CHAPTER 5

PREPARATION AND CHARACTERIZATION OF LIPOSOMAL DRY POWDER INHALER

5.1 INTRODUCTION

In the treatment of pulmonary disorders and in some cases, for systemic action, inhalation is the preferred route of administration. Aerosols are an effective method of delivering therapeutic agents to the respiratory tract tissues and can be delivered by nebulizers, metered dose inhalers of dry powder inhalers (Finlay, 2001 and Gonda, 1990). Delivering small doses of active ingredient directly to the lung effectively localizes the drug there by maximizing therapeutic effect while minimizing unwanted side effects. Improving drug delivery to the pulmonary system has become an area of increasing interest among several disciplines. Liposome aerosols are promising vehicles for respiratory delivery of therapeutic drugs and have attracted the attention of many researchers, especially in the area of nebulizers (Taylor et al, 1989 and 1990) and dry powder inhalers (Schreier et al, 1994 and Ho, 1995). As the stability and leakage problems are associated with aqueous dispersions with nebulizers (Niven et al, 1992). An alternative approach has been considered delivery of liposomes by dry powder (Schreier et al, 1994, Ho, 1995 and Holzner et al, 1998) mainly based on the fact that liposomes can be more stable when dried by lyophilization (Mobley et al, 1994 and Sun et al, 1996). But the liposome powders as drug carriers for inhalation therapies should be micronized to particles of ~1-5 μm in diameter for efficient delivery to the lung. Thus micronization normally been achieved by jet-milling (Schreier et al, 1994, Holzner et al, 1998 and Minley, 1998) causes particles to break apart on colliding in a high velocity air-stream. Though lyophilization (freeze-drying) is considered as a promising means of extending the shelf-life of liposomes, both freezing and drying can lead to structural and functional changes in liposomes (Muldrew et al, 1990 and McGranth, 1984). Considerable attention has been devoted to the design of therapeutic aerosol inhalers to improve the efficiency of inhalation therapies (Timsina et al, 1995 and Tansey, 1994). Attention has also been given to the design of dry powder aerosol surface texture, regarding particularly the need to avoid particle aggregation, a phenomenon which considerably diminishes the efficiency of inhalation therapies owing to particle aggregation (French, 1996). Recently, the development of large porous particles, characterized by their low mass density and large geometric size, has received attention as a potential option for the efficient delivery of therapeutic drugs to the alveolated airways (Edwards et al, 1997 and Edwards et al, 1998). A novel approach of delivering liposomes in dry powder form

was investigated by Tejas et al (2002) to avoid the detrimental effects of lyophilization and jet-milling on leakage of the encapsulated drug.

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 and 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 (Edward et al, 1997).

This lyophilized 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 depending 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, 1995 and 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 and 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.

The principle requirement for the development of an effective and efficient dry powder system for pulmonary drug delivery is that the mass median aerodynamic diameters (MMAD) of particles comprising the generated aerosol cloud should be in the range 1.0-5.0 μm (Hickey, 1993). With liposome powders as drug carriers for inhalation therapies, the lyophilized precursor should be micronized to particles of 1.0-5.0 μm in diameter for efficient delivery to the lung. Also the performance of many current DPI formulations relies on the formation of ordered units between fine drug crystals and coarse/fine carrier particles where device emptying is problematic due to poor flow behaviour. To circumvent the potential negative effects of

lyophilization, jet-milling and to improve the flow behaviour the manufacturing of lyophilized liposomal dry powders with low density excipients presents an opportunity to optimize pulmonary deposition. In this study, we have aimed to make Liposomal Dry Powder Inhaler (LDPI) by lyophilization process with minimal drug leakage and powder characteristic that gives improved pulmonary deposition. The potential of this LDPI to facilitate pulmonary deposition with respect to particle size, density, moisture content and sorption and pulmonary deposition performance was evaluated as fine particle fraction (FPF) and dispersing efficiency were examined.

5.2 LYOPHILIZATION OF LIPOSOMES

Liposomes prepared by optimized process and formulation components (Chapter-4) were lyophilized with cryoprotectant to preserve the vesicular size and shape, hence the PDE.

5.2.1 INH liposomes

Liposomal pellets of INH liposome obtained after centrifugation of liposomal dispersions ($3.3 \times 10^6 g$, 2 hours) were suspended in solution containing either trehalose or sucrose or lactose alone and with or without hydrolyzed gelatine in mass ratio of lipid: sugar: hydrolyzed gelatin (1:1:0.05) as cryoprotectant for INH38 to INH45 formulations. The liposomal suspension with cryoprotectant was freezed overnight at -40°C, dried for 48h at 50mbar using a Heto Freeze-dry system (Hetodry, Denmark) The effect of use of cryoprotectant such as trehalose, sucrose, lactose alone and with or without hydrolyzed gelatine on particle size and PDR was studied. Similarly addition sequence of cryoprotectant (sucrose) (formulations INH46 to INH51) and mass ratios of lipid: sucrose: hydrolyzed gelatine (formulations INH52 to INH59) was optimized for PDR (Table 5.1). PDR of lyophilized liposomes were determined following dehydration-rehydration cycle. Further the preparation of the lyophilized liposomal dry powders with low density excipient (hydrolyzed gelatine) was optimized for the enhancement of flow properties, powder dispersion and deposition.

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

		Rehydrated liposome	Liposomal dry powder		
Batch No.	**Variable studied**	**PDR (%)***	**Volume mean diameter (μm)***	**Density (ρ) (g/cm^3)***	**MADt (μm)**
SELECTION OF CRYOPROTECTANT (1:1:0.5, LIPID: SUGAR:HG)					
INH38	Trehalose	25.73 ± 2.44	16.3 ± 3.4	0.64 ± 0.003	13.06
INH39	Trehalose	31.90 ± 1.12	16.2 ± 3.6	0.66 ± 0.004	13.16
INH40	Sucrose	36.02 ± 2.41	18.2 ± 5.4	0.76 ± 0.007	15.86
INH41	Sucrose	35.56 ± 2.45	18.4 ± 5.6	0.77 ± 0.008	16.16
INH42	Lactose	25.40 ± 1.62	18.7 ± 7.2	0.84 ± 0.003	17.14
INH43	Lactose	31.50 ± 2.13	18.5 ± 7.1	0.82 ± 0.004	16.75
INH44	**Sucrose with HG**	**57.63 ± 1.82**	**16.2 ± 4.3**	**0.15 ± 0.003**	**6.27**
INH45	**Sucrose with HG**	**56.90 ± 2.17**	**16.4 ± 4.5**	**0.16 ± 0.004**	**6.56**
PHASE OF CRYOPROTECTANT ADDITION (SUCROSE)					
INH46	External	56.63 ± 2.82			
INH47	External	58.80 ± 1.17			
INH48	Internal	54.63 ± 2.72			
INH49	Internal	58.72 ± 1.58			
INH50	**Both**	**72.28 ± 2.32**			
INH51	**Both**	**76.83 ± 2.41**			
MASS RATIO OF SUCROSE (LIPID: SUCROSE:HG)					
INH52	1 : 0.5:0.5	74.22 ± 2.44	14.3 ± 5.3	0.12 ± 0.002	4.95
INH53	1 : 0.5·0.5	77.83 ± 2.50	14.7 ± 5.2	0.12 ± 0.003	5.09
INH54	**1 : 1.0:0.5**	**88.59 ± 2.40**	**15.4 ± 4.9**	**0.17 ± 0.004**	**6.35**
INH55	**1 : 1.0:0.5**	**92.50 ± 1.98**	**15.6 ± 5.2**	**0.18 ± 0.003**	**6.61**
INH56	1 : 1.5:0.5	97.23 ± 2.54	16.3 ± 5.4	0.28 ± 0.004	8.63
INH57	1 : 1.5:0.5	95.10 ± 1.70	16.7 ± 5.6	0.27 ± 0.003	8.67
INH58	1 . 2:0.5	99.31 ± 1.44	15.4 ± 5.3	0.35 ± 0.006	9.11
INH59	1 · 2·0.5	94.24 ± 2.21	15.2 ± 5.6	0.36 ± 0.005	9.12

