CHAPTER 4

PREPARATION & CHARACTERIZATION OF LIPOSOMES

Liposomes are microscopic vesicles composed of phospholipid bilayers surrounding aqueous compartments as described by Bangham et al (1965). They consist of one or more bilayers. The driving force for bilayer assembly is the amphiphilic nature of phospholipid molecules. Phospholipid typically consists of a hydrophilic head group attached to two hydrophobic fatty acid chains. When suspended in an excess of aqueous solution, phospholipid molecules originate themselves in ordered bilayers so that the polar heads are hydrated and hydrophobic tails are excluded from the aqueous environment. Dependent on the selection of lipids, the preparation technique, and preparation conditions, liposomes can vary widely in size, number, position of lamellae e.g. multilamellar vesicles (MLVs) or Multivesicular vesicles (MVV), charge and bilayer rigidity (liquid-crystalline or gel state). These parameters influence the behavior of liposomes both in vivo and in vitro. The opsonization process, leakage profiles, disposition in the body and shelf life all depend on the type of liposome involved. Therefore it is important to select liposome constituents and the preparation technique carefully and to characterize the produced liposomes properly.

Many methods have been developed to prepare liposomes. There are at least fourteen major reported methods (Ostro et. al 1987 and Martin 1990). The seven most commonly employed method are lipid film hydration also referred as thin layer evaporation method (Bangham et al 1965), reverse phase evaporation technique (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.

In the preparation of liposomes intended to be freeze dried for pulmonary delivery there were two basic necessities, sufficient rigidity in the liposomal membrane to withstand drying with least leakage of the entrapped species (1) and second is liposomal size should be preferentially below 5μm (2)

Liposomes of Amikacin (AMK) and Amphotericin B (AMB) were prepared using thin layer evaporation technique (TFH) and reverse phase evaporation (REV) technique with membrane composition of saturated/unsaturated soyaphosphatidylcholine; cholesterol and either saturated soyaphosphatidylglycerol or stearylamine to prepare negatively and 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.

4.1 Experimental

4.1.1 Preparation of AMK liposomes

The liposomes of AMK were prepared by two methods like: Thin Film Hydration (TFH) and Reverse phase evaporation (REV) methods.

TFH method

Soya Phosphatidylcholine (Soya PC) (Lipoid S-100), or Hydrogenated soyaphosphatidylcholine (HSPC), cholesterol (CHOL) and either saturated soyaphosphatidylglycerol or stearylamine were dissolved in solvent mixture in 250 ml round bottom flask (Quick fit neck B-24) in different mass ratios (Table 4.1). Flask was

rotated in a rotary flash evaporator at 120-rpm under vacuum of 20 inches of Hg. The nitrogen atmosphere was maintained for 30 minutes to form smooth, uniform and dried film. Hydration of film was carried out by using AMK (10mg/ml) in 10mM Succinate buffer pH 6.5 containing 1mM EDTA under nitrogen atmosphere at room temperature for SPC & at 60°C for HSPC for 1 hours. The formed liposomal dispersion was extruded through 2μm polycarbonate membrane above the phase transition temperature i.e. at room temperature for SPC & at 60°C for HSPC for 5 cycles. Annealing of liposomes at room temperature was carried out for 2 hour after extrusion at room temperature for SPC & at 60°C for and separated from unentrapped drug by dialysis. The liposomal dispersion of AMK thus obtained was filled in amber colored vial under nitrogen atmosphere, sealed and stored in refrigerator until required for further experiments. The complete procedure is shown in Figure 4.1.

REV Method

Multilamellar vesicles (MLVs) of AMK were prepared by the modified REV technique (Cortesi et al, 1999) by optimizing both formulation variables like choice of organic solvent and ratio of aqueous phase to organic phase for proper orientation of vesicles and higher percent drug entrapment (PDE) (Table 4.2). Soya PC or HSPC, CHOL, α-tocopherol (1 percent of PC) and either SPG-3 or SA, were mixed with ethanol – ethyl acetate solvent system (1:1) and transferred to narrow neck tube with standard B-24 joint. REV cycles of 10 min at 10 in. of Hg, followed by 10 min at 15 in. of Hg and using AMK (10mg/ml) in 10mM Succinate buffer pH 6.5 containing 1mM EDTA (ratio of aqueous phase: organic phase to be 1:5) with intermittent vortexing. Liposomal dispersion was subjected to complete removal of last traces of organic solvent for 15 min

