CHAPTER 4

PREPARATION AND CHARACTERIZATION OF LIPOSOMES

4.1 INTRODUCTION

Many methods have been developed to prepare liposomes. There are at least fourteen major reported methods (Ostro, 1987 and Martin, 1990). The seven most commonly employed method are lipid film hydration also referred as thin layer evaporation method (THF) (Bangham et al., 1965), reverse phase evaporation technique (REV) (Sozoka and Papahadjopoules, 1978), rehydration-dehydration technique (Shew and Deamer, 1985), ethanol injection method (Batzri and Korn, 1975), ether infusion method (Deamer et al 1976), French press technique (Barenholz et al, 1979) and detergent dialysis technique (Kagawa and Racker, 1971). The difference between various methods of manufacture is in the way in which the membrane components are dispersed in aqueous media before being allowed to coalesce in the form of bilayer sheets. From pharmaceutical point of view, the three most important factors to be evaluated before selecting the method of preparation are the trapping efficiency, drug retention property and drug/lipid ratio (Betagiri et. al., 1993).

Trapping efficiency is one of the important parameters in selecting a method of preparation of liposomes. An optimum loading procedure would achieve trapping efficiency of 90% or more. This obviates the need for removal of unentrapped drug because loading doses of 10% or less of free drug can usually be tolerated. The procedures, such as dialysis and passage through exclusion columns, for removal of unentrapped drug are often time-consuming, tedious, expensive and recovery of unentrapped drug is usually difficult.

Many lipid compositions can be employed for liposomal delivery systems; however, stability and cost are important determinants. Thus, acidic (negatively charged) lipids such as phosphatidyl serine (PS), cardiolipin and phosphatidic acid (PA) are not preferred components as compared to phosphatidylcholine (PC) because of high costs and the often labile nature of these compounds. Similarly, the use of highly unsaturated lipids, such as soya phosphatidylcholine (SPC) or naturally occurring PS, phosphatidylethanolamine (PE) and cardiolipin should be avoided because of the considerable oxidation problems encountered. Thus, given similar loading and retention characteristics, liposomal systems composed of egg PC or hydrogenated varieties of egg or soya PC are more acceptable pharmaceutically.

Considering drug retention, it is unlikely that most drug-liposome formulations can exhibit sufficiently low leakage rates to allow retention times of one year or more.

However, if the trapping efficiencies are sufficiently high (e.g. 90% or more), unentrapped drug need not be removed. No leakage of drug would then occur on extended storage, because of the absence of transmembrane drug concentration gradients. The optimum drug/lipid ratio of a liposomal formulation will likely be dictated by the biological efficacy and toxicity of the preparation. From a pharmaceutical point of view, high drug/lipid ratios are obviously more economical.

In summary, optimum liposomal formulations will exhibit drug trapping efficiencies in excess of 90%, employ inexpensive and relatively saturated lipids such as hydrogenated soya phosphatidyl choline (HSPC) and cholesterol and exhibit the highest possible drug/lipid ratio which is consistent with maintained efficacy of the preparation.

Apart from these factors; other factors which need to be considered in selection of the methods of preparation include selection of methods which would avoid the use of organic solvents and detergents (which are difficult to remove), yield well-defined and reproducible liposomes and which are rapid and amenable to scale up procedures.

Any special applications of the liposomes to be prepared may also contribute in the selection of the appropriate method. In the preparation of freeze dried liposomes intended for pulmonary delivery there are two basic necessities, first, sufficient rigidity in the liposomal membrane to withstand drying with least leakage of the entrapped species and second, liposomal size should be preferentially below 5μm.

This chapter demonstrates the preparation of liposomes considering the above discussed factors. Liposomes of Isoniazid (INH) and Rifampicin (RFP) were prepared using TFH and REV technique with membrane composition consisting of saturated/unsaturated SPC, cholesterol with saturated soyaphosphatidylglycerol (SPG) or SA to prepare negatively or positively charged liposomes, respectively. Prepared liposomes were characterized for shape, size and size distribution, lamellarity, and PDE and for the contents of phosphatidylcholine and cholesterol. Optimization was carried our by selection of various formulations and process variables.

4.2 MATERIALS AND EQUIPMENTS

Isoniazid (INH) and Cholesterol (CHOL) were purchased from S. D. Fine Chemicals, India; Rifampicin (RFP) was Gift samples from Cadila Pharmaceuticals, India; Water (distilled) prepared in laboratory by distillation; Soyaphosphatidylcholine (SPC), Hydrogenated Soyaphosphatidylcholine (HSPC) and Hydrogenated soyaphosphatidylglycerol (SPG) were gift samples from Lipoid, GmbH, UK; Stearylamine (SA) was purchased from Sigma Chemical Co. (USA); Chloroform, Methanol, Triton X-100 were purchased from Qualigens, Mumbai; Ethanol was from Govt. supply, Baroda; Nuclepore Polycarbonate membrane 2 μm 25mm was purchased from Whatman, USA.

The equipments like rotary evaporator with vacuum pump and thermostatically controlled water bath and nitrogen purging facility (Superfit Equipments, India); Analytical balance (Precisa 205A SCS, Switzerland); pH meter (Systronics 335, India); Cyclomixer, three blade stirrer (Remi Scientific Equipments, Mumbai); Probe sonicator (RR-120, Ralsonics, Mumbai); Cooling Centrifuge (C-24), Water bath, Magnetic stirrer and heating mantle (Remi, Mumbai); Homogenizer, SD4C (Raliwolf Ltd. Mumabi); UV-Visible Spectrophotometer, (Shimadzu UV-1601, Japan); Infrared spectrophotometer (Shimadzu corporation, Japan); NMR (Advance DPX 200, Bruker Inc., Switzerland); Vacuum PumpF16, (Bharat Vacuum pumps, Banglore); Optical microscope with polarizer (BX 40, Olympus Optical Co. Ltd., Japan); Malvern particle size analyzer (Malvern Master sizer 2000 SM, U.K.); Differential Scanning Calorimeter (Mettler DSC 20, Mettler Toledo, Switzerland); Karl fisher Auto-titrater (Toshiwal Instruments (Bombay) Pvt. Ltd., Nasik) were used.

4.3 PREPARATION OF INH LIPOSOMES BY TFH

Multi lamellar vesicles (MLVs) of INH consisting of SPC/CHOL and HSPC/CHOL with SPG or SA were prepared by TFH technique (New, 1990). Briefly, the lipids were dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v) in a 250ml round bottom flask in different molar ratios (Table 4.1). The solvent was evaporated in the rotary flash evaporator, flask rotated at a speed of 120 rpm for 20min in a thermostatically controlled water bath (25°C/60°C) under vacuum (15 inch Hg). The thin dry lipid film thus formed was hydrated using aqueous hydrating medium at 25°C /60°C and the flask was rotated once again, at the same speed as before at ambient or 60°C temperature for another 30 min to obtain liposomal suspension. A flowchart

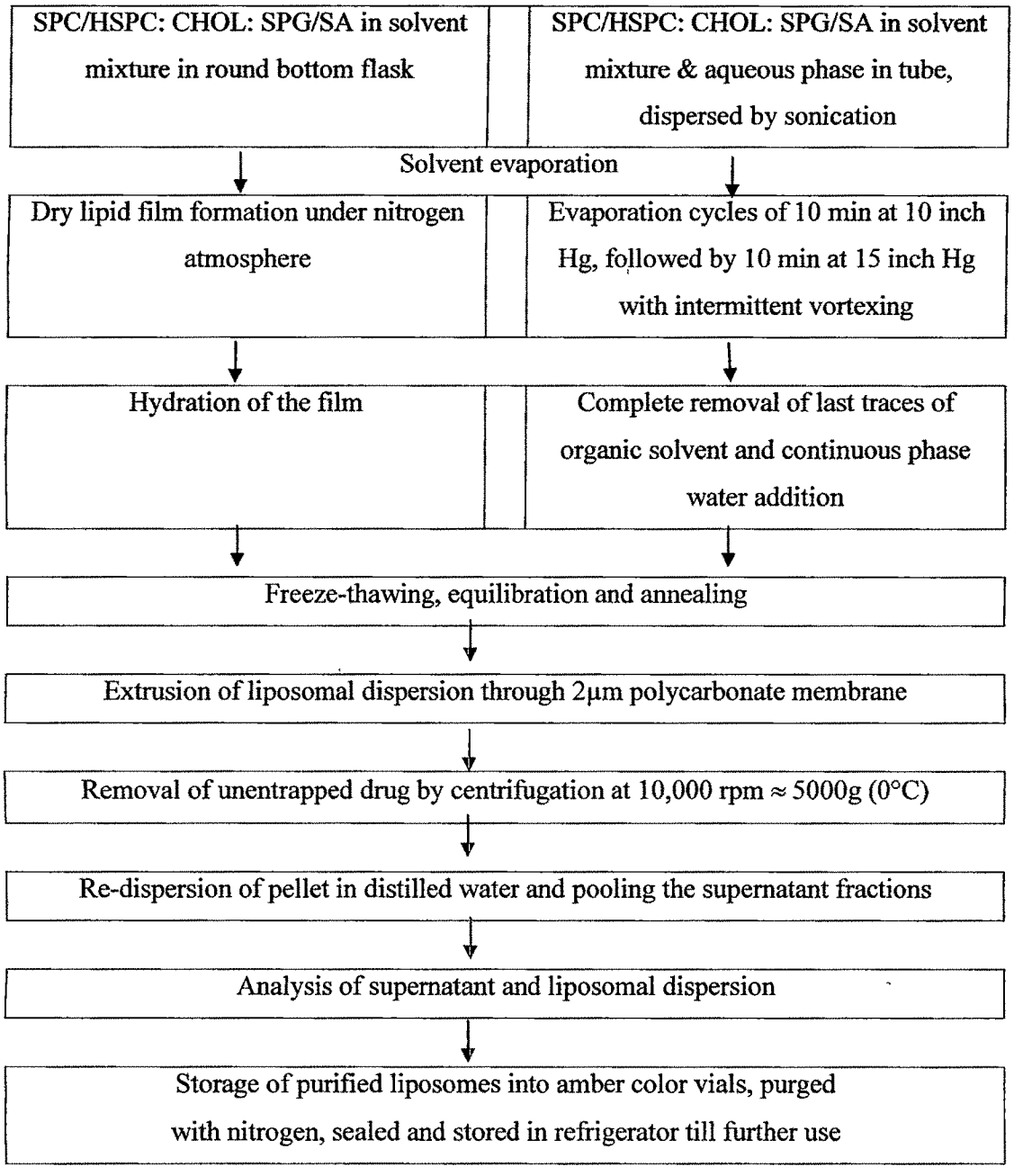
depicting the process is shown in scheme 4.1. The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment. A suitable method for particle size reduction and for removal of unentrapped drug was selected and optimized.