* Mean ± SEM (n = 6)

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

		Rehydrated liposome	Liposomal dry powder		
Batch No.	**Variable studied**	**PDR (%)***	**Volume mean diameter (μm)***	**Density (ρ) (g/cm^3)***	**MADt (μm)**
SELECTION OF CRYOPROTECTANT (1:1:0.5, LIPID: SUGAR:HG)					
RFP38	Trehalose	65.73 ± 2.42	17.4 ± 4.2	0.61 ± 0.003	13.59
RFP39	Trehalose	71.90 ± 2.12	17.2 ± 4.8	0.62 ± 0.002	13.54
RFP40	Sucrose	76.02 ± 2.11	21.8 ± 6.5	0.72 ± 0.003	18.49
RFP41	Sucrose	78.56 ± 2.41	20.8 ± 6.8	0.72 ± 0.003	17.65
RFP42	Lactose	65.40 ± 1.62	18.4 ± 7.6	0.77 ± 0.002	16.15
RFP43	Lactose	73.50 ± 2.14	19.4 ± 7.5	0.78 ± 0.003	17.13
RFP44	**Sucrose with HG**	**92.63 ± 2.82**	14.4 ± 5.7	0.18 ± 0.003	6.11
RFP45	**Sucrose with HG**	**89.90 ± 2.16**	15.4 ± 5.8	0.18 ± 0.003	6.53
PHASE OF CRYOPROTECTANT ADDITION (SUCROSE)					
RFP46	External	93.63 ± 2.42			
RFP47	External	88 80 ± 2.17			
RFP48	Internal	74.63 ± 2.32			
RFP49	Internal	78.72 ± 1.48			
RFP50	**Both**	**92.28 ± 2.12**			
RFP51	**Both**	**96.83 ± 2.31**			
MASS RATIO OF SUCROSE (LIPID: SUCROSE:HG)					
RFP52	**1 : 0.5:0.5**	**94.22 ± 2.34**	14.4 ± 5.7	0.12 ± 0.003	4.99
RFP53	**1 : 0.5:0.5**	**93.83 ± 2.40**	15.4 ± 5 8	0.12 ± 0.003	5.33
RFP54	1 : 1.0:0.5	98.59 ± 2.42	16.4 ± 5.7	0.17 ± 0.003	6.76
RFP55	1 : 1.0:0.5	96.50 ± 1.78	15.7 ± 5.8	0.18 ± 0.003	6.66
RFP56	1 : 1.5:0.5	97.23 ± 2.44	15 3 ± 5.7	0.28 ± 0.004	8.09
RFP57	1 : 1.5:0.5	98.10 ± 1.73	16.4 ± 5.8	0.27 ± 0.003	8.52
RFP58	1 : 2:0.5	96.31 ± 1.64	15.4 ± 5.7	0.38 ± 0.006	9.49
RFP59	1 : 2:0.5	97.24 ± 2.25	15.2 ± 5.8	0.36 ± 0.005	9.12

* Mean ± SEM (n = 6)

5.2.2 RFP liposomes

Liposomal pellets of RFP liposome obtained after centrifugation of liposomal dispersions (3.3 × $10^6 g$, 2 hours) were suspended in solution containing either trehalose or sucrose or lactose alone and with or without hydrolyzed gelatine in mass ratio of lipid: sugar: hydrolyzed gelatin (1:1:0.05) as cryoprotectant for RFP38 to RFP45 formulations. The liposomal suspension with cryoprotectant was freezed overnight at -40°C, dried for 48h at 50mbar using a Heto Freeze-dry system (Hetodry, Denmark). The effect of use of cryoprotectant such as trehalose, sucrose, lactose alone and with or without hydrolyzed gelatine on particle size and PDR was studied. Similarly addition sequence of cryoprotectant (sucrose) (formulations INH46 to INH51) and mass ratios of lipid: sucrose: hydrolyzed gelatine (formulations INH52 to INH59) was optimized for PDR (Table 5.2). PDR of lyophilized liposomes were determined following dehydration-rehydration cycle. Further the preparation of the lyophilized liposomal dry powders with low density excipient (hydrolyzed gelatine) was optimized for the enhancement of flow properties, powder dispersion and deposition.

5.3 METHOD OF PREPARATION OF LIPOSOMAL DRY POWDER INHALER FORMULATIONS

For the preparation of LDPI formulations, during preparation of liposomes by optimized TFH technique, INH and RFP liposomes were extruded through 2μm polycarbonate membrane for 3 cycles and 5 cycles respectively and separated from unentrapped drug by centrifugation (3.3 × $10^6 g$, 2 hours). To the liposomal concentrate aliquot of solution containing sucrose and hydrolyzed gelatin solution were added and lyophilized. The lyophilized liposomal cakes were passed through serious of sieves from 100 to 400 meshes (Jayanth Sieve, Mumbai, India). Aerosol formulations were prepared by tumble mixing sieved liposomal dry powders with different ratio and grades of lactose carrier. Powder blends were filled in a hard gelatin capsule (size, 2) and stored in a desiccator till further use.

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

5.3.1 INH LDPI formulation

INH LPDI formulations were prepared by blending a suitable carrier for pulmonary delivery (Lactose). The effect of carrier mass ratio, addition of fine carriers and the adding sequence of carrier were optimized for achieving desired flow and dispersion of the LPDI formulations.

5.3.1.1 Effect of carrier mass ratio

The sieved lyophilized liposomal powder containing 1000 ± 50 µg of INH (app. 5mg) was mixed with lactose carrier (63-90 µm sieved Pharmatose 325M) in varying mass ratios from 1:0 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 INH60 to INH69 formulations for fine particle fraction (Table 5.3).

5.3.1.2 Effect of adding fines

The sieved lyophilized liposomal powders were mixed with lactose carrier (63-90 µm Sieved Pharmatose 325M) containing 5% to 15% sieved Sorbolac 400 (#500 – 25µm) in mass ratios of lipid: lactose at 1:1 and these formulations were evaluated using TSI for INH70 to INH75 formulations for fine particle fraction (Table 5.3).

5.3.1.3 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 100# and 400 #) in a mass ratio of lipid: lactose at 1:1 (Formulation A) for INH72 and INH73 respectively. In another set of experiment, the fines (10% sieved Sorbolac 400 – 25µm) were first mixed with sieved lyophilized liposomes (through 100# and 400 #) and then with lactose carrier (63-90 µm Sieved Pharmatose 325M) at same ratio (Formulation B) for INH72 and INH73 respectively. These formulations were evaluated using TSI for fine particle fraction (Table 5.3).