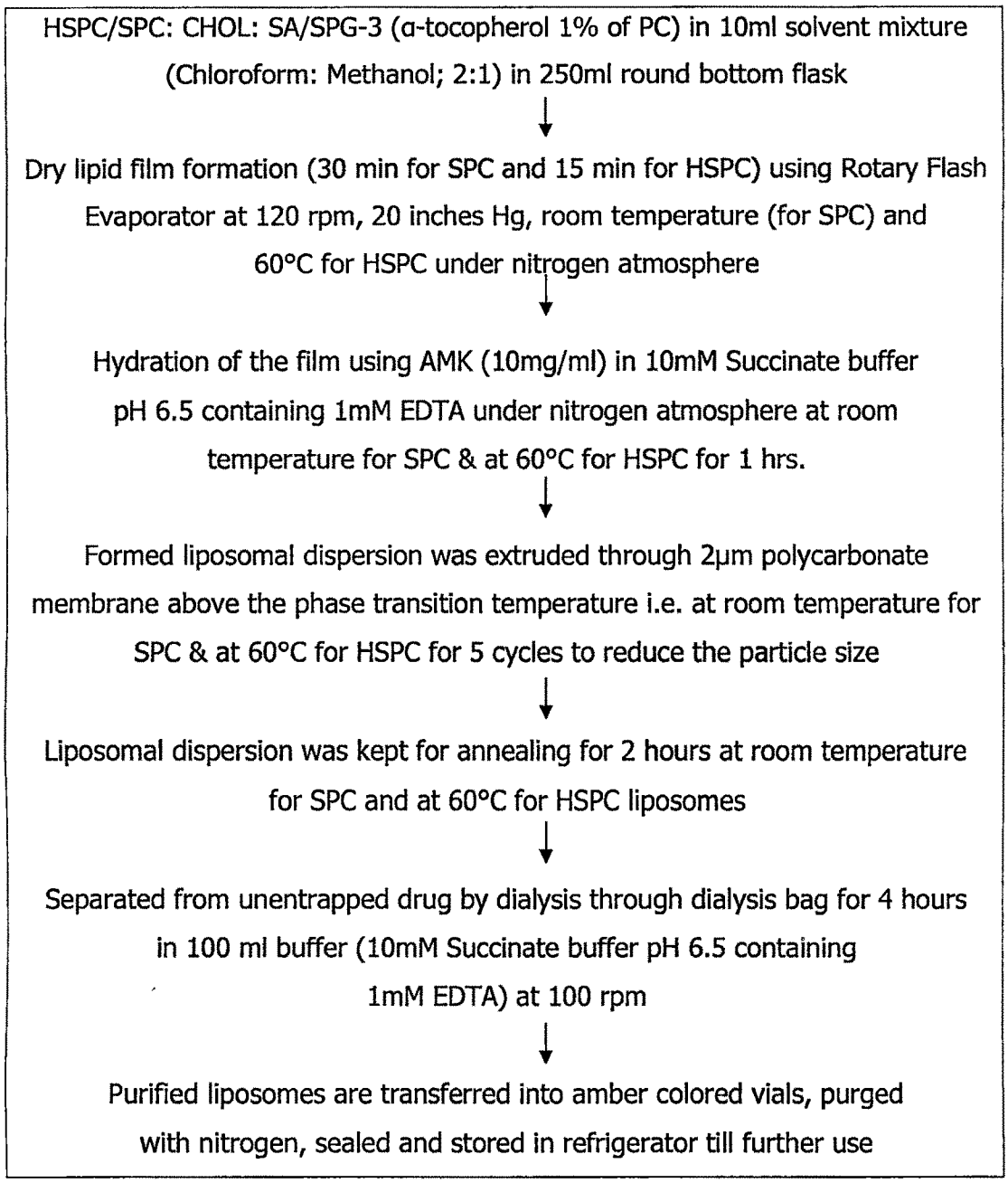

Figure 4.1 Flow diagram showing formulation technique in the preparation of AMK liposomes by TFH method

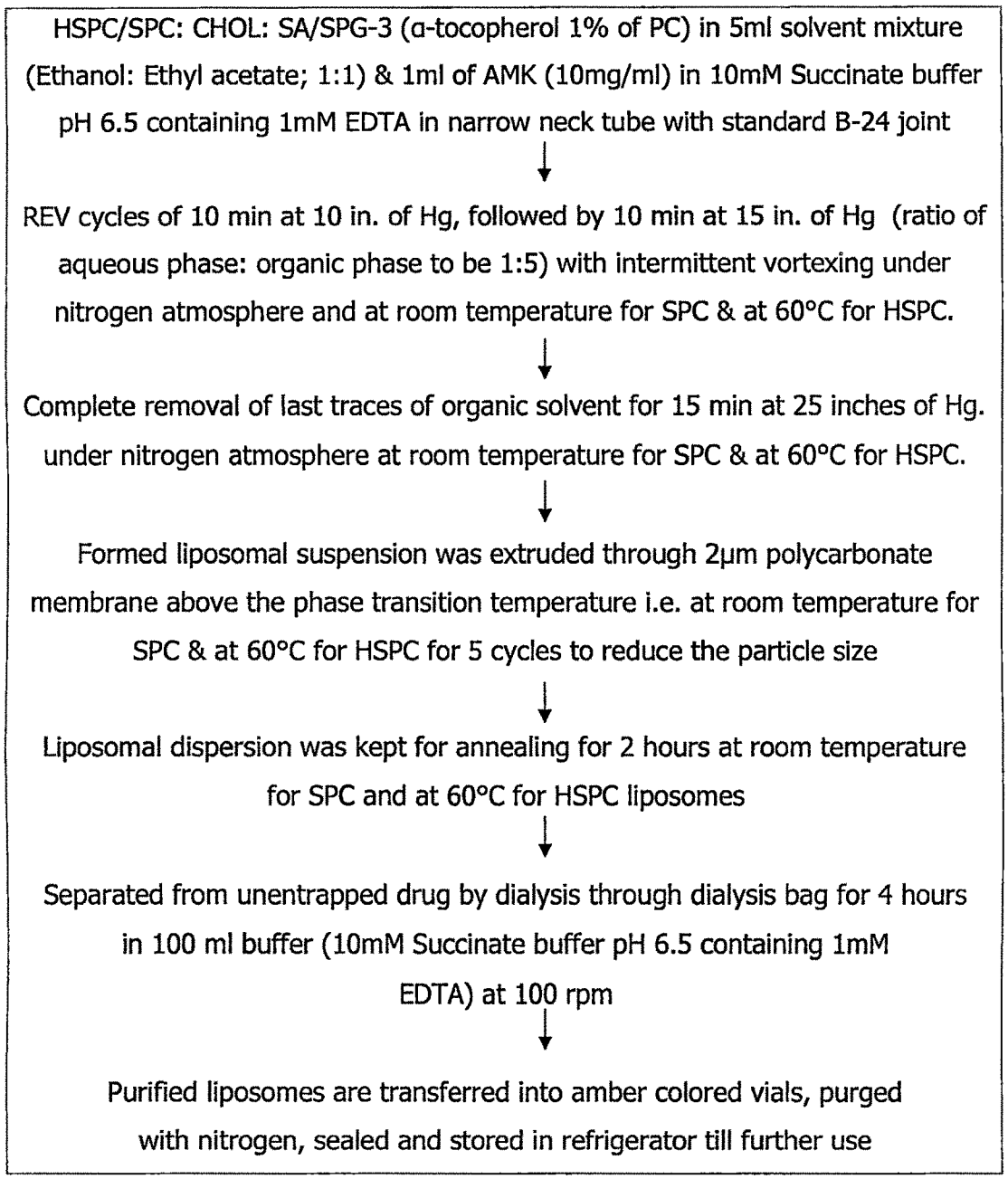

Figure 4.2 Flow diagram showing formulation technique in the preparation of AMK liposomes by REV method