4.4 PREPARATION OF INH LIPOSOMES BY REV

Multilamellar vesicles (MLVs) of INH consisting of SPC/CHOL and HSPC/CHOL with SPG or SA were prepared by the modified REV technique (Cortesi et al, 1999). Briefly, the lipids were dissolved in ethanol – ethyl acetate solvent system (1:1) and transferred to narrow neck tube with standard B-24 joint. Aqueous medium was added to the lipid solution with sonication to form a homogenous dispersion. The dispersion was evaporated for 10 min at 10 inch Hg, followed by 10 min at 15 inch Hg with intermittent vortexing. The ratio of aqueous: organic phase was 1:5. Liposomal dispersion was subjected to complete removal of last traces of organic solvent for 15 min at 20 inch Hg to obtain liposomal suspension. A flowchart depicting the above process is shown in scheme 4.1. The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment (Table-2). A suitable method for particle size reduction and for removal of unentrapped drug was selected and optimized.

4.5 DRUG LOADING

INH was loaded in the liposomes prepared by TFH or REV method by pre- and post-loading (trans-membrane) techniques. In pre-loading technique drug was added either in lipid film hydrating medium or in aqueous medium of REV dispersion system during the liposome formation. In post-loading technique the liposomal suspension was transferred to a suitable glass tube containing INH/INH–EDTA mixture. The dispersion was freezed at -50°C in a deep fridge for 1 h and thawed at 60°C for 15 min. The freeze-thawing (F/T) cycle was repeated thrice. The F/T dispersion was then equilibrated for about 2 h at room temperature/60°C for SPC/HSPC composition, respectively. A flowchart depicting the process is shown in scheme 4.1.



Scheme 4.1: TFH and REV process stages in the preparation of INH liposomes

Table 4.1: Optimization of liposomal INH preparation by TFH method

Batch No.	INH-EDTA: Lipid	PC: Cholesterol: Charge	Entrapment efficiency (%)*	Observation and Inference
Batches with SPC and SPG				
INH1	**1:1**	**1:0:0**	**29.30 ± 2.28**	Low entrapment efficiency
INH2	1:5	5:0:0	44.58 ± 2.22	-
INH3	1:10	10:0:0	66.92 ± 2.48	High entrapment efficiency, but low drug to lipid ratio
INH4	1:1	9:1:0	23.71 ± 2.18	-
INH5	**1:1**	**8:2:0**	**35.54 ± 2.42**	Optimal PC:CHOL ratio
INH6	1:1	7:3:0	35.81 ± 2.19	-
INH7	**1:1**	**7.9:2:0.1**	**49.77 ± 2.40**	Optimal PC:CHOL:Charge ratio
INH8	1:1	7.5:2:0.5	46.84 ± 2.47	-
INH9	1:1	7:2:1	46.29 ± 3.12	-
Batches with SPG and SA				
INH10	1:1	7.9:2:0.1	28.37 ± 1.91	Optimal PC:CHOL:Charge ratio, but low entrapment efficiency
INH11	1:1	7.5:2:0.5	26.94 ± 2.22	-
INH12	1:1	7:2:1	24.53 ± 2.92	-
Batches with HSPC and SPG				
INH13	**1:1**	**10:0:0**	**63.71 ± 2.14**	High entrapment efficiency with high drug to lipid ratio
INH14	1:1	9:1:0	38.54 ± 2.28	Uneven orientation of Liposomes with low entrapment efficiency
INH15	1:1	7:3:0	35.81 ± 2.81	Uneven orientation of Liposomes with low entrapment efficiency
INH16	1:1	9.9:0:0.1	58.40 ± 2.40	-
INH17	**1:1**	**9.5:0:0.5**	**64.16 ± 2.85**	Optimal PC:Charge ratio with high entrapment efficiency
INH18	1:1	9:0:1	65.22 ± 2.64	-
Batches with HSPC and SA				
INH19	1:1	9.9:0:0.1	48.96 ± 2.88	Optimal PC:Charge ratio, but low entrapment efficiency and poor stability
INH20	1:1	9.5:0:0.5	47.74 ± 2.73	-
INH21	1:1	9:0:1	46.28 ± 2.94	-

* Mean ± SEM (n = 6)

Table 4.2: Optimization of liposomal INH preparation by REV method

Variable	Batch No.	HSPC: CHOL: Charge	Entrapment efficiency (%)*	Observation and Inference
Choice of organic solvent (Ratio of aqueous phase to organic phase was 1:5)				
Ethyl	INH22	10:0:0	35.41 ± 2.21	Uneven orientation of Liposomes
acetate	INH23	9.5:0:0.5	32.73 ± 2.15	
Ethanol	INH24	10:0:0	34.85 ± 1.52	Uneven orientation of Liposomes
	INH25	9.5:0:0.5	36.09 ± 2.24	
Ethyl	INH26	10:0:0	63.07 ± 2.17	Good vesicle formation
acetate :	INH27	9.5:0:0.5	67.93 ± 2.58	
Ethanol				
(1:1)				
Ratio of aqueous phase to organic phase				
1:2	INH28	10:0:0	30.48 ± 2.73	Uneven orientation of Liposomes
	INH29	9.5:0:0.5	38.92 ± 1.95	
1:3	INH30	10:0:0	36.07 ± 2.02	Uneven orientation of Liposomes
	INH31	9.5:0:0.5	37.93 ± 2.63	
1:4	INH32	10:0:0	54.29 ± 2.86	Uneven orientation of Liposomes
	INH33	9.5:0:0.5	56.20 ± 2.02	
1:5	**INH34**	10:0:0	66.71 ± 1.97	Optimal solvent ratio with good
	INH35	9.5:0:0.5	68.54 ± 1.40	vesicle formation
1:6	INH36	10:0:0	66.37 ± 2.33	No major change in vesicle
	INH37	9.5:0:0.5	67.90 ± 1.81	formation and entrapment efficiency