5.3.2 RFP LDPI formulation

RFP LPDI formulations were prepared by blending a suitable carrier for pulmonary delivery (Lactose). The effect of carrier mass ratio, addition of fine carriers and the adding sequence of carrier were optimized for achieving desired flow and dispersion of the LPDI formulations.

5.3.2.1 Effect of carrier addition

The sieved lyophilized liposomal powder containing 500 ± 50 µg of RFP (app. 5mg) was mixed with lactose carrier (63-90 µm sieved Pharmatose 325M) in varying mass ratios from 1:0 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 RFP60 to RFP69 formulations for fine particle fraction (Table 5.4).

5.3.2..2 Effect of adding fines

The sieved lyophilized liposomal powders were mixed with lactose carrier (63-90 µm Sieved Pharmatose 325M) containing 5% to 15% sieved Sorbolac 400 (#500 – 25µm) in mass ratios of lipid· lactose at 1:1 and these formulations were evaluated using TSI for RFP70 to RFP75 formulations for fine particle fraction (Table 5.4).

5.3.2.3 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 100# and 400 #) in a mass ratio of lipid: lactose at 1:2 (Formulation A) for RFP72 and RFP73 respectively. In another set of experiment, the fines (10% sieved Sorbolac 400 – 25µm) were first mixed with sieved lyophilized liposomes (through 100# and 400 #) and then with lactose carrier (63-90 µm Sieved Pharmatose 325M) at same ratio (Formulation B) for RFP72 and RFP73 respectively. These formulations were evaluated using TSI for fine particle fraction (Table 5.4).

Table 5.3 Optimization of INH LDPI formulation

Batch No.	Variable studied	Emission (%)*	FPF (%)*
Effect of lipid: lactose ratio			
INH60	1: 0	67.5 ± 4.2	27.5 ± 2.2
INH61	1: 0	66.8 ± 5.1	26.8 ± 2.1
INH62	**1: 1**	**79.7 ± 6.3**	**29.8 ± 2.3**
INH63	**1: 1**	**78.3 ± 5.4**	**28.3 ± 2.4**
INH64	1: 2	82.5 ± 5.0	22.4 ± 2.0
INH65	1: 2	83.8 ± 4.5	21.3 ± 1.5
INH66	1: 4	85.7 ± 4.6	14.5 ± 1.6
INH67	1: 4	82.3 ± 5.8	12.9 ± 1.8
INH68	1: 6	84.2 ± 5.1	14.7 ± 2.1
INH69	1: 6	83.9 ± 6.2	13.8 ± 2.2
Effect of fine lactose (lipid: lactose ratio was 1:1)			
INH70	**5 %**	**79.4 ± 4.2**	**29.4 ± 2.2**
INH71	**5 %**	**78.5 ± 4.4**	**34.6 ± 2.4**
INH72 (Form-A)	10 %	74.8 ± 5.7	24.8 ± 1.7
INH73 (Form-A)	10 %	76.3 ± 4.3	26.2 ± 2.3
INH72 (Form-B)	10 %	74.8 ± 5.7	18.7 ± 2.4
INH73 (Form-B)	10 %	76.3 ± 4.3	16.8 ± 1.4
INH74	15 %	75.2 ± 5.7	23.1 ± 1.7
INH75	15 %	78.2 ± 6.2	21.2 ± 2.2

* Mean ± SEM (n = 6)

Table 5.4 Optimization of RFP LDPI formulation

Batch No.	Variable studied	Emission (%)*	FPF (%)*
Effect of lipid: lactose ratio			
RFP60	1: 0	57.3 ± 5.2	24.8 ± 2.1
RFP61	1: 0	56.4 ± 5.6	22.7 ± 1.4
RFP62	1: 1	69.7 ± 6.2	23.3 ± 2.3
RFP63	1: 1	68.4 ± 5.9	25.5 ± 2.1
RFP64	**1: 2**	82.4 ± 5.2	**25.1 ± 3.0**
RFP65	**1: 2**	84.8 ± 4.5	**23.6 ± 2.2**
RFP66	1: 4	83.6 ± 5.6	14.5 ± 2.3
RFP67	1: 4	84.2 ± 5.2	13.2 ± 2.7
RFP68	1: 6	86.2 ± 4.9	16.4 ± 2.4
RFP69	1: 6	85.4 ± 5.7	11.9 ± 2.3
Effect of fine lactose (lipid: lactose ratio was 1:2)			
RFP70	**5 %**	**89.4 ± 5.2**	**29.2 ± 2.6**
RFP71	**5 %**	**88.5 ± 4.9**	**24.9 ± 2.5**
RFP72 (Form-A)	10 %	84.8 ± 5.7	25.3 ± 1.8
RFP73 (Form-A)	10 %	86.4 ± 5.3	19.6 ± 1.5
RFP72 (Form-B)	10 %	76.4 ± 4.6	17.4 ± 1.8
RFP73 (Form-B)	10 %	78.3 ± 4.4	14.3 ± 2.3
RFP74	15 %	87.6 ± 5.2	20.1 ± 1.9
RFP75	15 %	88.1 ± 6.0	14.6 ± 2.3

* Mean ± SEM (n = 6)

5.4 LDPI FORMULATION CHARACTERIZATION

The LDPI formulations were characterized for the following physico-chemical properties.

5.4.1 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, 1965). The angle of repose was calculated by inverting tangentially the ratio of height and radius of the formed pile (Table 5.5).

5.4.2 Compressibility index

Compressibility index (CI) of powder as a measure of flow and dispersibility were measured by methods as described in United States Pharmacopoeia, (2001)

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.

The CI for INH (70 & 71) and RFP (70 & 71) LDPI formulations are summarized in Table 5.5.

5.4.3 Particle size determination

Particle sizes of rehydrated liposomes of LDPI formulations, particle sizes of LDPI and the Mass median Aerodynamic diameter of LDPI formulations were determined.

5.4.3.1 Rehydrated liposomal particle size determination

The vesicle size of rehydrated liposomes was determined by laser diffraction using Malvern particle size analyzer (Malvern Master sizer 2000 SM, U.K.). The LDPI formulations were rehydrated and diluted with distilled water. Diluted liposome suspension was added to the sample dispersion unit-containing stirrer and stirred at 2000 rpm in order to reduce the interparticle aggregation, and laser obscuration range was maintained between 10-20%. The mean liposomal size is recorded in Table 5.5 for INH (70 & 71) and RFP (70 & 71).

5.4.3.2 Liposomal Dry Powder particle size determination

Particle sizes distributions of liposomal dry powders were measured using laser diffraction particle size analyzer (HELOS, Sympatec GmbH, Germany). The size analysis was carried out by dry powder dispersing method at a pressure of 2 bars and feed rate of 70%. Each measurement was performed in triplicate. The size

distributions were expressed in terms of the volume mean diameter (VMD) and the diameters below which 10% and 90% by volume of the particles in the powder resided. The mean liposomal dry powder size is summarized in Table 5.5 for INH (70 & 71) and RFP (70 & 71).

The theoretical mean aerodynamic diameter (MADt) was determined by the following formula (Gonda, 1991):

d_{aer} (MADt) = $\sqrt{\rho}$ x d (VMD) where, ρ is tapped density in units of g/cm^{3}and d is volume mean diameter in micron.