at 20 in. of Hg. The formed liposomal dispersion was extruded through 2μm polycarbonate membrane above the phase transition temperature i.e. at room temperature for Soya PC & at 60°C for HSPC. Annealing of liposomes at room temperature was carried out for 2 hour after extrusion and separated from unentrapped drug by dialysis. The liposomal dispersion of AMK thus obtained was filled in amber colored vial under nitrogen atmosphere, sealed and stored in refrigerator until required for further experiments. The complete procedure is shown in Figure 4.2

4.1.2 Preparation of AMB liposomes

The liposomes of AMB were prepared by two methods like: Thin Film Hydration (TFH) and Reverse phase evaporation (REV) methods.

TFH method

Drug (5mg), Soya Phosphatidylcholine (Soya PC) (Lipoid S-100), or Hydrogenated soyaphosphatidylcholine (HSPC), cholesterol (CHOL) and either saturated soyaphosphatidylglycerol (SPG-3) or stearylamine (SA) were dissolved in solvent mixture (Chloroform: Methanol, 1:1) in 250 ml round bottom flask (Quick fit neck B-24) in different mass ratios as shown in (Table 4.3). Flask was rotated in a rotary flash evaporator at 120-rpm under vacuum of 20 inches of Hg. The nitrogen atmosphere was maintained for 30 minutes to form smooth, uniform and dried film. Hydration of film was carried out by using 10mM Tris buffer pH 6.5 containing 1mM EDTA under nitrogen atmosphere at room temperature for SPC & at 60°C for HSPC for 1 hrs. The formed liposomal dispersion was extruded through 2μm polycarbonate membrane above the phase transition temperature i.e. at room temperature for SPC & at 60°C for HSPC. Annealing of liposomes at room temperature was carried out for 3 hour after extrusion at

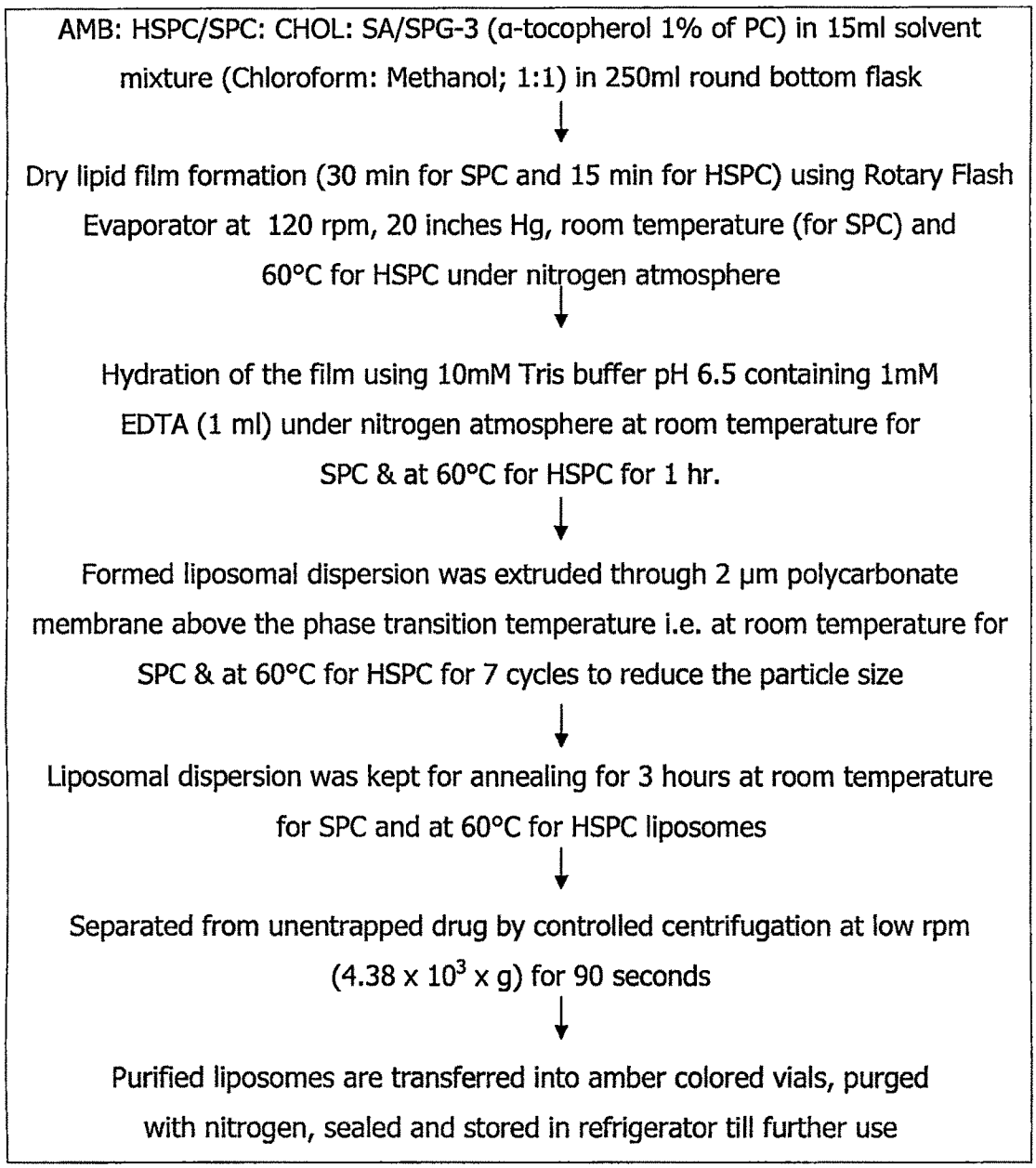

Figure 4.3 Flow diagram showing formulation technique in the preparation of AMB liposomes by TFH method

