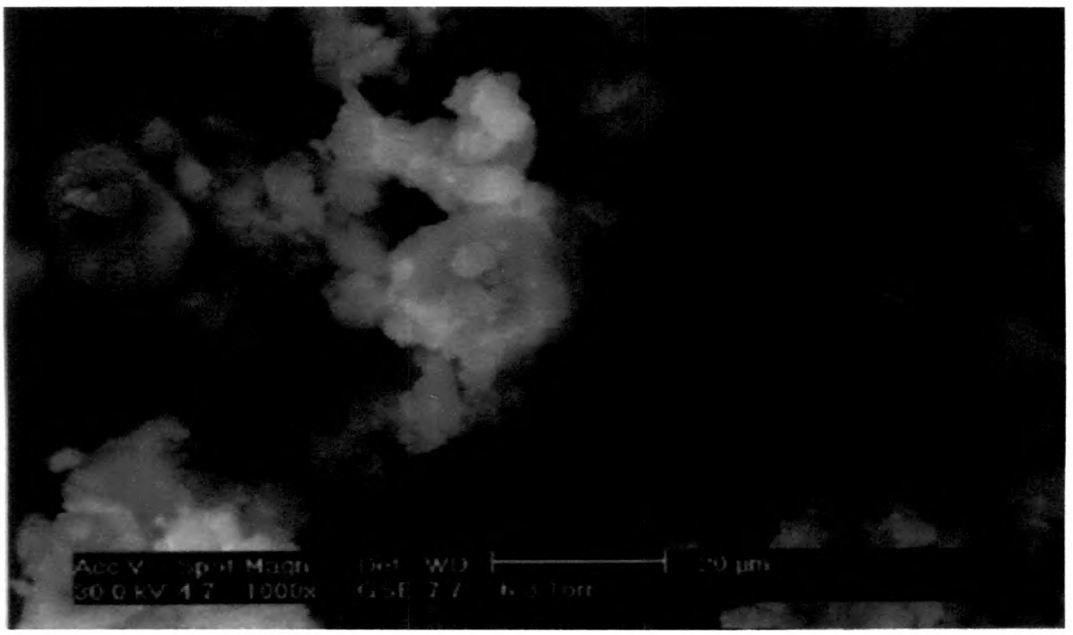

Figure 5.3 SEM photomicrographs of AMK69 (1000X)

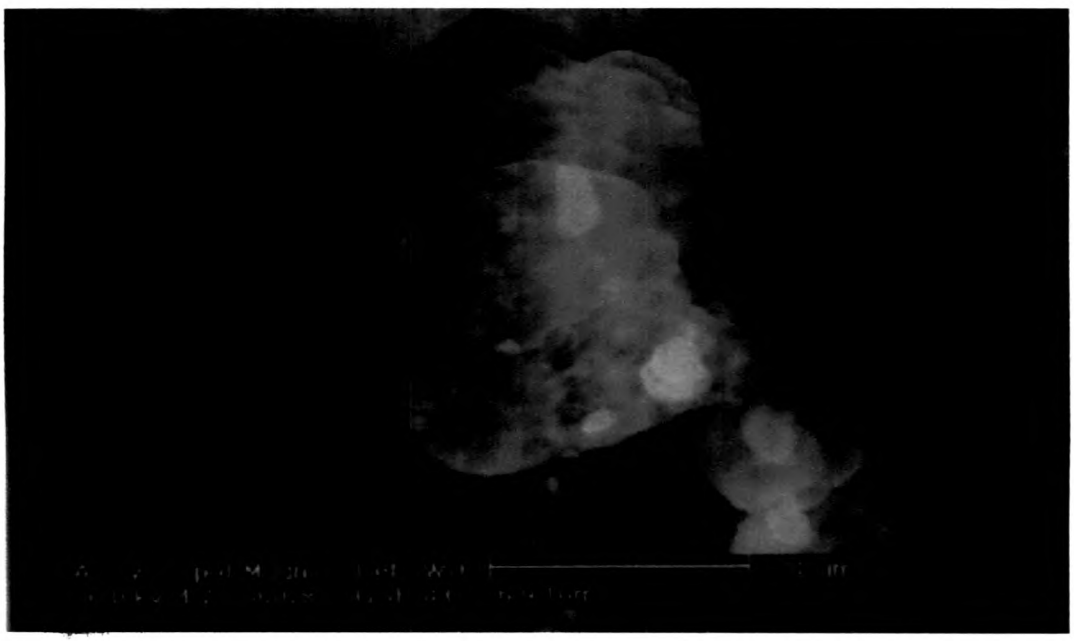

Figure 5.4 SEM photomicrographs of AMK70 (3000X)