5.4.3.1 Mass Median Aerodynamic particle size determination

The Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) were measured using Inhaler Module Sympatec laser diffraction particle size analyzer (HELOS, Sympatec GmbH, Germany). Aerodynamic diameter is defined as the diameter of a unit density sphere having the same settling velocity, generally in air, as the particle. It is an indicator of the aerodynamic behaviour of the particle and is dictated by particle shape, density and geometric size, all of which can affect the deposition of the drug in the lung. GSD is defined as the ratio of the diameter for 84.1% cumulative probability to that of a 50% probability or 15.9% and 50% and is a measure of aerosol polydispersity. The size analysis was carried out by dry powder dispersing method at a pressure of 2 bars and feed rate of 70%. Each measurement was performed in triplicate. The MMAD of LDPI formulations are summarized in Table 5.5 for INH (70 & 71) and RFP (70 & 71).

5.4.4 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 morphological characteristics of liposomal dry powder before lyophilization and liposomal vesicles after hydration of liposomal dry powder formulations.

5.4.5 SEM photomicrographs

Scanning electron microscopy of the representative LDPI formulation was carried using Environmental SEM, Philips XL30, The Netherlands.

5.4.6 Fine particle fraction

The twin stage impinger (TSI) (Apparatus A, British Pharmacopoeia) was used to obtain the FPF values. For INH LDPI formulations, the volume of capturing solvent (Methanol) in the upper (stage 1) and lower (stage2) were 7 and 30ml respectively in TSI (B.P. Apparatus A) (1993) while for RFP LDPI formulations, the volume of capturing solvent (Chloroform) 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 60 ± 2 L/min and 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 air flow rate of 60 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 methanol (for INH) or chloroform (for RFP) and analyzed to measure the amount of drug retained as described in chapter 3 (Section 3.3.6 for INH and Section 3.4.4 for RFP). 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 60 L/min. Each capsule contained a powder mass equivalent to nominal dose of 1000 ± 50 μg INH (INH70 and INH71) and 500 ± 10 μg of RFP (RFP70 and RFP71). 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 INH and Table 5.6 for RFP formulations). As a control, a marketed preparation (Asthalin Rotacaps, Cipla Ltd., India) containing salbutamol sulphate powder was used and the FPF determined at 60 L/min flow rate using Rotahaler (Cipla, India) as delivery device (Table 5.5). Further the fine particle fraction of potential LDPI formulations were also measured using Next Generation Impactor (NGI) (Copley Scientific, UK). 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.

5.4.7 Residual water content and moisture sorption 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 analysis. The reagent was standardized with addition and determination of known quantity of water (250mg). Firstly, 40ml of methanol was added into the titration vessel and titrated with the reagent to determine the amount of water present in the samples. The water content determined for the LDPI and its formulations are recorded in Table 5.5.

The moisture sorption attributes of the formulation was carried out by finding the moisture uptake at different relative humidity at fixed time interval (Campen and Zografi, 1983).

5.4.8 Percent drug retained

Percent drug retained (PDR) 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 centrifugation method described in chapter 3 (Section 3.3.6 for INH and Section 3.4.4 for RFP). The mean percent drug remained entrapped with its standard error of mean, for six determinations are shown in Table 5.1 (INH) and Table 5.2 (RFP).

5.5 STATISTICAL ANALYSIS

Each batch was prepared six times and data from all experiments are expressed as mean ± SEM unless specified. 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.

5.6 RESULTS AND DISCUSSION

Liposomes prepared by TFH method were re-suspended into the hydrating medium with cryoprotectant and freeze dried. Liposomes with cryoprotectant were frozen slowly in deep freezer (-40°C), rather than quickly submerging the samples in boiling nitrogen, to achieve higher retention of the entrapped drug (Edwoud et el, 1997). In the process of lyophilization the formulation variables were optimized to obtain dry powder of liposome with maximum PDR. In the initial experiments, the selection of cryoprotectant, the phase of its addition and the lipid to cryoprotectant mass ratio were carried out. The optimized lyophilized powder of liposomes formulation was further processed for preparing dry powder inhaler. LDPI formulations were prepared by tumble mixing sieved liposomal dry powders with different ratio and grades of lactose carrier. The effect of carrier mass ratio, addition of fine carriers and the adding sequence of carrier were optimized for achieving desired flow, dispersion and in vitro deposition characteristics of the LPDI formulations.

5.6.1 Optimization of lyophilization of liposomes

The process and formulations were optimized for lyophilization of liposomes to obtained dry powders suitable for inhalation and the results are recorded in Table 5.1 – 5.2 and the discussion are as follows:

5.6.1.1 Selection of cryoprotectant

Liposomal dispersion of composition INH13, INH17, RFP13 and RFP16 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 solution containing either trehalose or sucrose or lactose alone and with or without hydrolyzed gelatin in mass ratio of lipid: sugar: hydrolyzed gelatin (1:1:0.05). PDR of liposomes following dehydration-rehydration cycle was determined. The particle size, tapped density and theoretical mean aerodynamic diameter was determined for the liposomal dry powder and compared. The results are shown in Table 5.1 (INH) and 5.2 (RFP) reveal that sucrose with hydrolyzed gelatin resulted in highest PDR (INH44, 57.63 %; INH45 56.90 %; RFP44, 92.63 % and RFP45, 89.90 %) as compared to Trehalose, sucrose and lactose (Calculated 'F'= 339.03, tabulated 'F' = 3.09, at 3 degree of freedom, P = 0.05). Different sugars have markedly different effect on stability, Madden and Co-workers (1985). 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 lyophilization. Leopold and Vertucci (1987) have suggested that sugars that tend to crystallize more readily may not be as effective and so lactose despite of being disaccharides are not effective compared to sucrose. Most noticeably desired liposomal powder density and theoretical mean aerodynamic size was obtained by inclusion of low density excipient (Hydrolyzed gelatin) compared to powders made from Trehalose, sucrose and lactose alone. The results are shown in Table 5.1 and 5.2 for INH and RFP liposomal dry powders respectively.

5.6.1.2 Phase of cryoprotectant addition

To study the effect of presence of cryoprotectant only in internal and external and in both aqueous phase, INH and RFP liposomes were prepared using hydrating medium containing with and without sucrose (1:1 mass ratio of lipid: sucrose). In the experiments (INH46, INH 47, RFP46 and RFP47) sucrose was present only in the external phase during freeze-drying. For the presence of cryoprotectant only in internal phase the pellet was re-suspended into the medium without sucrose for both drugs (INH48, INH 49, RFP48 and RFP49). 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 1:1) (INH50, INH 51, RFP50 and RFP51). When sucrose was present only on the inside and outside of the vesicles, less PDR was achieved Table – 5.1 (INH) and Table – 5.2 (RFP). In comparison to these, when sucrose was present on both side of bilayer; INH50, (72.28 %), INH 51, (76.83%), RFP50 (92.28 %) and RFP51 (96.83 %) maximum PDR 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 and 1989). It can be concluded that during lyophilization 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. However the lamellarity and size of liposomes are expected to change these requirements. With the above assumption it was concluded that the liposomal vesicles when lyophilized in presence of sucrose retain their contents without disrupting the bilayer structures.

5.6.1.3 Mass ratio of lipid: sucrose

The liposomal dispersions were sequentially diluted with sucrose solution so as to obtain mass ratio, lyophilized and the PDR obtained after rehydration are recorded in Table 5.1 and 5.2 for INH and RFP 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 1:1 for INH and mass ratio of 1:0.5 for RFP was found to be optimum with high PDR (INH54, 88.59 %; INH55, 92.50 %; RFP52, 94.22 % and RFP53, 97.83 %). Further increase in the lipid to sucrose mass ratio did not show significant improvement in PDR of lyophilization for both drugs (Calculated 'F'= 19.09, tabulated 'F' = 2.78, at 3 degree of freedom, P= 3.09). 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-frying.