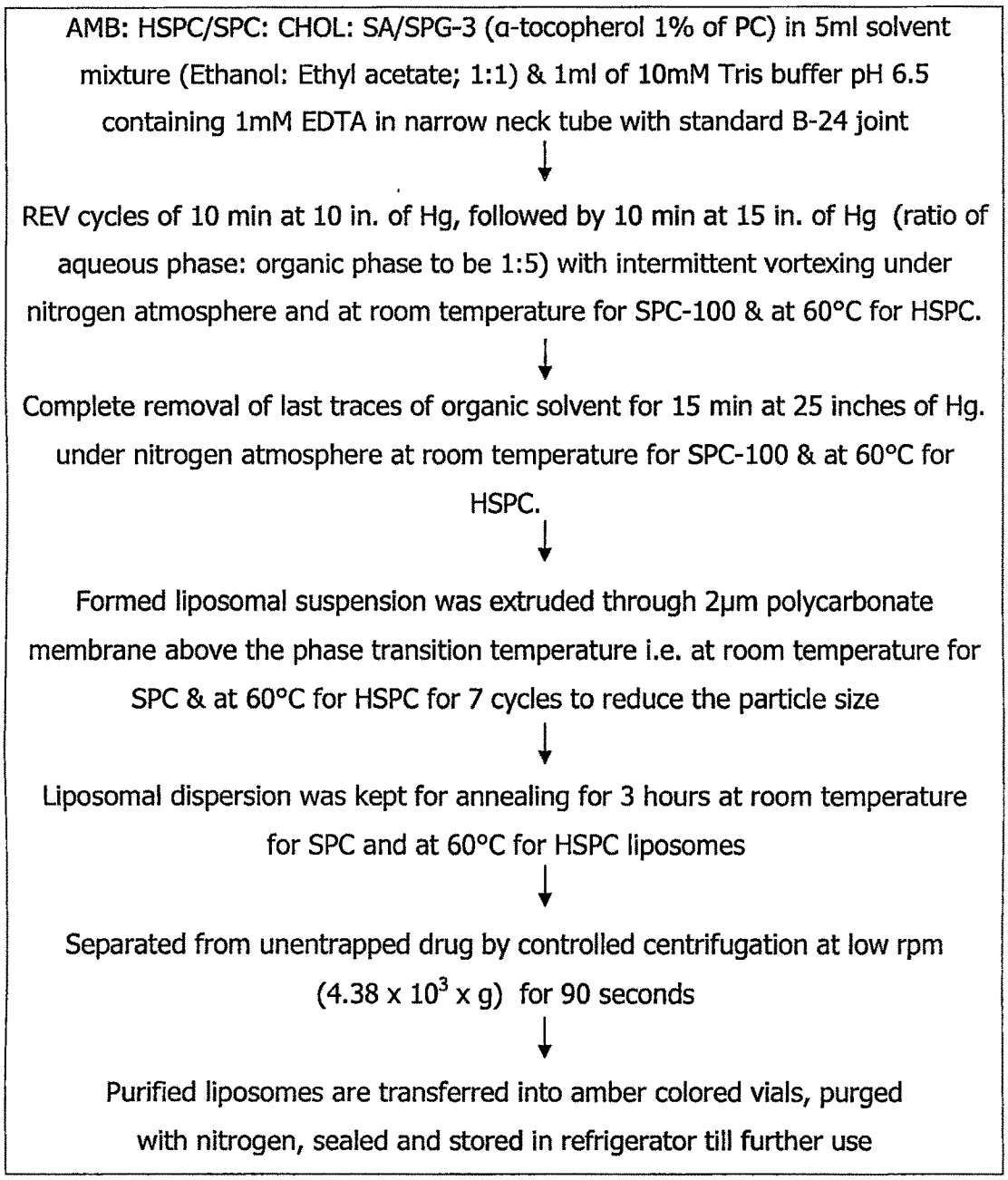

Figure 4.4 Flow diagram showing formulation technique in the preparation of AMB liposomes by REV method

room temperature for SPC & at 60°C for HSPC and separated from unentrapped drug by controlled centrifugation at slow speed. The liposomal dispersion of AMB thus obtained was filled in amber colored vial under nitrogen atmosphere, sealed and stored in refrigerator until required for further experiments. The complete procedure is shown in Figure 4.3.

REV Method

Multilamellar vesicles (MLVs) of AMB were prepared by the modified REV technique (Cortesi et al, 1999) by optimising both formulation variables like choice of organic solvent and ratio of aqueous phase to organic phase for proper orientation of vesicles and higher percent drug entrapment (PDE) (Table 4.4). Drug (5mg), Soya PC or HSPC, CHOL, α-tocopherol (1 percent of PC) and either SPG-3 or SA, were mixed with ethanol – ethyl acetate solvent system (1:1) and transferred to narrow neck tube with standard B-24 joint. REV cycles of 10 min at 10 in. of Hg, followed by 10 min at 15 in. of Hg and using 10mM Tris buffer pH 6.5 containing 1mM EDTA (ratio of aqueous phase: organic phase to be 1:5) with intermittent vortexing. Liposomal dispersion was subjected to complete removal of last traces of organic solvent for 15 min at 20 in. of Hg. The formed liposomal dispersion was extruded through 2μm polycarbonate membrane above the phase transition temperature i.e. at room temperature for Soya PC & at 60°C for HSPC. Annealing of liposomes at room temperature was carried out for 3 hour after extrusion and separated from unentrapped drug by controlled centrifugation. The liposomal dispersion of AMB thus obtained was filled in amber colored vial under nitrogen atmosphere, sealed and stored in refrigerator until required for further experiments. The complete procedure is shown in Figure 4.4.