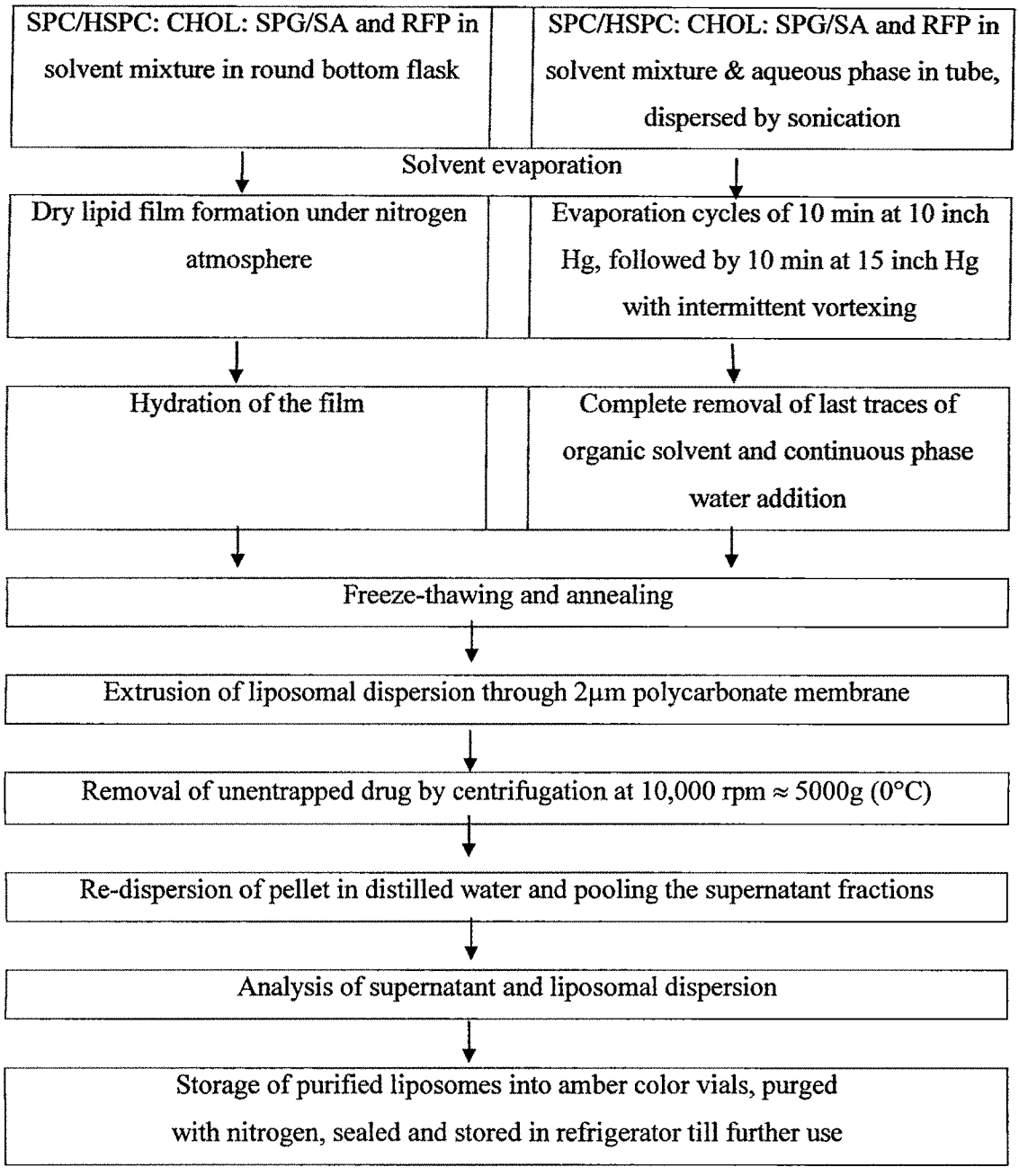
* Mean ± SEM (n = 6)

4.6 PREPARATION OF RFP LIPOSOMES BY TFH

Multi lamellar vesicles (MLVs) of RFP consisting of SPC/CHOL and HSPC/CHOL with SPG or SA were prepared by TFH technique (New, 1990). Briefly, the lipids and RFP were dissolved in a mixture of chloroform and methanol (ratio 2:1 v/v) in a 250ml round bottom flask in different molar ratios (Table 4.3). The solvent was evaporated in the rotary flash evaporator, flask rotated at a speed of 120 rpm for 20 min in a thermostatically controlled water bath (25°C/60°C) under vacuum (15 inch Hg). The thin dry lipid film thus formed was hydrated using aqueous hydrating medium at 25°C /60°C and the flask was rotated once again, at the same speed as before at ambient or 60°C temperature for another 30 min to obtain liposomal suspension. A flowchart depicting the process is shown in scheme 4.2.The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment.

4.7 PREPARATION OF RFP LIPOSOMES BY REV

Multilamellar vesicles (MLVs) of RFP consisting of SPC/CHOL and HSPC/CHOL with SPG or SA were prepared by the modified REV technique (Cortesi et al, 1999). Briefly, the lipids and RFP were dissolved in ethanol – ethyl acetate solvent system (1:1) and transferred to narrow neck tube with standard B-24 joint. Aqueous medium was added to the lipid solution with sonication to form a homogenous dispersion. The dispersion was evaporated for 10 min at 10 inch Hg, followed by 10 min at 15 inch Hg with intermittent vortexing. The ratio of aqueous phase: organic phase was 1:5. Liposomal dispersion was subjected to complete removal of last traces of organic solvent for 15 min at 20 inch Hg to obtain liposomal suspension. A flowchart depicting the process is shown in scheme 4.2.The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment (Table 4.4).



Scheme 4.2: TFH and REV process stages in the preparation of RFP liposomes

Table 4.3: Optimization of liposomal RFP preparation by TFH method

Batch No.	RFP: Lipid	PC: Cholesterol: Charge	Entrapment efficiency (%)*	Observation and Inference
Batches using Soya PC (S-100) with SPG-3 as charge inducer				
RFP1	1:5	10:0:0	52.94 ± 1.42	Low entrapment efficiency, but high drug to lipid ratio
RFP2	1:10	10:0:0	75.27 ± 1.88	Optimal drug to lipid ratio with high entrapment efficiency
RFP3	1:15	10:0:0	76.64 ± 2.15	-
RFP4	1:10	9:1:0	73.58 ± 1.48	Optimal lipid to CHOL ratio with high entrapment efficiency
RFP5	1:10	8:2:0	65.44 ± 1.53	-
RFP6	1:10	7:3:0	58.70 ± 1.95	-
RFP7	1:10	8.9:1:0.1	79.32 ± 1.24	Optimal lipid to charge ratio with high entrapment efficiency
RFP8	1:10	8.5:1:0.5	74.78 ± 1.88	-
RFP9	1:10	8:1:1	70.65 ± 1.49	-
Batches using Soya PC (S-100) with SA as charge inducer				
RFP10	1:10	8.9:1:0.1	48.22 ± 1.29	Poor entrapment efficiency due to SA-drug interaction
RFP11	1:10	8.5:1:0.5	35.91 ± 1.62	-do-
RFP12	1:10	8:1:1	30.37 ± 2.04	-do-
Batches using HSPC with SPG-3 as charge inducer				
RFP13	**1:10**	**10:0:0**	**83.71 ± 2.15**	**Optimal lipid to CHOL ratio with high entrapment efficiency**
RFP14	1:10	9:1:0	78.54 ± 2.25	-
RFP15	1:10	7:3:0	65.81 ± 2.88	-
RFP16	**1:10**	**9.9:0:0.1**	**88.80 ± 2.12**	**Optimal lipid to charge ratio with high entrapment efficiency**
RFP17	1:10	9.5:0:0.5	82.18 ± 1.98	
RFP18	1:10	9:0:1	85.69 ± 1.87	
Batches using HSPC using SA as charge inducer				
RFP19	1:10	9.9:0:0.1	50.06 ± 1.44	Poor entrapment efficiency due to SA-drug interaction
RFP20	1:10	9.5:0:0.5	49.34 ± 1.58	-do-
RFP21	1:10	9:0:1	38.20 ± 1.73	-do-

* Mean ± SEM (n = 6)

Table 4.4: Optimization of liposomal RFP preparation by REV method

Variable	Batch No.	HSPC: Cholesterol: Charge (SPG-3)	Entrapment efficiency (%)*	Observation and Inference
Choice of organic solvent (Ratio of aqueous phase to organic phase was 1:5)				
Ethyl acetate	RFP22	10:0:0	45.22 ± 1.92	Uneven orientation of Liposomes
	RFP23	9.9:0:0.1	53.92 ± 2.30	
Ethanol	RFP24	10:0:0	50.38 ± 2.44	Uneven orientation of Liposomes
	RFP25	9.9:0:0.1	48.72 ± 2.24	
Ethyl acetate: Ethanol (1:1)	RFP26	10:0:0	78.47 ± 2.36	Good vesicle formation
	RFP27	9.9:0:0.1	76.90 ± 3.02	
Ratio of aqueous phase to organic phase				
1:2	RFP28	10:0:0	62.37 ± 2.62	Uneven orientation of Liposomes
	RFP29	9.9:0:0.1	55.30 ± 1.87	
1:3	RFP30	10:0:0	78.52 ± 2.38	Uneven orientation of Liposomes
	RFP31	10:0:0	76.88 ± 3.03	
1:4	**RFP32**	**10:0:0**	**86.31 ± 2.14**	**Optimal solvent ratio with good vesicle formation**
	RFP33	**9.9:0:0.1**	**88.46 ± 2.28**	
1:5	RFP34	10:0:0	85.42 ± 1.90	No major change in vesicle formation and entrapment efficiency
	RFP35	9.9:0:0.1	87.92 ± 1.83	
1:6	RFP36	10:0:0	85.11 ± 2.17	No major change in vesicle formation and entrapment efficiency
	RFP37	9.9:0:0.1	88.08 ± 2.48	

* Mean ± SEM (n = 6)

4.8 PARTICLE SIZE REDUCTION AND SEPARATION OF UNENTRAPPED DRUG.

The suitable method adapted for the size reduction and separation of unentrapped drug for INH liposomes are extrusion through membrane filters and centrifugation method, respectively. Briefly, the size of liposomal dispersion was reduced by extruding through 2 μm polycarbonate track-etch membrane filters at 60°C (Nucleopore, Whatman Inc. New Jersey, USA). The unentrapped drug was removed from the liposomal suspension by centrifugation at 10,000rpm for 30min at 0°C temperature. The supernatant was collected and the pellet was re-suspended in fresh distilled water. The process was repeated thrice to remove all unentrapped drug. The supernatant fractions thus collected were pooled and analyzed for drug content. The liposomal dispersion of INH and RFP thus obtained was filled in amber colored vial under nitrogen atmosphere, sealed and stored in refrigerator until required for further experiments. A flowchart depicting the process is scheme 4.1 & 4.2. The liposomal compositions and process parameters were optimized to achieve maximum drug entrapment.