However, it was observed that further increase in the lipid to sucrose mass ratio leads to increase in the density of liposomal powder, hence higher MADt that leads to reduction in the powder flow and dispersion characteristics. Hence, attempt was made to improve the flow, dispersion and deposition characteristics of lyophilized liposomal dry powder by inclusion of low density excipient (hydrolyzed gelatin). When hydrolyzed gelatin alone was used, lower drug retention was observed; hence it was used along with other cryoprotectant. In presence of hydrolyzed gelatin inclusion of minimal level of sugar showed enhanced cryoprotectant activity due to avoidance of fusion induced drug leakage for a selected lipid composition of vesicles during freeze-drying (Table – 5.1 & 5.2). This may be due to reduction in lipid phase transitions in vesicles by sugars in complex mixtures of phospholipids. This suggests

that the role of sugar in preserving dry liposomes is not only by inhibition of fusion but also it avoids the phase transition during the rehydration step (Crowe et al, 1989) thus minimizes drug leakage. It is evident that fusion between vesicles can be minimized by inclusion of minimal levels of sugars along with the low density excipient (hydrolyzed gelatin) used. Inclusion of hydrolyzed gelatin for the freeze-drying of liposome shows that it not only modifies the bulk properties of the formulation but also prevents the leakage of trapped solute from liposomal vesicles during freeze-drying. Thus the inclusion of hydrolyzed gelatin improved the liposomal dry powder flow, dispersion and deposition characteristics as compared to the use of sugars alone as cryoprotectant. The results are discussed in section characterization of LDPI formulations (5.6.3).

5.6.2 Optimization of LDPI formulation

The liposomal dry powder made from optimized lyophilized process was formulated with suitable carriers in various mass ratios for pulmonary delivery to achieve maximum FPF.

5.6.2.1 Effect of carrier particle ratio

To formulate LDPI formulations, series of experiments were conducted (Section 5.4.1). The LDPI formulations were optimized to get maximum FPF with drug to carrier ratios. The variables studied and the results are summarized in table – 5.3 and 5.4 for INH and RFP respectively. Lyophilized liposomes when formulated as LDPI formulation without using any carrier particles resulted in low emission (device retention) due to poor powder flow and thus resulted in low FPF value. This corroborates 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 various lipid: lactose mass ratio from 1:1 to 1:6 and its effect on FPF were studied. The data revealed the optimum lipid: lactose mass ratio of 1:1 for INH LDPI formulations (FPF; INH62 – 29.8 % and INH63 – 28.3 %) and 1:2 for RFP LDPI formulations (FPF; RFP64 – 25.1 % and RFP65 – 23.6 %). Optimum concentration of carrier is required to achieve good flowability, dispersibility and detachment of liposomal dry powder from carrier particles. Though, the liposomal dry powder without carrier particles produced similar FPF, but the emission (INH60 67.5 % and RFP60 57.3 %) was found to be low due to poor powder flowability. However, improvement in emission was observed to be optimal with

inclusion of carrier particles (INH62 (1:1) 79.7 % and RFP64 (1:2) 82.4 %), but the FPF was found to decrease significantly with further increase in carrier concentration above the optimal level (Calculated 'F'= 73.95, tabulated 'F' = 2.25, at 7 degree of freedom, P = 0.05). This behavior of lower FPF values with higher carrier concentration (1:4 – 1:6) was due to dominance of adhesive forces within the ordered units. Thus with higher carrier particle concentration more surface for drug-carrier interaction and reduction in FPF. Though there was decrease in FPF there was no significant difference in emission was observed with higher carrier particle concentration (Calculated 'F'= 1.36, tabulated 'F' = 2.25, at 7 degree of freedom, P = 0.05).

5.6.2.2 Effect of fine particle addition and addition sequence

One strategy shown to increase re-dispersion of the drug into the respirable aerosol is the use of a smaller particle size carrier (Steckel and Muller, 1997). Hence the effect of adding fines (sieved Sorbolac-400) in 5%, 10% & 15% proportion with carrier (63-90μm) keeping final lipid: lactose mass ratio of 1: 1 was evaluated. The effect of fine particle addition on FPF results are summarized in table – 5.3 and 5.4 for INH and RFP respectively. With 5% fine lactose (Sarbolac-400) added to LDPI formulation showed higher FPF (INH70 29.4 %, INH71 34.6%, RFP70 29.2 % and RFP71 24.9 %). However further increase in the fine lactose concentration to 10 & 15% resulted in lower FPF values. This result corroborate with results of Staniforth, (1996) that the high-energy adhesion sites (HA) of lactose may bind strongly to the fine carrier particles and low-energy adhesion sites (LA) may allow the formation of more reversible bonds with liposomal drug. Thus results in efficient detachment of liposomal drug from the carrier. Similar observation was seen during the evaluation of the effect of adding sequence of fines to the liposomal formulation. Blending the fines (5% Sorbolac 400) first with carrier (63-90 μm) resulted in emission and FPF (Table 5.3 and 5.4). From the results it was concluded that addition of fine carrier to coarse lactose binds to high-energy adhesion sites (HA) of lactose strongly and leaving low-energy adhesion sites (LA) for liposomal drug particles that may allow the formation of more reversible bonds with liposomal drug. Liposomal drug powder adheres to carrier particles as seen in scanning electron microscopy photographs of INH LDPI formulations (Figure 5.3 and 5.4).

5.6.3 Characterization of LDPI formulations

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, 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. The various physico-chemical properties of liposomal dry powder after freeze-drying and LDPI formulations results are recorded in Table 5.5.

5.6.3.1 Angle of repose and compressibility index

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 32.1 to 39.7 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.

The flowability and floodability expressed by angle of repose (32.1 to 39.7°), compressibility index (17.1 to 24.3) 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 for the optimized LDPI formulations (INH70, INH71, RFP70 and RFP71) and assure optimal dispersion in stream of air upon inhalation.

5.6.3.2 Particle size characterization

The liposomal size of rehydrated liposomes was determined by laser diffraction using Malvern particle size analyzer (Malvern Master sizer 2000 SM, U.K.). The aggregation and fusion of liposomes induced drug leakage during lyophilization could be evidenced by the effect of cryoprotectant on the liposomal particle size (Fig-1 & 2). The results in the graph indicate that incorporation of cryoprotectant in lyophilized liposomes maintained the particle size of lyophilized/rehydrated liposomes due to

limited fusion and aggregation of liposomes. It is concluded from this that freeze-drying – rehydration causes considerable leakage of drug from the lyophilized liposome matrix and the leakage are a function of the type and concentration of cryoprotectant. It was observed that sugar and hydrolyzed gelatin mixture cryoprotectant gave maximum drug retention. It is evident that fusion between vesicles can be minimized even with minimal levels of sugars and inclusion of low density excipients, which not only modifies the bulk properties of the formulation but also the leakage of trapped solute from liposomal vesicles during freeze-drying. There was no significant change in the liposome size before and after freeze-drying observed for optimized formulations (Table - 5.6). Thus it could be concluded that after freeze drying according to this protocol provided sufficient cryoprotection to the liposomes.