All the batches were prepared with 10mg (AMK) and 5mg (AMB) with molar proportion of Soya PC or HSPC, Cholesterol and Soya Phosphatidylglycerol (SPG) or Stearylamine

(SA) calculated based upon their molecular weights to give negatively or positively charged liposomes respectively.

4.2 Separation of unentrapped drug from liposomes

For amikacin, free (unentrapped) drug was done by dialysis technique as described by Hayat et al. (1970). This technique is a modification of basic dialysis technique and permits the use of smaller volumes of dialyzing material. The liposomal dispersion (5ml) was transferred to dialyzing tube (Sigma, Molecular weight cut-off 12000 Dalton), sealed at both ends. It was held suspended into 250ml glass beaker containing 125ml-hydrating medium surrounding the sac for 4 hours while dialyzing medium was stirred at 100 rpm with magnetic needle. During dialysis, the unentrapped drug will pass to the dialyzing medium while entrapped drug in liposomes will remain in the dialysis tube.

For Amphotericin B, 5ml of Amphotericin B liposomal suspension was filled into glass centrifuge tubes of 15ml capacity and centrifuged in bench centrifuge at a very slow speed of 1000 rpm ($4.38 \times 10^3 \times g$) for 90 seconds. Drug crystals will sediment to form pellet at bottom of the tube and supernatant constituted purified liposomal dispersion.

4.3 Chemical analysis

4.3.1 Percent drug entrapment

To determine percent drug entrapment in amikacin liposomes modified Bligh-dyer two-phase extraction procedure was used. Liposomal dispersion (0.2ml) was transferred to a calibrated glass centrifuge tube and volume was made up to 2ml with saturated sodium chloride solution, to it 2ml of chloroform was added and vortex vigorously. The tubes were centrifuged at $4.38 \times 10^3 \times g$ for 10 minutes. The lower chloroform layer was transferred to 10ml volumetric flask through bed of anhydrous sodium sulphate

previously saturated with chloroform. The extraction was repeated twice with 2ml of chloroform and combined extract was retained for PC and Cholesterol analysis. Aqueous extract was further processed to obtain the amikacin concentration in linearity range and analysed by Spectrophotometric method described in chapter 3 section 3.3.1. Entrapment efficiency (Percent drug entrapped) (PDE) is expressed as percentage of the drug initially added. The mean percent drug entrapment for five batches with its standard error of mean has been reported in Table 4.1 – 4.4.

For Amphotericin B, 0.1ml of liposomal suspension was dissolved in mixture of DMSO: Methanol (1:1) and further processed to obtain Amphotericin B concentration in linearity range of method and analysed spectrophotometrically as described in chapter 3 section 3.4.1.

4.3.2 Percent drug unentrapped

For analysis of unentrapped amikacin, 1ml of dialyzing medium surrounding the dialysis sac was analysed for drug content by spectroscopic method as described in chapter 3 section 3.3.1. For analysis of unentrapped amphotericin B, sediment obtained by controlled centrifugation was solubilized directly in mixture of DMSO: Methanol (1:1) and analysed as described in Chapter 3 section 3.4.1.

4.4 Physical analysis

4.4.1 Trapped volume

Liposomal dispersion (5ml) after separation of unentrapped drug was centrifuged at 3.6 x 10^6 x g for 30 minutes to get a tight pellet. The supernatant was decanted off from it to remove every drop of excess fluid (including some liposomes if necessary). The pellets were solubilized in 0.1% Triton-X-100 in methanol (10ml). A small aliquot (0.5ml) was

removed for the quantification of PC (Chapter 3, section 3.3.5 and section 3.4.3) and the remainder was used to obtain water content by Karl Fischer method (Veego, India). Commercially available pyridine free reagent was used for the purpose of analysis. The reagent was standardized with known quantity of water (250mg). Before adding sample, 40ml of methanol was added into the titration vessel and titrated with the reagent to an audiovisual end point to consume any moisture that may be present. The trapped volume as µl per µmoles of PC is summarized in Table 4.6.

4.4.2 Laser light scattering measurement

The liposome size of unextruded and extruded liposomes after 5 passes were determined by laser diffraction technique using Mastersizer (Malvern Instruments ltd. UK) operating at a beam length of 2.40 mm and range of lens at 300 mm. The liposomal dispersions were concentrated before analysis so further diluted with the hydrating medium to a factor of 10,000. The mean liposomal size with their respective size range is summarized in Table 4.7 for AMK and AMB liposomes.

4.4.3 Photomicrography

All the batches of the liposomes prepared were viewed under Olympus (BX40F4, Japan) with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity.

4.5 Statistical analysis

Each batch was prepared five times and data from all experiments are expressed as mean ± SEM unless specified. Process variables were studied by comparing PDE of two batches having all other variables same. PDE was expressed as the percentage of the drug

initially added. Similarly, the PDR is relative to the drug initially entrapped. Effective index is the geometric mean of the total emitted dose and FPF, represented by the equation (Hino et al, 1998):

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

Where, DF is the device fraction.