4.9 LIPOSOME CHARACTERIZATION

The liposomes were characterized for the following physico-chemical properties.

4.9.1 Trapped volume

Liposomal dispersion (5ml) was centrifuged at 3.6 x 10^6g for 30 min to get a tight pellet at the bottom of the centrifuge tube. The supernatant was decanted off to remove every drop of excess fluid (including some liposomes if necessary). The pellets were solubilized in 10 ml of 0.1% Triton-X-100 solution in methanol. A small aliquot (0.5ml) was removed for the quantification of phospholipids (Chapter 3, section 3.4.1.4) and the remainder was used to determine water content by Karl Fischer method (Karl Fischer titrater, Veego, India). 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 trapped volume in μl per μmoles of phospholipids was calculated, which is recorded in table - 4.7.

4.9.2 Liposome size

The liposome size of unextruded and extruded liposomes was determined by laser diffraction technique using Malvern particle size analyzer (Malvern Master sizer 2000 SM, U.K.). Diluted liposome suspension was added to the sample dispersion unit-containing stirrer and stirred at 2000 rpm in order to avoid interparticle aggregation. The laser obscuration range was maintained between 10-20%. The average particle size was measured after performing the experiment in triplicate. The mean particle size of the various liposomal formulations before extrusion and after extrusion through 0.2μm Nuclepore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA) is shown in table - 4.8. The particle size distribution pattern of the liposomal formulations after extrusion is shown in the particle size analysis data (Table-4.8).

4.9.3 Shape and lamellarity

The liposomes were viewed under the Olympus optical microscope (BX40F4, Japan). The provision of dark background and attachment of polarizing lens was used to study the liposome shape and lamellarity.

4.9.4 Percent drug entrapment

To determine percent drug entrapment (PDE) free and entrapped drug was measured. The free INH and RFP (unentrapped) in the liposomal dispersion were separated by ultracentrifugation method as described by (New, 1990a). Briefly, the liposomal dispersion was centrifuged at 10,000 rpm ≈ 5000g (0°C) and the supernatant was removed without disturbing the liposomal pellet. The liposomal pellet was redispersed with known aliquots of distilled water and the centrifugation cycle was repeated and the pooled supernatant was analyzed for the free drug content by the procedure described in sections 3.4.3.4 & 3.4.4.4 for INH and RFP, respectively.

The entrapped INH and RFP in liposomal dispersion were determined by modified Bligh-dyer two-phase extraction procedure. Liposomal dispersion 0.2ml was transferred to a calibrated glass centrifuge tube. The volume was made up to 2ml with saturated sodium chloride solution and 2ml of chloroform was added with vigorous vortexing. The tubes were centrifuged at 4.38 x 10^3g for 10 min. The lower chloroform layer was transferred to 10ml volumetric flask through a bed of anhydrous sodium sulphate previously saturated with chloroform. The extraction was repeated twice with 2ml of chloroform and combined extract was retained for phospholipid and

CHOL analysis. Aqueous extract was further diluted to obtain the INH concentration in linearity range for Spectrophotometric analysis described in chapter 3 section 3.4.3.2. PDE is expressed as:

$$PDE = \frac{\text{drug entrapped}}{\text{drug entrapped + free drug}} \times 100$$

4.10 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.

4.11 RESULTS AND DISCUSSION

Liposomes of INH and RFP were prepared by the selected TFH and REV methods that are optimized to achieve maximum PDE. INH being hydrophilic drug the entrapment in to liposome was made either by dissolving the drug in aqueous hydrating medium for THF method or in aqueous dispersed phase for REV method. While RFP being amphiphilic drug entrapment in to liposomes involved co-evaporation of the lipid and drug from the solvent system in a round bottom flask in TFH method and the REV method involved emulsification of organic phase containing lipid and drug with aqueous phase followed by evaporation of organic phase under vacuum was adopted. First of all the optimization and selection of various process and formulation variables for THF and REV methods were carried out followed by the selection of suitable method for the particle size reduction and separation of unentrapped drug was conducted. The results are summarized and discussed in the following sections.

4.11.1 Optimization of TFH and REV methods process variables

Process variables, viz. vacuum conditions for dry film formation, hydration time, and speed of rotation of flask were optimized. The effect of one variable was studied at a time keeping other variables constant. The results are recorded in Table 4.5 from which the following conclusions are drawn:

1. The vacuum required for solvent evaporation to form a uniform thin film was raised from 15 inch Hg to 25 inch Hg. The low vacuum (15 inch Hg) was found to be insufficient for the complete removal of the solvent mixture. The presence of residual solvent may lead to physical destabilization of liposomes

by interfering with the co-operative hydrophobic interactions among the phospholipid methylene groups that hold the structure together (Martin et al, 1990). The vacuum of 20 inches of Hg for 30 min and 60 minutes for INH and RFP respectively was found to be optimum for complete evaporation of solvent mixture and producing more translucent lipid film. However, for complete solvent removal of residual solvent (post film formation) the flask was attached to manifold of a lyophilizer exposed to high vacuum overnight for the preparation of empty and INH containing liposomes. In case of RFP liposome preparation, higher vacuum (25 inch Hg) resulted in rapid evaporation of the solvent system leading to crystallization of the drug (RFP) due to its comparatively less solubility in the solvent mixture and hence resulted in poor percent drug entrapment in the liposomes. This is in agreement with the findings of Martin et al (1990) that differential solubilities of amphiphilic components of bilayer and drug in organic solvents are often encountered and must be taken into consideration in order to avoid crystallization of a single component during solvent-stripping operations.

In REV method of preparation, the organic solvents were removed under partial vacuum (10 inch Hg) at 20-30°C. The initial low vacuum was maintained and carefully controlled using a nitrogen gas bleed to remove bulk of the organic solvents and then increased gradually (15 inch Hg) for complete solvent stripping as described by Betageri et al, 1993. Followed by the vacuum of 10 inch Hg at high speed (180 rpm) with intermittent vortexing for 10 min cycle was adopted for complete removal of solvent.

2. Speed of rotation: In TFH method, the speed of rotation of flask was increased from 60 rpm to 180 rpm. Rotation of 60 rpm resulted in thick incompletely dried film and presence of residual solvents. While at 180-rpm speed, a dry film with varying thickness was produced with a thicker film at periphery and thinner film at the center. A speed of 120 rpm was found to be adequate to give thin, uniform and completely dry film. Hence, 120-rpm speed of rotation of flask was selected to be optimum for both liposomal preparations.
3. Hydration time: In TFH method, the lipid film was hydrated from 30 minutes to 3 hours before size reduction. An optimal hydration time was required for complete conversion of planner bilayers to spherical liposomes. Lower hydration time led to a non-uniform shape and size of the liposomes and also

the un-hydrated part posed difficulty in size reduction by extrusion. The hydration time beyond 1 h resulted in no further improvement. Hence, 1 hr hydration time was found to be optimum for the INH and RFP liposome preparation.

In REV method, the critical step was phase inversion when most of the organic solvent was removed and a gel was formed. It needed vigorous vortexing to convert the viscous fluid state to liposomes. The collapse of gel spontaneously coincided with the conversion of the w/o emulsion into the liposomal form. When water content dropped considerably and/or higher concentration of lipids were used, MLV-REV was formed in which a large aqueous core was surrounded by many phospholipid bilayers (Pidgeon et al, 1986). To the inverted gel a small amount of water was added to the gel as continuous phase water (CPW). The addition of CPW was attempted to optimize drug entrapment that intended to be bulk water to suspend or convert the lipids into liposomes at the gel stage. Supplying bulk water to the gel reduced the need for core water to move and become bulk water during the final stage of liposome formation and thus resulted in higher PDE (Pidgeon et al, 1987).

Table 4.5: Selection of process parameters by TFH method for INH and RFP liposomes.