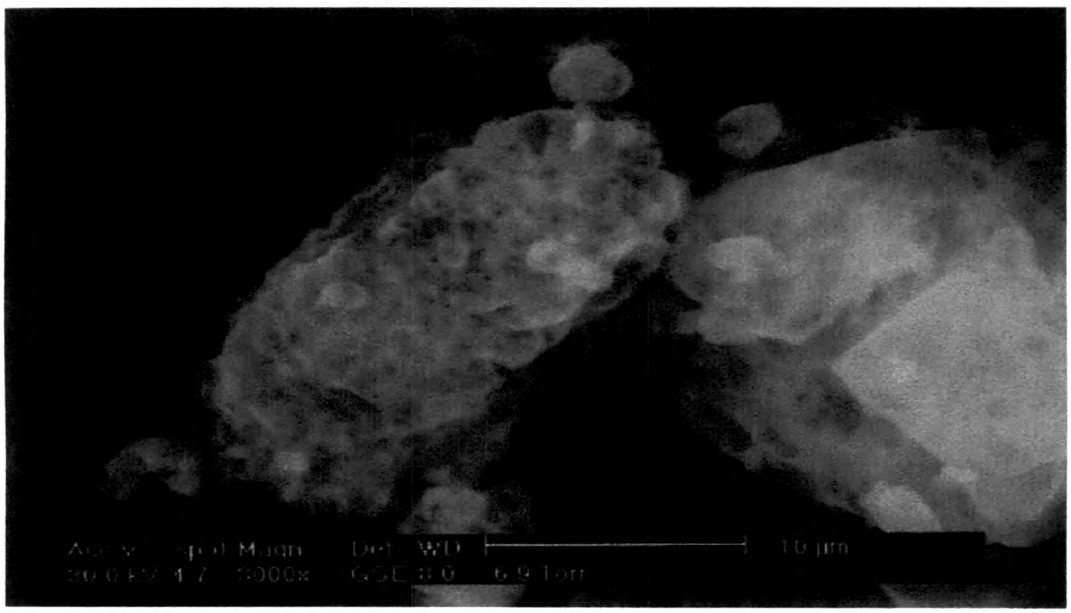

Figure 5.5 SEM photomicrographs of AMB77 (3000X)

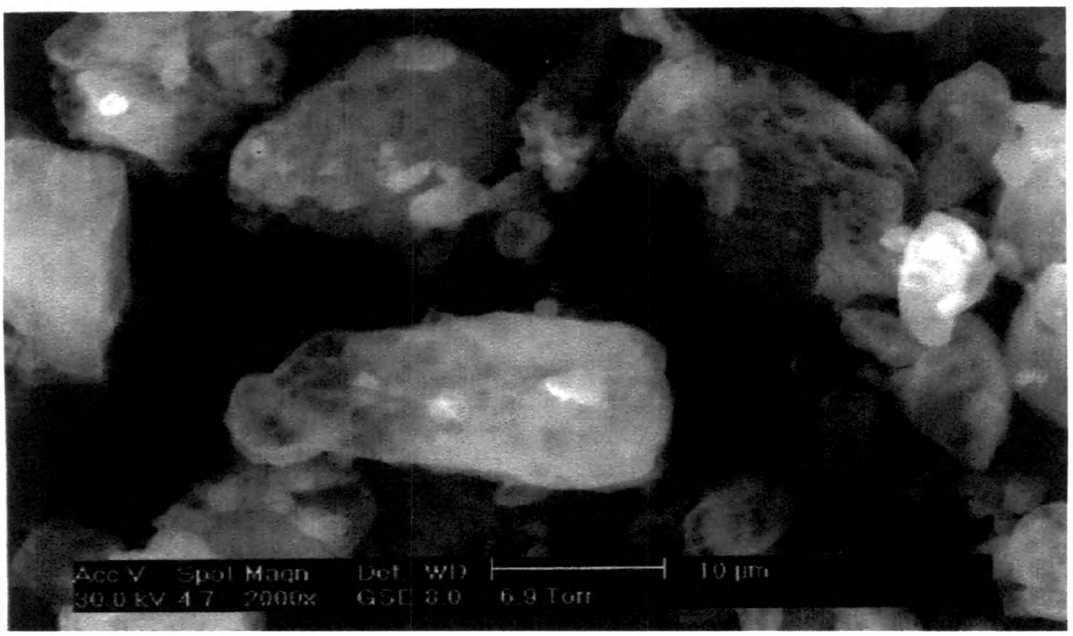

Figure 5.6 SEM photomicrographs of AMB78 (2000X)