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

4.6 Results and discussion

Liposomes were prepared by the reported thin film hydration (TFH) and Reverse phase evaporation (REV) technique and both techniques were compared to achieve maximum drug entrapment of AMK and AMB. For LAMB, TFH method involved co-evaporation of the lipid and drug from the solvent system in a round bottom flask while for AMK evaporation of lipid phase containing PC and Cholesterol along with SA or SPG-3 to form thin uniform film which was hydrated with AMK containing Succinate buffer (pH 6.5) alongwith 1mM EDTA as hydration medium. REV method involved emulsification of organic and aqueous phase containing lipid and drug followed by evaporation of organic phase under vacuum for both drugs. α – tocopherol (1%) was added to prolong the characteristic induction phase of auto-oxidation and thus the shelf life of the liposomes (Hunt et al, 1981). The purpose of addition of 1mM EDTA was to chelate heavy metals and hence inhibition of free radical chain reaction. For separation of unentrapped AMK dialysis method was adopted because of AMK is water soluble while separation of unentrapped AMB was done by controlled centrifugation method at low g value. The major difference here was instead of attempting to pellet liposomes drug

Table 4.1 Optimization of liposomal AMK preparation by TFH method

Batch No.	AMK: Lipid	PC: Cholesterol: Charge	Entrapment efficiency (%) Mean ± SEM	Free AMK Mean ± SEM*
Batches using Soya PC (S-100) using SPG-3 as charge inducer				
AMK1	1:2	2:0:0	19.30 ± 2.18	76.81 ± 2.46
AMK2	1:3	3:0:0	34.58 ± 2.20	61.30 ± 2.54
AMK3	1:5	5:0:0	46.92 ± 2.46	48.52 ± 2.16
AMK4	1:10	10:0:0	53.71 ± 2.16	42.60 ± 2.51
AMK5	1:3	2:1:0	58.54 ± 2.48	34.72 ± 2.64
AMK6	1:3	1.5:1.5:0	35.81 ± 2.89	61.20 ± 2.22
AMK7	1:3.05	2:1:0.05	69.77 ± 2.50	25.81 ± 2.35
AMK8	1:3.1	2:1:0.10	76.84 ± 1.67	17.24 ± 2.40
AMK9	1:3.15	2:1:0.15	64.29 ± 1.88	31.43 ± 2.06
Batches using Soya PC (S-100) using SA as charge inducer				
AMK10	1:3.05	2:1:0.05	71.37 ± 1.95	24.76 ± 1.87
AMK11	1:3.1	2:1:0.10	78.94 ± 2.20	16.51 ± 1.79
AMK12	1:3.15	2:1:0.15	68.53 ± 1.97	25.37 ± 2.57
Batches using HSPC with SPG-3 as charge inducer				
AMK13	1:3.05	2:1:0.05	78.40 ± 2.00	17.28 ± 2.37
AMK14	**1:3.1**	**2:1:0.10**	**84.16 ± 1.85**	**11.50 ± 2.21**
AMK15	1:3.15	2:1:0.15	75.22 ± 2.64	21.40 ± 2.54
Batches using HSPC using SA as charge inducer				
AMK16	1:3.05	2:1:0.05	80.96 ± 1.88	15.58 ± 2.40
AMK17	**1:3.1**	**2:1:0.10**	**88.74 ± 1.73**	**7.65 ± 1.91**
AMK18	1:3.15	2:1:0.15	76.28 ± 1.94	18.70 ± 2.11

Table 4.2 Optimization of liposomal AMK preparation by REV method

Variable	Batch No.	PC: Cholesterol: Charge*	Entrapment efficiency (%) Mean ± SEM	Free AMK Mean ± SEM*	Observation and Inference
Choice of organic solvent (Ratio of aqueous phase to organic phase was 1:3)					
Ethyl acetate	AMK19	2:1:0.1	65.41 ± 2.31	28.67 ± 2.55	Liposomes were not properly oriented
	AMK20	2:1:0.1	62.73 ± 2.05	30.94 ± 3.27	
Ethanol	AMK21	2:1:0.1	64.85 ± 1.53	29.61 ± 3.02	Drug leakage from liposomes
	AMK22	2:1:0.1	66.09 ± 2.25	27.11 ± 2.83	
Ethyl acetate : Ethanol (1:1)	AMK23	2:1:0.1	76.07 ± 2.07	18.26 ± 3.09	Good vesicle formation
	AMK24	2:1:0.1	77.93 ± 2.61	15.82 ± 2.72	
Ratio of aqueous phase to organic phase					
1:2	AMK25	2:1:0.1	60.48 ± 2.73	33.82 ± 2.54	Less PDE
	AMK26	2:1:0.1	58.92 ± 1.95	36.40 ± 2.22	
1:3	AMK27	2:1:0.1	76.07 ± 2.02	21.64 ± 2.81	Good vesicle formation
	AMK28	2:1:0.1	77.93 ± 2.63	17.68 ± 2.20	
1:4	AMK29	2:1:0.1	84.29 ± 2.86	12.80 ± 1.83	Increased PDE
	AMK30	2:1:0.1	86.20 ± 2.02	08.42 ± 2.44	
1:5	**AMK31**	2:1:0.1	96.71 ± 1.97	-----	Good PDE and good vesicle formation
	AMK32	2:1:0.1	98.54 ± 1.40	-----	
1:6	AMK33	2:1:0.1	96.37 ± 2.33	00.81 ± 0.26	No major change in vesicle formation and PDE
	AMK34	2:1:0.1	97.90 ± 1.81	00.55 ± 0.15	

* SPG-3 for AMK 19, AMK 21, AMK 23, AMK 25, AMK 27, AMK 29, AMK 31 and AMK33

& SA for AMK 20, AMK 22, AMK 24, AMK 26, AMK 28, AMK 30, AMK 32, AMK 34 respectively