Parameters	Observation	Inference	
		INH	RFP
VACUUM			
15 " of Hg	Aggregation of liposomes	Poor solvent removal	
20 " of Hg	**Distinct separated liposomes**	**Suitable**	
25 " of Hg	Poor drug entrapment	Precipitation of lipid/drug	
SOLVENT EVAPORATION TIME (28" of Hg)			
15 minutes	Solvent residual	-----	-----
30 minutes	**Complete solvent removal**	**Suitable**	-----
60 minutes	**Complete solvent removal**	-----	**Suitable**
120 minutes	No further improvement	-----	-----
SPEED OF ROTATION			
60 rpm	Thick lipid film formation	-----	-----
120 rpm	**Uniform lipid film formation**	**Suitable**	**Suitable**
180 rpm	Thin but non-uniform film formation	-----	-----
HYDRATION TIME			
30 minutes	Non uniform shape and size distribution	-----	-----
60 minutes	**Uniform size distribution**	**Suitable**	**Suitable**
120 minutes	No further improvement	-----	-----
FREEZE-THAW CYCLE (-50°C for 1h - 30°C/60°C for 1/2h)			
1st	Non-uniform liposomes	-----	-----
2nd	Non-uniform liposomes	-----	-----
3rd	**Uniformly oriented liposomes**	**Suitable**	**Suitable**
4th	Non-uniform liposomes	-----	-----
NO. OF EXTRUSION CYCLES			
3 cycles	**Non uniform distributed liposomes**	**Suitable**	-----
5 cycles	**Uniform distributed liposomes**	-----	**Suitable**
7 cycles	Uniform distributed liposomes	-----	-----
SEPARATION OF UNENTRAPED DRUG			
Mini-column centrifugation	Very tenuous method with limited capacity	-----	-----
Dialysis	Easy, but time consuming and possible drug leak	-----	-----
Ultra centrifugation	**Easy and fast convenient method**	**Suitable**	**Suitable**
Protamine aggregation	Easy and fast but destructive method. Also not suitable for charged liposomes	-----	-----

4.11.2 Optimization of THF and REV methods formulation variables

Formulation variables, like composition of solvent system, solvent system volume, volume of hydration medium, drug: lipid ratio, composition of lamellae (PC: CHOL: Charge ratio), choice of organic solvent combination for REV method and ratio of aqueous phase to organic phase, were optimized. The results are recorded in Table 4.1 – 4.6 from which the following conclusions are drawn:

1. Composition of solvent system: The solvent system composition should be such that it prevents precipitation of formulation components during solvent stripping process. For TFH method, organic solvent system of chloroform: methanol was used for dissolving the formulation components like SPC/HSPC, CHOL, and charged lipids SPG/SA. The solvent ratio of 2:1 was observed to be optimal for both INH and RFP liposomal preparations.
2. Volume of solvent system: For the THF method, the organic solvent system chloroform: methanol (2: 1) from 5ml to 30 ml was used for dissolving the formulation components like SPC/HSPC, CHOL, SPG/SA and drug (RFP). The optimum solvent system required for both drugs was 10ml for a lipid/drug concentration of 100mg. The lower volume of the solvent system, 5ml resulted in lesser surface exposure and formation of thicker film. Thus increase in solvent system was found to be directly related to increase in the surface area of the formed film and an increased surface area resulted into thinner and uniform film formation thereby a net increase in the drug entrapment value (Payne et al, 1986). Further increase in the solvent system did not show any improvement in film formation and in drug entrapment. It may be due to physical limitation added by the size of the round bottom flask (250ml) and the hydrophilic or hydrophobic nature of drug also affected the volume of solvent required in order to prevent crystallization during solvent striping (Martin et al, 1990).
3. Volume of hydration medium: The optimum volume of hydration medium is required to ensure complete hydration of the planar bilayers to form the spherical liposomes. For hydrophilic drugs like INH, 1 ml of hydration volume for a lipid concentration of 100-200mg was found optimal to form uniform spherical liposomes by TFH evaporation method. Further increasing the hydration volume lead no further increase in PDE. It may be due to that the entrapment efficiency of water soluble is depend on the trapped volume, hence

for a given lipid concentration the trapped volume do not change by change in the hydration volume. However for RFP, 2ml of hydration volume for a lipid/drug concentration of 100mg was found optimal, RFP being amphiphilic leaks or gets dissolved into the higher hydrating medium. Hence, further increase in the hydration volume showed decreased PDE.

4. Drug: lipid ratio: Increase in the lipid proportion relative to drug led to the increase in the drug entrapment from 29.30 % (INH1, 1:1) to 66.92 % (INH3, 1:10), and from 52.94 % (RFP1, 1:5) to 76.64 % (RFP3, 1:15) (Calculated 'F'= 433.10, tabulated 'F' = 3.68, at 2 degree of freedom, P = 0.05). With increase in quantity of lipids, more number of liposomes per ml of the liposomal dispersion was formed, resulting into increased drug entrapment (Schneider et al, 1994). But the proportionate increase in % drug entrapment is compensated with proportionate increase in lipids i.e. to use more lipids to entrap constant amount of drug. Hence, lower drug: lipid ratio was selected for both drugs (INH 1:1 and RFP 1:10).
5. Composition of lamellae (PC: CHOL: Charge ratio): With increase in CHOL content up to certain level showed increase in drug entrapment for both drugs (INH4, 9:1:0, 23.71 % to INH5, 8:2, 35.54 % and RFP4, 9:1, 73.58 % (Calculated 'F'= 61.03, tabulated 'F' = 3.68, at 2 degree of freedom, P = 0.05). This may be due to increased bilayer stability by incorporation of cholesterol in to the bilayers (Betageri et al, 1993). Further increasing the CHOL proportion resulted in decrease/no improvement in drug entrapment (INH6, 7:3, 35.81 and RFP4, 9:1, 73.58 % to RFP6, 7:3, 58.70 %). In case of water soluble drug (INH) the probable reason may be due to its higher permeability through membrane bilayer. Whereas in case of amphiphilic drug (RFP) it is due to drug and CHOL both competes with PC molecules for orientation into the bilayer membranes and results in decreased PDE.

   Similarly inclusion of negative and positive charge in the liposomal membrane resulted in increase in drug entrapment. This may be due to increase in interlamellar repeat distance between successive bilayers in the MLVs, swelling the structure with a greater proportion of the aqueous phase (INH7, 7.9:2:0.1, 49.77 % (SPG-3), & INH10, 7.9:2:0.1 (SA), 78.94 %). The presence of charged lipids also reduces the likelihood of aggregation following the formation of MLVs (Betageri et al, 1993). The optimal lipid to CHOL to

charge ratio was found to be 7.9.2:0.1 and 9.5:0:0.5 for INH liposome and 8.9:1:0.1 and 9.9:0:0.1 for RFP liposome made from SPC and HSPC respectively. Further increase in charge had no significant increase in PDE (Calculated 'F'= 3.19, tabulated 'F' = 3.68, at 2 degree of freedom, P = 0.05) As INH is hydrophilic, % increase in drug entrapment was more in INH compared to RFP due to increase in interlamellar distance between two lamellae of MLV led to more increase in aqueous compartment and thus increase INH entrapment. Though in case of INH the entrapment in presence of SA increased, but due to poor stability (aggregation) it was not selected for further studies. Also RFP with SA showed poor entrapment efficiency due to SA-drug interaction.

6. Choice of organic solvent for REV method: The organic solvents, such as diethyl ether or methanol, employed in the liposome preparation although usually removed by evaporation, may remain as traces in the final formulation posing a possible risk for human health. It can also lead to inadequate stability of the vesicles (Cortesi et al, 1999). Use of other organic solvents like ethyl acetate and ethanol can solve such problem. Ethanol forms monophasic system upon contact with aqueous phase while ethyl acetate forms biphasic system (emulsion) upon contact with aqueous phase. When ethyl acetate was used alone, it resulted in distorted spherical vesicles due to formation of unstable biphasic system upon contact with aqueous phase. Use of ethanol alone resulted in high PDE due to formation of monophasic system upon contact with aqueous phase. However, drug leakage was observed due to presence of traces of ethanol that lead to disruption of bilayer. In case of ethyl acetate: ethanol (1:1) combination, proper spherical vesicles and high PDE was observed. Combination of these organic solvents with aqueous phase forms stable emulsion, which is pre-requisite for REV (Betageri et al, 1993).
7. Aqueous phase to organic phase ratio for REV method: When aqueous phase to organic phase ratio was raised from 1:2 to 1:5, marked increase in the PDE was observed i.e. INH28 (Neutral) 30.48 % to INH34 (Neutral) 66.71 % and INH29 (SPG-3) 38.92 % to INH35 (SPG-3) 68.54 %. Similarly for RFP also; RFP28 (Neutral) 62.37 % to RFP32 (Neutral) 86.31 % and RFP29 (SPG-3) 55.30 % to RFP33 (SPG-3) 88.46 %. Further increase in the organic phase did not result in to increase in PDE (Calculated 'F'= 1.85, tabulated 'F' = 3.09, at

3 degree of freedom, P = 0.05). Thus organic phase ratio is most important for proper emulsification and formation of fine droplet surrounded by phospholipids i.e. liposomes, with uniform size, shape and high PDE.