The volume mean diameter (VMD) particle size of liposomal dry powder after freeze-drying was measured and the results are recorded in table – 5.5. The VMD for the potential liposomal dry powders were in the range of 15.4 -17.4 μm. Though, the VMD of liposomal dry powders were observed to be > 5 μm the administration of the low density particles (less than about 0.4 g/cm^3) to the lung by aerosolization permits deep lung delivery of relatively large diameter therapeutic aerosols, for example, greater than 5 μm in mean diameter. This is evident from the calculated MADt are in the respirable range table - 5.1 & 5.2 (INH44 6.27, INH45 6.56, RFP44 6.11 and RFP45 6.53 μm). Calculated MADt are in relationship with the obtained MMAD of potential LDPI formulations (INH70 4.23, INH71 4.53, RFP70 4.12 and RFP45 4.71 μm).

Table 5.5 Characterization of LDPI formulations

Variable studied	Isoniazid*		Rifampicin*	
	INH70	**INH71**	**RFP70**	**RFP71**
Residual water content (%) (Liposomal dry powder)	1.6 ± 0.8	1.9 ± 0.9	1.4 ± 1.0	1.5 ± 0.7
Residual water content (%)	5.6 ± 0.8	5.9 ± 0.9	4.4 ± 1.0	5.5 ± 0.7
Angle of Repose (°)	32.1 ± 0.4	39.7 ± 0.4	38.3 ± 0.6	34.7 ± 0.5
Compressibility index	17.8 ± 2.4	21.5 ± 1.8	24.3 ± 2.4	19.9 ± 2.0
Mean particle size (μm) (rehydrated liposomes)	2.75 ± 0.23	2.93 ± 0.30	2.86 ± 0.33	2.83 ± 0.32
Mean particle size (μm) (Liposomal Dry Powder)	15.4 ± 3.8	16.4 ± 4.2	17.4 ± 3.6	15.4 ± 3.2
MMAD (μm)	4.23 ± 0.33	4.53 ± 0.21	4.12 ± 0.26	4.71 ± 0.32
GSD	1.9 ± 0.05	1.8 ± 0.03	2.2 ± 0.04	2.4 ± 0.06
FPD (μg)	359.4 ± 8.2	322.9 ± 7.6	126.5 ± 5.5	148.0 ± 7.4
FPF (%)	35.9 ± 0.8	32.2 ± 0.8	26.3 ± 0.6	29.6 ± 0.7
Emission (%)	78.9 ± 2.0	82.4 ± 1.7	84.1 ± 1.6	81.7 ± 1.9
Dispersibility	45.5 ± 1.6	39.1 ± 1.4	31.3 ±1.0	36.2 ± 0.6
Effective Index	53.2 ± 1.3	51.5 ± 1.7	47.0 ± 1.6	49.2 ± 1.3

Control: Ashthalin Capsules (Cipla Ltd., India):

FPF = 27.1 ± 2.0, EI = 48.6 ± 1.7 at 60 l min^{-1}.

* Mean ± SEM (n = 3)

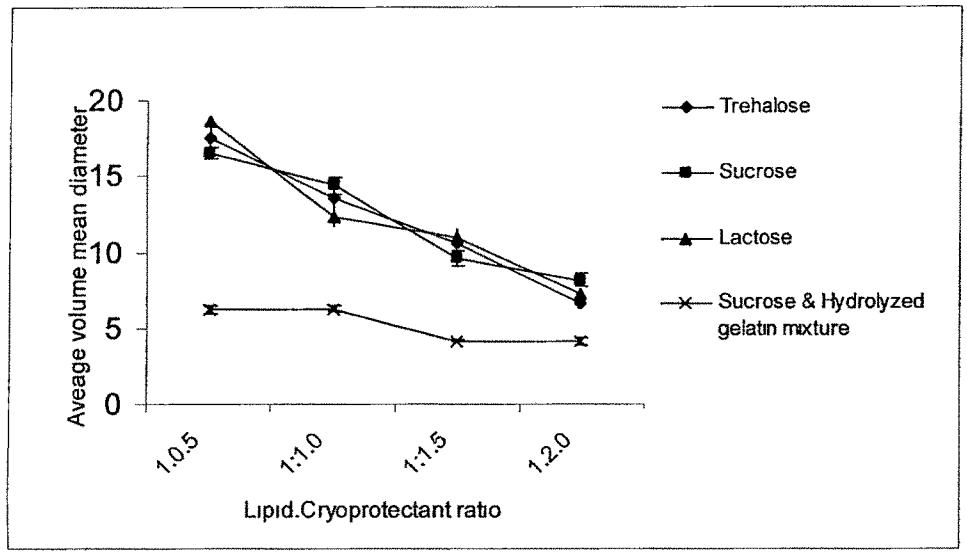

Figure – 5.1: Effect of cryoprotectant type/concentration on mean particle size of rehydrated INH LDP

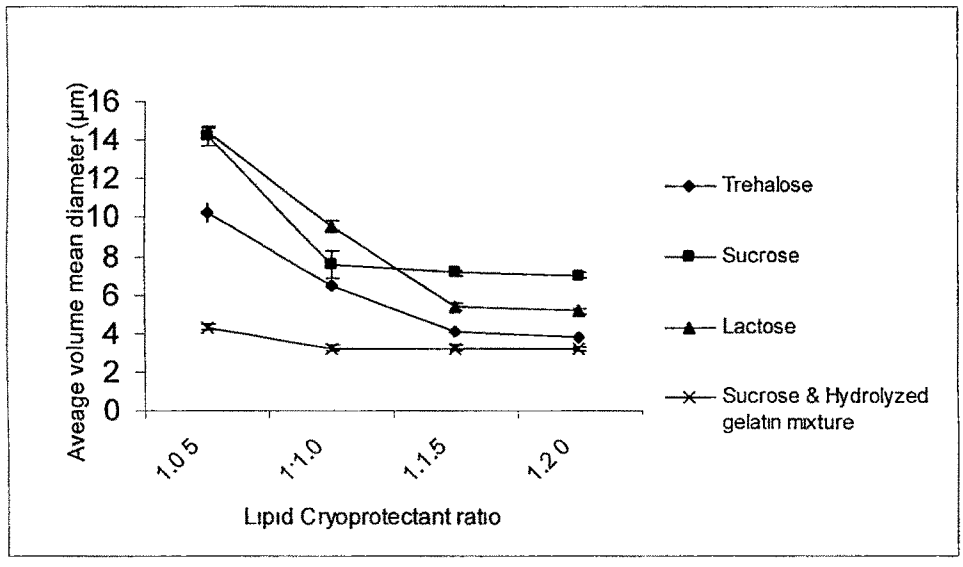

Figure – 5.2: Effect of cryoprotectant type/concentration on mean particle size of rehydrated RFP LDP

Table 5.6 Particle size range of rehydrated LDPI formulations

Batch No.	Volume mean diameter (μm)*	< 10% (μm)*	< 90% (μm)*
Pre extrusion			
INH70	2.12 ± 0.06	0.55 ± 0.02	25.79 ± 2.24
INH71	1.84 ± 0.04	0.59 ± 0.04	24.84 ± 4.42
RFP70	2.14 ± 0.02	0.63 ± 0.03	25.61 ± 4.26
RFP71	2.21 ± 0.03	0.53 ± 0.02	25.27 ± 3.37
Post-extrusion			
INH70	2.31 ± 0.03	0.65 ± 0.03	27.39 ± 3.14
INH71	2.16 ± 0.04	0.72 ± 0.05	28.75 ± 4.27
RFP70	2.54 ± 0.02	0.67 ± 0.04	31.31 ± 4.36
RFP71	2.61 ± 0.05	0.59 ± 0.07	29.17 ± 3.97

*Mean ± SEM (n=6)

5.6.3.3 Photomicrography and SEM photomicrographs

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 dry powder before lyophilization and liposomal vesicles after hydration of liposomal dry powder formulation are shown in figure 5.3 and 5.4 respectively. The prepared liposomes were found to be multilamellar and identified by Maltese crosses in liposomal photomicrographs.