Table 4.3 Optimization of liposomal AMB preparation by TFH method

Batch No.	AMB: Lipid	PC: Cholesterol: Charge	Entrapment efficiency (%) Mean ± SEM	Free AMB Mean ± SEM*
Batches using Soya PC (S-100) using SPG-3 as charge inducer				
AMB1	1:10	10:0:0	50.95 ± 1.43	47.14 ± 1.86
AMB2	1:20	20:0:0	61.27 ± 1.84	33.60 ± 1.54
AMB3	1:30	30:0:0	73.60 ± 2.05	23.81 ± 1.61
AMB4	1:10	9:1:0	53.58 ± 1.38	42.50 ± 2.05
AMB5	1:10	8:2:0	55.44 ± 1.52	40.22 ± 1.83
AMB6	1:10	7:3:0	58.70 ± 1.95	38.17 ± 1.55
AMB7	1:10	6:4:0	54.37 ± 1.58	41.33 ± 2.00
AMB8	1:10	5:5:0	45.22 ± 1.86	53.97 ± 1.75
AMB9	1:10.1	7:3:0.1	60.32 ± 1.24	35.66 ± 1.82
AMB10	1:10.3	7:3:0.3	64.78 ± 1.88	31.00 ± 1.59
AMB11	1:10.5	7:3:0.5	70.65 ± 1.49	24.16 ± 1.17
AMB12	1:10.7	7:3:0.7	73.43 ± 1.85	21.80 ± 2.04
AMB13	1:11	7:3:1	76.06 ± 1.73	18.76 ± 1.95
Batches using Soya PC (S-100) using SA as charge inducer				
AMB15	1:10.3	5:5:0.3	58.22 ± 1.29	37.68 ± 1.82
AMB16	1:10.5	5:5:0.5	65.91 ± 1.62	30.47 ± 1.67
AMB17	1:10.7	5:5:0.7	60.37 ± 2.04	36.72 ± 2.01
Batches using HSPC with SPG-3 as charge inducer				
AMB19	1:10.3	7:3:0.3	74.80 ± 2.11	21.03 ± 2.07
AMB20	1:10.5	7:3:0.5	82.18 ± 1.98	14.85 ± 1.80
AMB21	1:10.7	7:3:0.7	85.69 ± 1.87	09.86 ± 2.00
Batches using HSPC using SA as charge inducer				
AMB22	1:10.3	5:5:0.3	70.06 ± 1.43	25.27 ± 1.73
AMB23	1:10.5	5:5:0.5	79.34 ± 1.56	15.61 ± 1.50
AMB24	1:10.7	5:5:0.7	68.20 ± 1.83	27.16 ± 1.42

Table 4.3 Optimization of liposomal AMB preparation by REV method

Variable	Batch No.	PC: Cholesterol: Charge*	Entrapment efficiency (%) Mean ± SEM	Free AMB Mean ± SEM*	Observation and Inference
Choice of organic solvent (Ratio of aqueous phase to organic phase was 1:3)					
Ethyl acetate	AMB25	7:3:0.5	50.22 ±1.93	44.25 ± 1.86	Vesicles were not properly oriented
	AMB26	1:1:0.1	43.92 ±2.40	63.28 ± 2.15	
Ethanol	AMB27	7:3:0.5	60.38±2.49	34.67 ± 1.88	Drug leakage from vesicles
	AMB28	1:1:0.1	58.72±2.14	35.19 ± 2.04	
Ethyl acetate: Ethanol (1:1)	AMB29	7:3:0.5	78.47±2.36	17.64 ± 1.57	Good Vesicles formation
	AMB30	1:1:0.1	76.90±3.02	17.92 ± 2.43	
Ratio of aqueous phase to organic phase					
1:2	AMB31	7:3:0.5	62.37±2.62	32.48 ± 1.83	Less PDE
	AMB32	1:1:0.1	55.30±1.87	28.69 ± 2.01	
1:3	AMB33	7:3:0.5	78.52±2.38	16.80 ± 1.72	Good Vesicles formation
	AMB34	1:1:0.1	76.88±3.03	18.23 ± 2.57	
1:4	AMB35	7:3:0.5	86.31±2.14	09.20 ± 1.63	Increased PDE
	AMB36	1:1:0.1	80.46±2.29	15.55 ± 1.87	
1:5	**AMB37**	**7:3:0.5**	**95.84±1.50**	-----	Good PDE and good vesicle formation
	AMB38	**1:1:0.1**	**87.92±1.33**	**06.58 ± 1.16**	
1:6	AMB39	7:3:0.5	95.11±2.27	-----	No major change in vesicle formation and PDE
	AMB40	1:1:0.1	87.08±1.48	06.73 ± 1.40	

* SPG-3 for AMB25, AMB 27, AMB 29, AMB 31, AMB 33, AMB 35, AMB 37, AMB 39 & SA for AMB 26, AMB 28, AMB 30, AMB 32, AMB 34, AMB 36, AMB 38, AMB 40

crystals were sedimented. This basic difference in density was explored for the separation of unentrapped drug from liposomal dispersion and to pellet the drug. Microscopy under dark background was used to confirm the presence of crystalline drug. This method was used only for AMB where no other techniques worked efficiently.

4.6.1 Influence of process parameters

Process variables viz. dry film formation time, vacuum, hydrating medium, hydration time, speed of rotation of flask, method of size reduction and method of separation of unentrapped drug from liposomes were optimized to prepare liposomes of AMK and AMB. The effect of one variable was studied at a time keeping other variables same and each experiment was repeated six times. The results shown in Table 4.5 reveal following conclusions:

1. The vacuum used for drying of film at room temperature for SPC and 60°C for HSPC containing liposomes was raised from 15 inches of Hg to 25 inches of Hg. 15 inches of Hg was found to be inefficient for the complete removal of the solvent mixture and hence resulted in aggregation of liposomes on hydration. It may be difficult to remove the last traces of solvent from the lipid film in cases where sufficient vacuum is not attainable. The presence of this 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 25 inches of Hg has resulted in rapid evaporation of the solvent system leading to crystallization of the drug due to its comparatively less solubility in the solvent mixture and hence results in poor drug entrapment for hydrophobic drugs 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.