4.11.3 Drug (INH) loading

Initially attempts were made to entrap and retain INH in to liposomes by pre-loading technique. It was observed that for a drug substance with high water solubility and small molecular weight was difficult to entrap and retain the entrapped drug within the liposome. Further attempts to increase the amount of INH retention in to liposomes, like use of synthetic saturated phospholipids and pH gradient gave little success. Hence attempts made to complex INH with suitable complexing agents to increase the molecular size and hence the retention within the liposomes.

4.11.3.1 Preparation of INH-Etheylene Diamine Tetra Acetic acid complex

In order to achieve higher entrapment and retention of INH within liposomes by increasing the INH molecular size, an novel INH-EDTA complex (4:1) was made by dissolving EDTA into a heated (60°C) supersaturated solution of INH. The solubility of EDTA as such in water is only 2g/L, whereas on formation of complex with INH its solubility increased to 10-20 folds. The DSC, IR and NMR spectrum of the INH, EDTA alone, and INH-EDTA complex were taken (Figure – 4.1, 4.2 & 4.3). It was observed that the peak corresponds to EDTA or its functional group (-COOH) was absent in the DSC, IR and NMR spectra confirms formation of INH-EDTA complexation. The formed complex was used in pre- and post-loading (trans-membrane) of drug into liposomes. The trans-membrane loading technique gave better PDE than pre-loading. Hence the process of trans-membrane technique was further optimized.

4.11.3.2 Optimization of transmembrane loading of INH with in liposomes.

For drug delivery purpose the loading efficiency (weight of drug entrapped over the total weight of drug involved in entrapment) is usually not critical because the non-entrapped drug can generally be recovered and reused afterwards, hence the important factor is rather the ratio of useful entrapped drug against the weight of the lipids used for entrapment. The techniques of the prior art for drug loading with in liposomes by passive entrapment (Bangham et al, 1965), dehydration and rehydration treatment (Kirby & Gregoriadis, 1984), higher concentration of solute (Minchey et al., 1989),

transmembrane ionic/pH gradient are complicated (Beaumier et al., 1982), expensive and not generally applicable to all types of drugs. However, transmembrane drug loading (osmotically) controlled permeation process avoids tedious and expensive pretreatments of the film forming lipids. In brief, for transmembrane drug loading, one prepares empty liposomes by any available method that the aqueous phase entrapped therein is only pure water or very dilute solutions of non-ionic substances or electrolytes. Drugs to be encapsulated are incubated for a required period of time, at a temperature above the lipid phase transition temperature. After achieving the equilibrium (where the concentration of the encapsulated substances is the same inside and outside the liposomes) the liposomes are extruded and centrifuged to separate the unentrapped drug.

In the transmembrane drug loading process variables, viz. liposome formation and incubation temperature, duration of incubation and lipid concentration, were optimized. The effect of one variable was studied at a time keeping other variables same. MLV liposomes were prepared in purified water at various temperatures and were incubated at various temperatures with INH-EDTA solution (200mg INH/ml) for various time periods using various lipid (forming/incubation) concentrations. The results recorded in Table 4.6 reveal following conclusions:

1. The optimal temperature for liposome formation was observed to be 60°C (HSPC and SPG-3 in a 9.5:0.5 molar ratio).
2. The optimal temperature for incubation was in the range 50-60°C (HSPC and SPG-3 in a 9.5:0.5 molar ratio).
3. The optimal duration of incubation was in the range 30-60 minutes (HSPC and SPG-3 in a 9.5:0.5 molar ratio).
4. The optimal lipid concentration during liposomal formation was of 25-100mg/ml and the lipid concentration during incubation has no influence on the trapping capacity.

The advantage of transmembrane drug loading was that the lipid concentration in the aqueous carrier used for incubation had no significant influence on the internalization capacity. Hence by concentrating the liposomes in the aqueous carrier favorably influence the entrapment yield and reduce the amount of residual non entrapped substances to be recovered and reused. However for the ease of extrusion of liposome a lipid concentration of 100mg/ml was selected in our experiments.

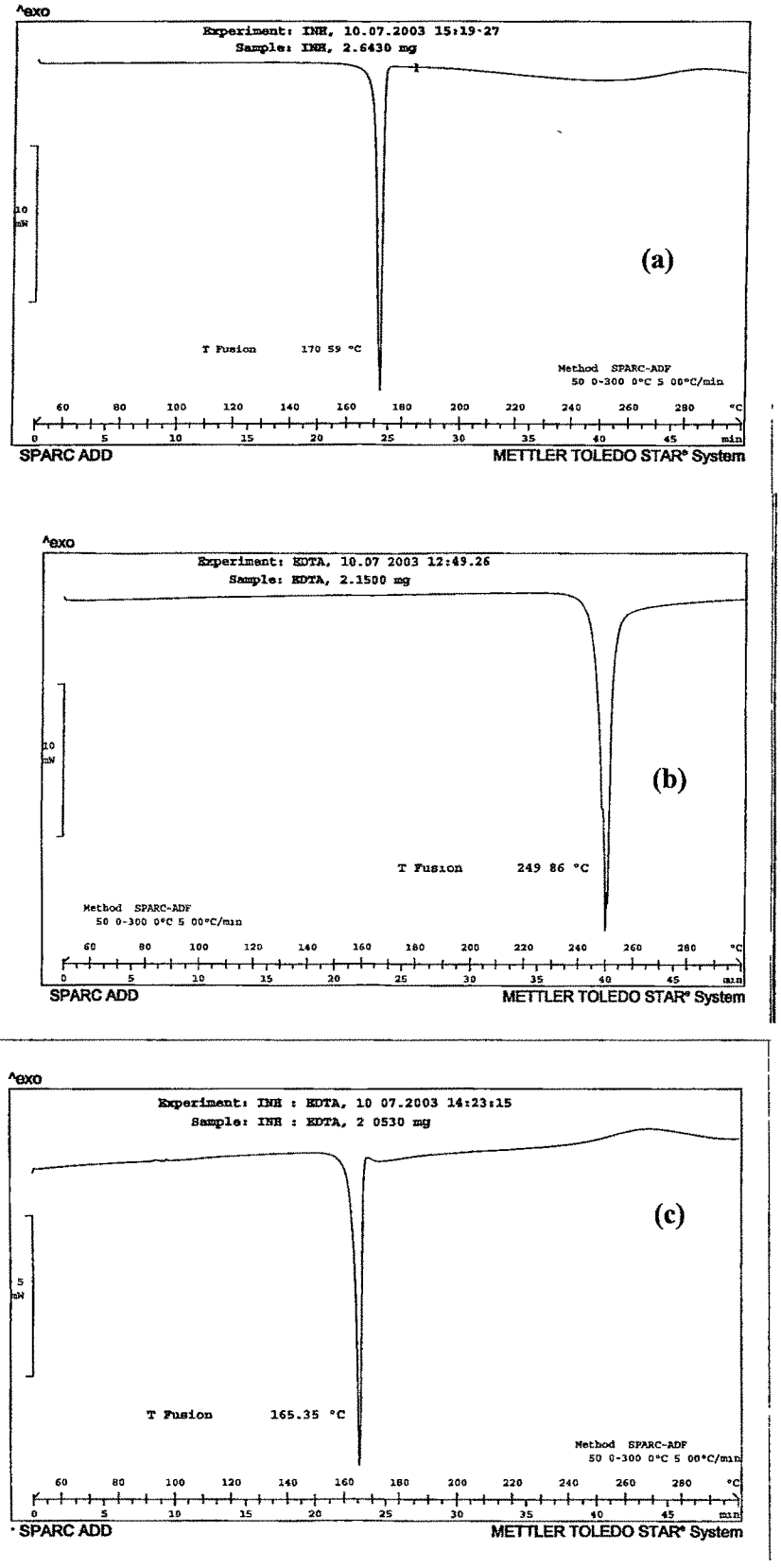


Figure 4.1: DSC thermograph a) INH; b) EDTA; c) INH: EDTA complex arrow showing the absence of EDTA peak.