The LDPI formulations (INH70 and RFP70) were viewed by Scanning Electron Microscopy (Philips XL30 ESEM, The Netherlands) and shown in figure 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.

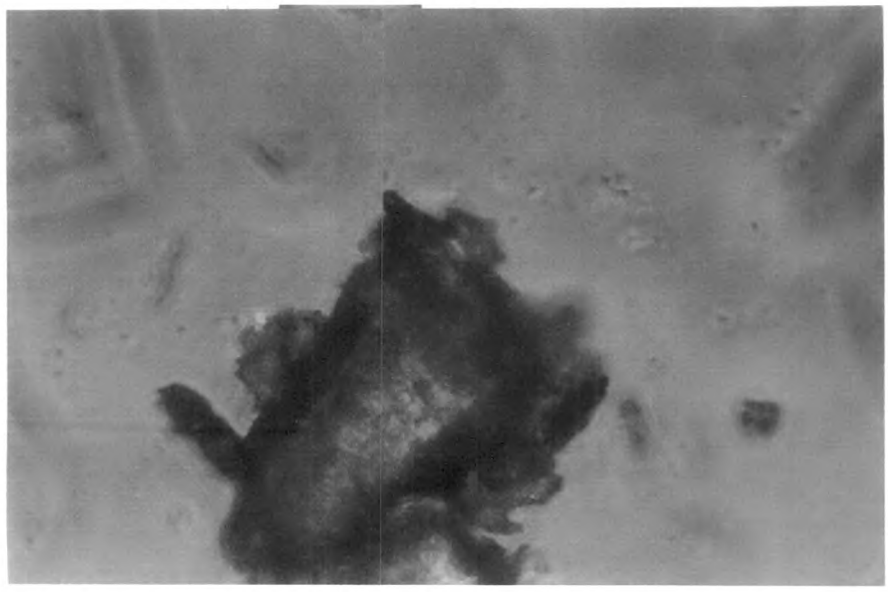

Figure 5.3: Photomicrograph of liposomal dry powder formulation under polarized light

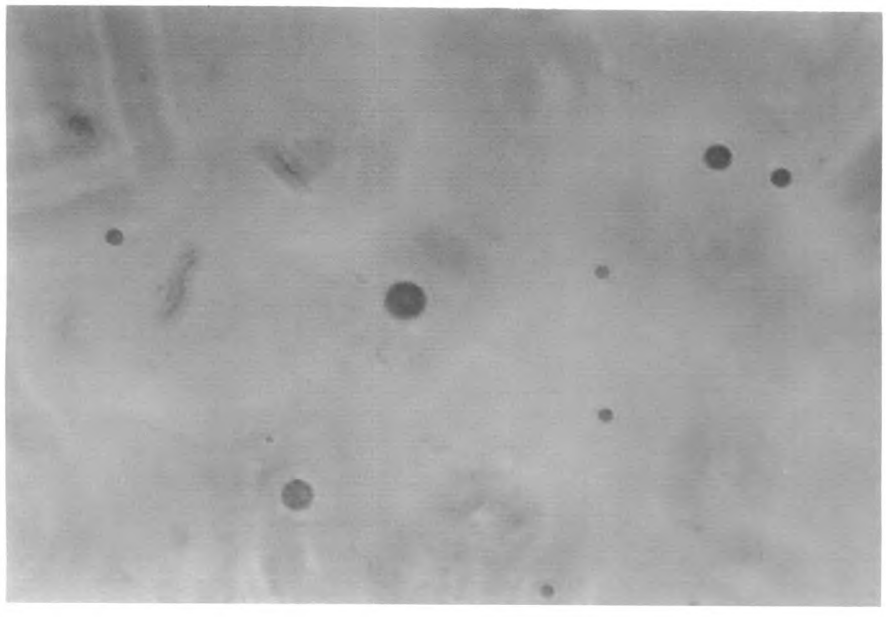

Figure 5.4: Photomicrograph of liposomal vesicles after hydration of liposomal dry powder formulation under polarized light

Figure 5.5 SEM photomicrographs of INH70

Figure 5.6 SEM photomicrographs of RFP70

5.6.3.4 Fine particle fraction

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) official in British Pharmacopoeia (1993) was used to obtain FPF values as reported in Table 5.5. 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 deposit in the lungs upon inhalation. The particles passing to the lower impingement chamber (Stage 2) are respirable and considered as FPF (<6.4 μm). The FPD (μg), FPF (%), Dispersibility (%) and Emission (%) at 60 L/min flow rate using Rotahaler (Cipla, India) as dispersing device are shown in Table 5.4. Ideal LDPI formulation should provide small device fraction (effective emission from the device) and large FPF when inhaled. The FPF values (Table 5.5) for the LDPI formulation falls in the range of 26.3 % (RFP70) to 35.9 (INH70), which is comparable with that of the marketed control value (27.1%).

Although the twin stage impinger has the reputation of being a robust instrument it is unable to measure the distribution of particle size of an aerosol. Hence the potential LDPI formulation particle size distribution and fine particle fraction were measured using Next Generation Impactor (NGI). The percent depositions on various stages of NGI are shown in figure – 5.7. It is apparent that the particle size distribution of INH and RFP LDPI was found to be in the stages of known cut-off diameter desired for lung deposition. The FPF obtained by NGI were comparable to TSI measurements 32.11 % for INH70, 29.95 % for INH71, 24.71 % for RFP70 and 27.42 % for RFP71 LDPI formulations.

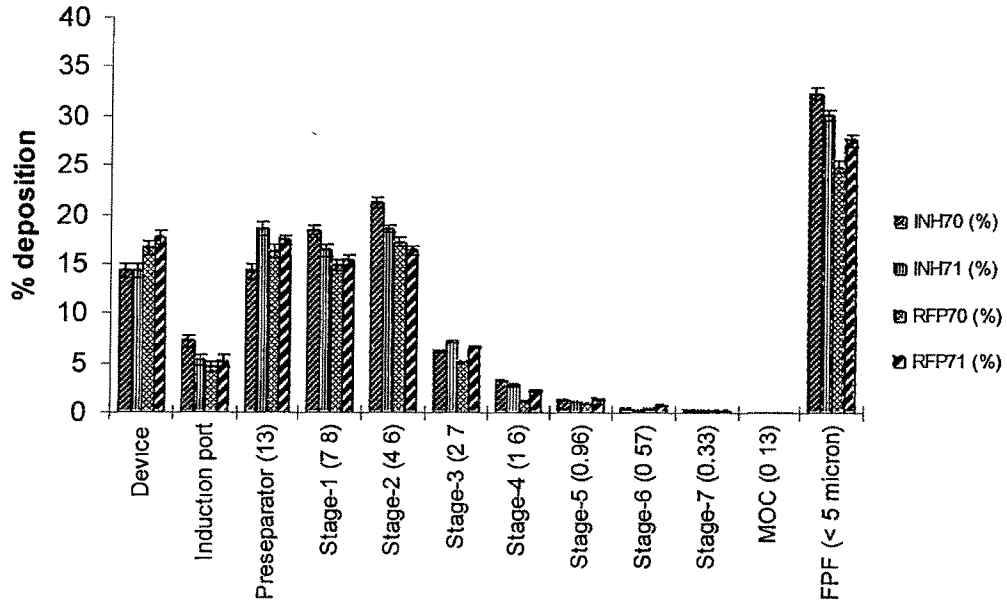

Figure - 5.7: In vitro deposition of INH and RFP LDPI formulations in NGI; Numbers in the parenthesis of x – axis is the effective cutoff diameter in micron.