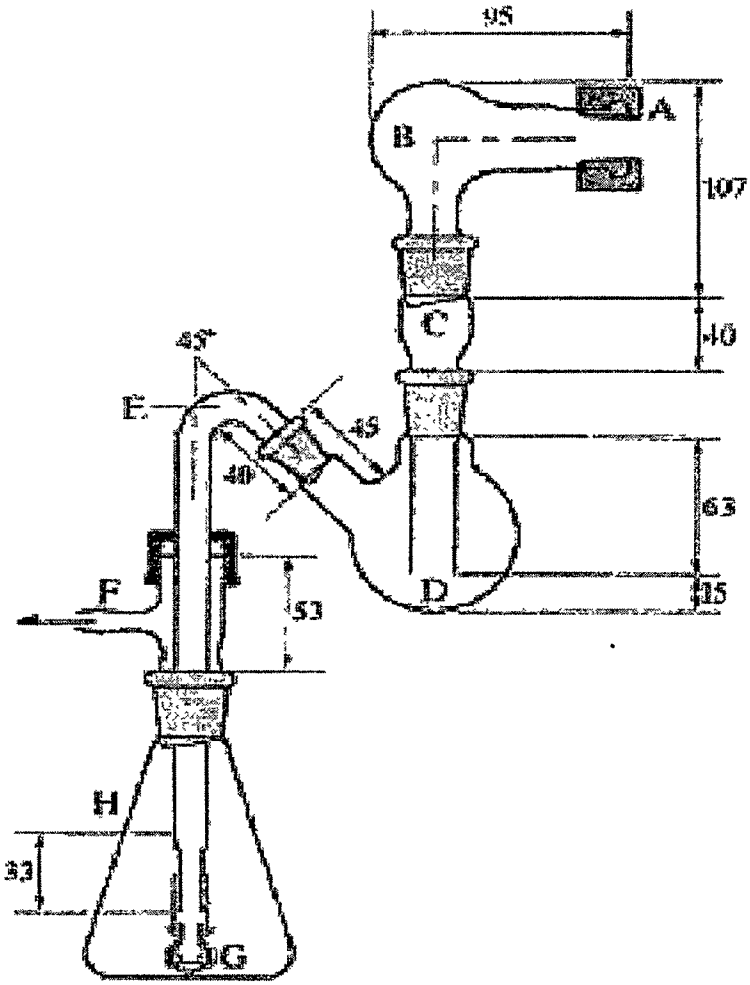

Figure 5.7 Schematic of the apparatus showing the standard glass twin stage impinger.

The following components are indicated, A. Mouthpiece adapter, B. Throat (round bottomed flask), C. Neck (Modified glass adaptor), D. Upper impingement chamber (Modified round bottomed flask), E. Coupling tube, F. Screwthread side arm adapter, G. Lower jet assembly and H. Lower impingement chamber.

The percent emission of AMK69 (negatively charged LDPI) was found to be better (88.9% v/s 84.4%) than the AMK70 (positively charged LDPI) and lower FPF compared to positively charged LDPI formulation (AMK70) (25.9% v/s 29.2%). It suggests efficient dispersion of AMK69 from the device but deposition of more proportion of the dispersed powder in the upper respiratory tract (Hino et al, 1998). On the contrary, the percent emission of AMB77 (negatively charged LDPI) was found to be better (89.1% v/s 83.1%) than the AMB78 (positively charged LDPI) suggestive of more effective emission of the liposomal drug but interestingly deposition of more proportion of the dispersed powder in the lung (FPF values-25.3% v/s 19.6%) than AMK LDPI formulations. It may be due to turbo-electrification or charge generation in liposomal powder during dispersion via the Rotahaler. Difference among both drugs LDPI formulations may be due to carrier particle size difference. AMK LDPI contains 63-90 μm sieved Pharmatose 325M as carrier; while AMB LDPI formulation contains Sorbolac 400 (<32 μm NLT 90%) as carrier. It can be concluded from these observations that carrier particle size plays important role in liposomal drug lung deposition from LDPI in presence of charge on liposomal surface. Higher device fractions of positively charged LDPI formulations (AMK70 and AMB78) are suggestive of some interaction within device it self.

Thus LDPI formulations have been prepared with high efficiency of lyophilization with optimized cryoprotectant in proper mass ratio to retain the drugs. These formulations (AMK69, AMK70, AMB77 andAMB78) were further studied for drug leakage during stability studies according to ICH guidelines.

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