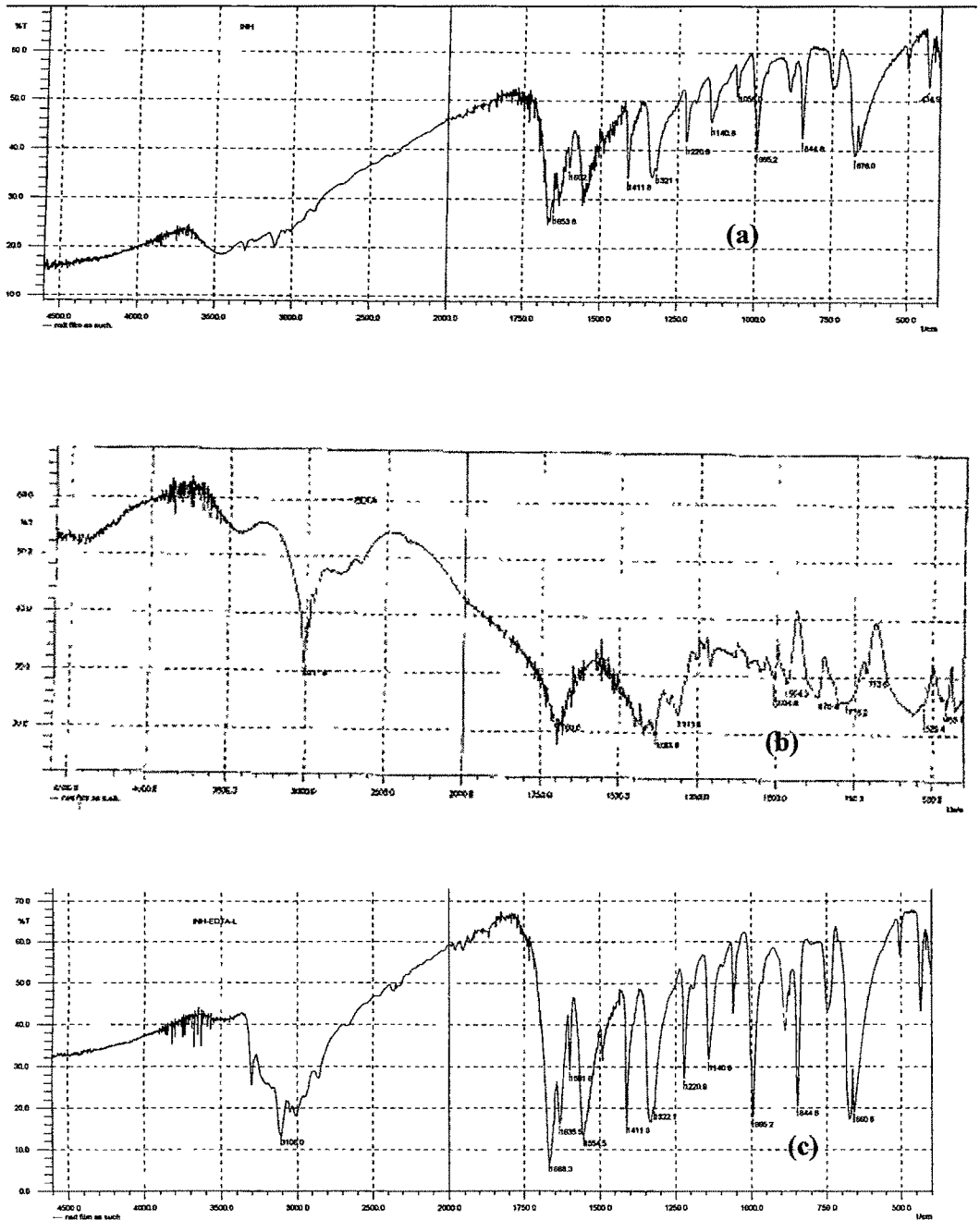


Figure 4.2: IR spectrum a) INH; b) EDTA; c) INH: EDTA complex.

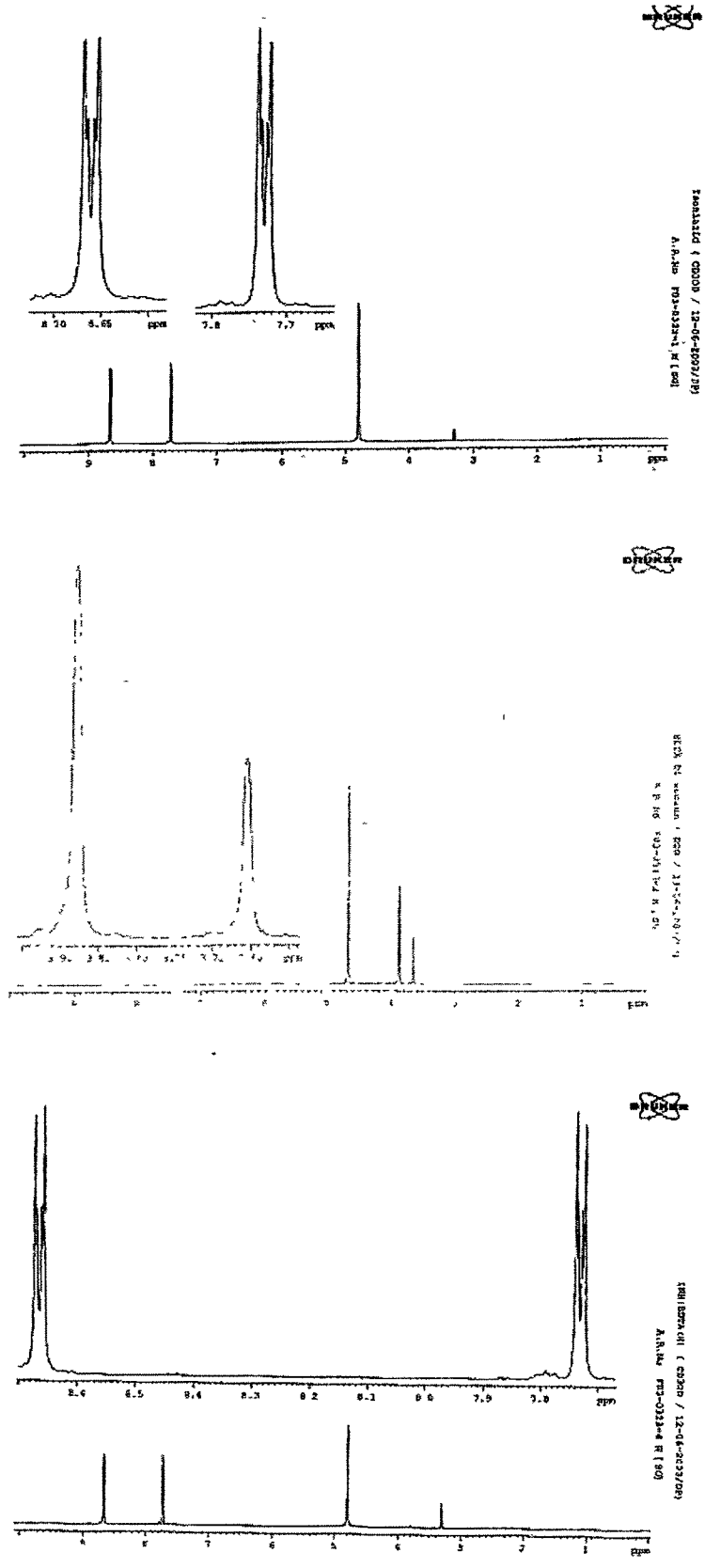


Figure 4.3: NMR spectrum a) INH; b) EDTA; c) INH: EDTA complex.

Table 4.6: Effect process and formulation variables on trans-membrane loading of INH.

Parameters	Results mg INH/mg lipid
Incubation temperature	
Temperature of liposome formation (°C)	
55	**1.1**
60	**1.3**
65	**1.2**
70	**0.8**
75	**0.7**
Temperature of liposome incubation (°C)	
40	**1.0**
50	**1.2**
60	**1.3**
70	**1.1**
80	**1.0**
Duration of incubation at 60°C (min.)	
15	**1.0**
30	**1.2**
45	**1.2**
60	**1.2**
90	**1.2**
120	**1.2**
Lipid concentration	
Lipid concentration at formation (mg/ml)	
25	**1.2**
50	**1.2**
100	**1.3**
150	**0.8**
200	**0.7**
Lipid concentration during incubation (mg/ml)	
25	**1.2**
50	**1.2**
100	**1.3**
150	**1.3**
200	**1.2**

4.11.4 Selection of method for liposome size reduction and separation of unentrapped drug

Selection of size reduction and separation of unentrapped drug from liposomes methods were optimized to prepare liposomes of INH and RFP.

4.11.4.1 Liposomal particle size reduction method

Extrusion was carried out for achieving the desired size mechanically breaks the liposomes to reduce size and in doing so distorts the orientation of the preformed bilayer. In order to regain the physical and conformational stability of the bilayer membrane, freeze-thawing was carried out for an appropriate time period and cycle. A freeze-thaw cycle of liposomes were done by freezing the liposomal dispersion at -50°C for 1h and thawing it at 30°C/60°C for 1/2h. The 3 times freeze-thaw cycle was found to be optimal for proper shape with proper orientation (confirmed by microscopy) and drug equilibrium.

Extrusion of liposomes using 2 μm polycarbonate membranes was used to reduce the size of liposomes below 5 μm suitable for pulmonary delivery. Number of extraction cycles is important for achieving uniform size distribution of the liposomes was optimized. For INH liposomes, after 3 cycles, uniform size distributed of liposomes were obtained (VMD 2.0 ± 0.2 for INH34 & 1.9 ± 0.3 for INH35). For RFP liposomes, 5 cycles were found to be optimum (VMD 1.8 ± 0.2 for RFP32 & 2.0 ± 0.3 for RFP33).