5.6.3.4 Residual water content and moisture sorption determination

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

The percent moisture sorption (% gain) of liposomal dry powder with various cryoprotectants for INH (38, 40, 42 & 44) and RFP (38, 40, 42 & 44) are shown in figure - 5.8 & 5.9 respectively. Liposomal dry powder containing RFP and INH had similar moisture sorption behavior. Liposomal dry powder made using sucrose, trehalose and lactose alone had shown higher tendency to absorb water into their amorphous structure. This was similar to the observations by Naini et al (1995). While LDP made using sugar and hydrolyzed gelatin mixture had showed lesser tendency to absorb water. This behavior might be that the hydrolyzed gelatin has a low tendency to absorb water into their amorphous structure and inhibits recrystallisation as seen with samples containing 50% protein and 50% trehalose reported by French et al (1995).

5.6.5 Percent drug retained

The percent drug retained obtained for optimized liposomal dry powder after rehydration are recorded in table -5.1 & 5.2 for INH and RFP respectively. The results showed insignificant difference between PDE to PDR of liposomal dispersion after freeze-drying.

Thus LDPI formulations have been prepared with high efficiency of lyophilization with optimized cryoprotectant in proper mass ratio to retain the drugs. The batches namely INH70, INH71, RFP70 and RFP71 were selected for the further characterization studies. The formulation components of these potential batches are shown in detail in Table 5.7. These formulations were further studied for drug leakage during stability studies according to ICH guidelines.

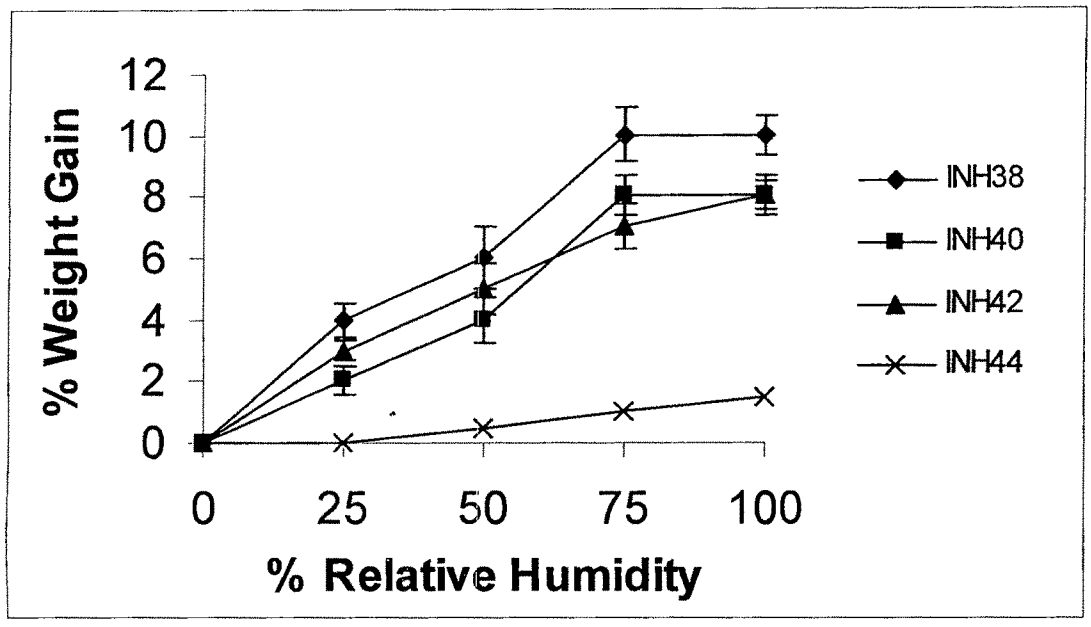

Figure 5.8: INH liposomal dry powder moisture sorption

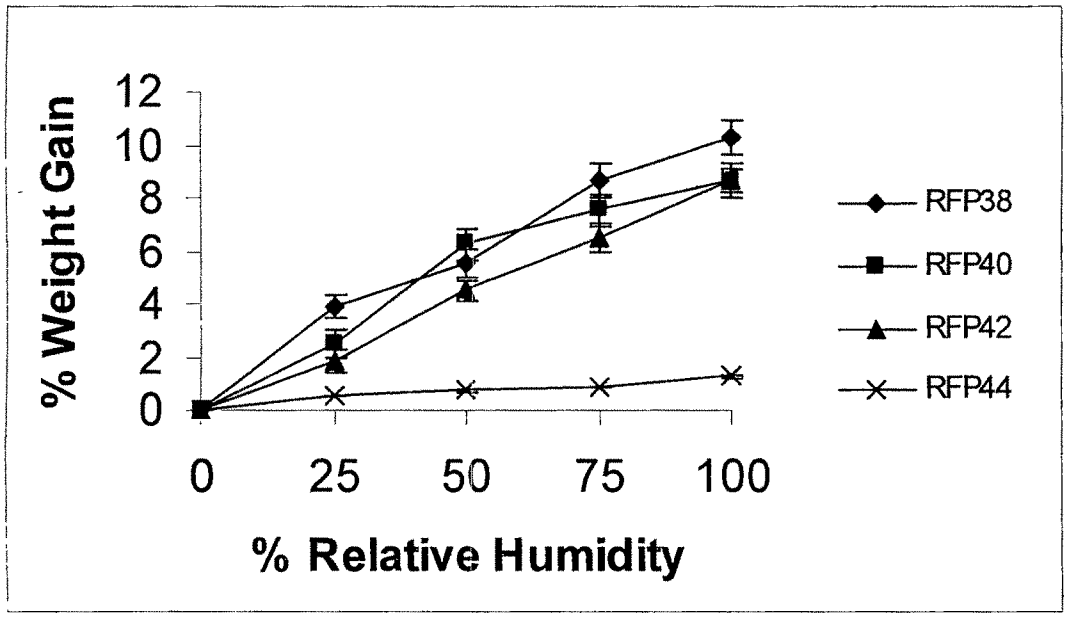

Figure 5.9: RFP liposomal dry powder moisture sorption

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

Batch No.	Drug (µg)	HSPC (mg)	SPG-3 (µg)	Sucrose (mg)	Hydrolyzed gelatin	Final Weight of liposomal dry powder	Lactose (mg)		Total (mg)
							Coarse	Fine	
INH70	1000	1.78	N.A.	2.78	1.39	5.05	9.59	0.51	15.15
INH71	1000	1.69	90.00	2.78	1.39	4.80	9.12	0.48	14.40
RFP70	500	6.26	N.A.	3.38	3.38	13.16	25.00	1.32	39.48
RFP71	500	5.86	60.00	3.21	3.21	12.20	23.18	1.22	36.60

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