4.11.4.2 Selection of method for separation of unentrapped drug

Separation of unentrapped drug (Betageri et al, 1993): Separation of unincorporated drug from liposomes can be achieved either by 'gel filtration' (mini-column centrifugation), ultra centrifugation, protamine aggregation, dialysis or controlled centrifugation at low speed. Gel filtration was done by the method described by (Fry et al, 1978) using Sephadex G-50 as the gel material. Gel filtration was found to be very tenuous method with limited capacity and was not feasible for the entire formulation purification. Slight modification in the procedure was required for each specific liposome. Ultra centrifugation (Montenegro et al, 1996) at higher G value ($5.33 \times 10^6 \times g$) was easy and faster method suitable for separation of unentrapped drug. Dialysis method was time consuming and wad observed that drug leaks during the dialysis period. Protamine aggregation was destructive approach and its use is restricted for the determination of the drug entrapment and could not be used for the

separation of the liposomal dispersion. Also, this method was not suitable for positively charged liposomal systems and was only suitable for negatively charged or neutral liposomes. The entrapment values obtained by high speed centrifugation method were found similar to that obtained with protamine aggregation/dialysis confirms the accuracy of the process. It was further confirmed microscopically to reassure total drug separation.

TFH method for the preparation of INH and RFP liposomes were selected because TFH method has produced similar/more drug entrapment than REV method and the process were simpler than REV. Further SPC containing liposomes were found to give less drug entrapment than HSPC containing liposomes. Also, from the viewpoint of stability, HSPC liposomes will be more stable compared to SPC liposomes. Thus TFH method was further employed for the liposome preparation containing HSPC in the bilayer composition as phosphatidylcholine component.

4.11.5 Characterization of liposomes

4.11.5.1 Trapped volume

The trapped volume of liposome preparation is normally expressed as the trapped volume per lipid and can vary from 0.5 µl per µ mole for some MLV and SUV systems to as much as 30 µl per µ mole of certain LUV system. For the mean liposomal size in the range of 1.33 to 1.80 µm, the estimated trapped volume (if unilamellar) as described by (Hope et al, 1990), would be approximately 40 to 50 µl per µmole. These may be compared with the measured value of 3.92 to 5.12 µl per µ mole, which is almost 10 times less then the expected value. In MLVs the presence of every lamellae would replace equivalent entrapped volume with a sphere of lamellae of 4 µm thickness (Blaurock et al, 1982), therefore the entrapped volume for MLVs will be many fold less than to that of a unilamellar vesicles of the same size. Based upon these observations it was concluded that the large majority of the vesicles produced by extrusion were still multilamellar. Trapped volume 3.92 – 5.12 µl per µ mole for INH liposome was proportionally related to the entrapped water soluble drug (Table 4.7) and was present predominantly in the inner aqueous compartment. For RFP being hydrophobic, it is only a parametric study, before going for the study of liposome behavior in physical or biological system.

Table 4.7: Trapped volumes of liposomal dispersions prepared with optimal process and formulation variables

Batch No.	Trapped Volume * (µl / µ mole)
Isoniazid	
INH 13	5.12 ± 0.18
INH 17	3.92 ± 0.32
Rifampicin	
RFP 13	4.82 ± 0.14
RFP 16	5.16 ± 0.22

* Mean ± SEM (n=6)

Table 4.8: Particle size range of liposomal batches at various stages of preparation

Batch No.	Volume mean diameter (µm)*	< 10% (µm)*	< 90% (µm)*
Pre extrusion			
INH13	17.2 ± 4.8	5.48 ± 0.8	88.47 ± 1.6
INH17	20.8 ± 6.8	7.78 ± 0.6	76.37 ± 3.4
RFP13	19.4 ± 7.5	6.87 ± 0.3	82.44 ± 2.8
RFP16	15.4 ± 5.8	8.46 ± 0.4	81.84 ± 4.2
Post-extrusion			
INH13	2.12 ± 0.06	0.55 ± 0.02	25.79 ± 2.24
INH17	1.84 ± 0.04	0.59 ± 0.04	24.84 ± 4.42
RFP13	2.14 ± 0.02	0.63 ± 0.03	25.61 ± 4.26
RFP16	2.21 ± 0.03	0.53 ± 0.02	25.27 ± 3.37

*Mean ± SEM (n=6)

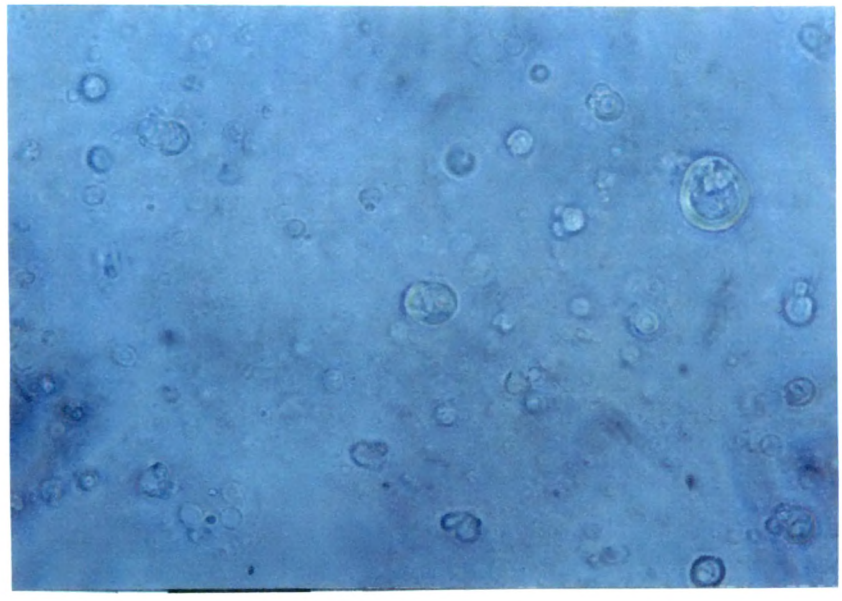


Figure 4.4: Photomicrographs showing liposomal vesicles under normal light.

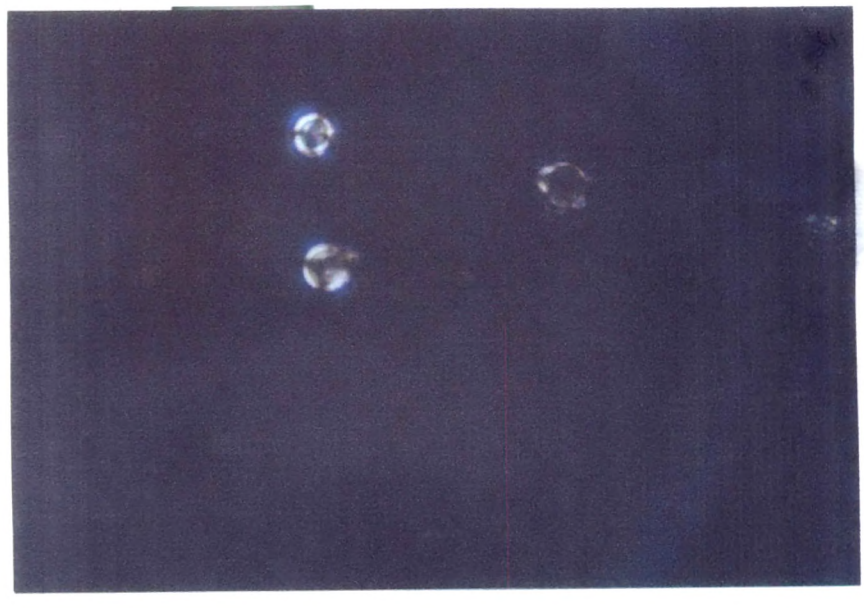


Figure 4.5: Photomicrographs showing liposomal vesicles under dark background and attachment of polarizing lens.

4.11.5.2 Liposome size

The vesicle size before extrusion and after extrusion was determined by laser diffraction using Malvern particle size analyser (Malvern Master sizer 2000 SM, U.K.). Liposome prior to extrusion had a greater mean size and broader size distribution, however, upon extrusion it acquired a narrower range of distribution and a mean liposomal size is below 5 μm (Table 4.8).

4.11.5.3 Photomicrography

All the batches of the liposomes prepared were viewed under Olympus (BX 40F4, Japan) with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity. A photograph in normal light is shown in figure 4.4 for RFP 13. The multilamellar vesicles after viewing it in polarizing attachment with Olympus shows the presence of Maltese crosses, which are characteristics of multilamellar liposomes (Hofland et al, 1994).

4.11.5.4 Percent drug entrapment

The mean PDE obtained during the optimization of liposomal INH and RFP by THF and RFP methods are reported in Table 4.1 – 4.4. Batches with higher PDE (INH13 – 63.71, INH – 64.16, RFP13 – 83.71 and RFP16 – 88.80) were selected for the development of liposomal dry powder inhaler formulations.

TFH method for the preparation of INH and RFP liposomes containing HSPC as bilayer component, size reduced by extrusion process and purified by high speed centrifugation method was selected for further development of liposomal dry powder.

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