The vacuum of 15 inches of Hg was found to be optimum as regard to complete evaporation of solvent mixture producing more translucent film that on microscopic examination showed longitudinal striations and high percent of drug entrapment in the liposomes on rehydration.

In REV method of preparation, the organic solvents were removed under partial vacuum produced at 20-30°C. The vacuum is usually maintained a low initially and carefully controlled using a nitrogen gas bleed to regulate the vacuum to remove bulk of the organic solvents and then increased cautiously to complete solvent stripping (Betageri et al, 1993). The vacuum cycle of 10 inches of Hg, followed by 10 min at 15 in. of Hg with intermittent vortexing at high speed was developed. A critical step in the preparation procedure of REV occurs when most of the organic solvent has been removed. A gel is formed that needs vigorous vortexing to be converted into a viscous fluid state with in the liposomes. The collapse of gel supposedly coincides with the conversion of the w/o emulsion into the liposomal form. When water content drops considerably and/or higher concentration of lipids are used, MLV-REV will be formed in which a large aqueous core is surrounded by many phospholipid bilayers (Pidgeon et al, 1986). Finally to remove last traces of organic solvent, vacuum cycle of 20 inches of Hg for 15 minutes will suffice the purpose of removal of traces of organic solvents.

Table 4.5 Effect of process variables on formation of liposomal AMK and AMB

Parameters	Observation	Inference	
		AMK	AMB
VACUUM			
15 " of Hg	Aggregation of liposomes	-----	-----
20 " of Hg	**Distinct separated liposomes**	**Suitable**	**Suitable**
25 " of Hg	Poor drug entrapment	-----	-----
HYDRATION TIME			
30 minutes	Non uniform shape and size distribution	-----	-----
60 minutes	**Uniform size distribution**	**Suitable**	**Suitable**
120 minutes	Fragmentation of liposomes on extrusion	-----	-----
180 minutes	Fragmentation of liposomes on extrusion	-----	-----
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	-----	-----
No. of Extrusion cycles			
3 cycles	Non uniform distributed liposomes	-----	-----
5 cycles	**Uniform distributed liposomes**	**Suitable**	-----
7 cycles	**Uniform distributed liposomes**	-----	**Suitable**
ANNEALING TIME			
30 minutes	Non-uniform liposomes	-----	-----
1 hour	**Uniformly oriented liposomes**	**Suitable**	-----
2 hours	Drug leakage observed	-----	------
4 hours	Drug leakage observed	-----	-----
5 hours	**Uniformly oriented liposomes**	-----	**Suitable**
6 hours	-----	-----	-----
SEPARATION OF UNENTRAPED DRUG			
Mini-column centrifugation	Very tenuous method with limited capacity	-----	-----
Dialysis	**Easy & convenient method**	**Suitable**	
Ultra centrifugation	Easy and fast method but possibility of drug leakage due to fusion at high speed	-----	-----
Protamine aggregation	Easy but destructive method. Also not suitable for charged liposomes	-----	-----
Controlled centrifugation at low speed	**Suitable method**	-----	**Suitable for hydrophobic drugs**

Thus the vacuum cycle was optimized for the preparation of MLV-REV liposomes with high drug entrapment for both liposomal formulations of AMK and AMB.

2. Hydration time of the lipid film before size reduction was increased from 30 minutes to 3 hours. It was observed that increasing the hydration time beyond 1 hrs resulted in fragmentation of liposomes on extrusion due to increased fragility of the liposomal membrane whereas a lower hydration time led to a non-uniform shape and size of the liposomes. It may be due to improper conversion of planner bilayers to spherical liposomes. Hence, 1 hr hydration time was found to be optimum for the liposome preparation of both drugs.
3. Speed of rotation: 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 prepared liposomes were unstable due to the presence of residual solvents. While at 180-rpm speed, produced a dry film with varying thickness, which results in non-uniform distribution of particle size distribution. At 120-rpm speed found to be adequate in increasing the surface are for evaporation of solvents to give thin, uniform and completely dry film. Hence, 120-rpm speed of rotation of flask was selected to be optimum.
4. No. of extrusion cycles: The purpose of extrusion by using 2 μm polycarbonate membranes was to reduce the size of liposomes for pulmonary delivery below 5 μm. No. of cycles is also important for proper uniform size distribution of the liposomes. For AMK liposomes, after 5 cycles, liposomes were uniformly distributed (VMD 2.0 ± 0.2 for AMK31 & 1.9 ± 0.3 for AMK32). For AMB

liposomes, 7 cycles were found to be optimum (VMD 1.8 ± 0.2 for AMB37 & 2.0 ± 0.3 for AMB38).

5. Annealing time: 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, annealing time is carried out for an appropriate time period. For hydrophilic drug like AMK, will require short annealing time while for hydrophobic drugs like AMB will require more annealing time. For AMK liposomes annealing time from 30 minutes to 2 hours was checked and found that at 1 hr time, liposomes were in proper shape with proper orientation (confirmed by microscopy). For AMB liposomes, annealing time was found to be 5 hours may be because, being hydrophobic drug, AMB orients itself into the lamellae of the liposomes and since the external environment being aqueous, a prolongation in hydration time provides for a sufficient time for the drug to get entrapped into the lamellae. Further increase in annealing time to 8 hours did not showed significant change in entrapment and so annealing time was set at 5 hours.
6. Separation of unentraped 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 is not

feasible if the entire formulation is to be purified. Also, slight modification in the procedure is required for each specific liposome. Ultra centrifugation (Montenegro et al, 1996) at higher G value ($5.33 \times 10^6 \times g$) is a faster method yet rigorous process accounting for low drug entrapment (AMK4; 30.48 %). Loss of drug may be due to the breakage and fusion of liposomes (Heeremans et al, 1995) and thus low drug entrapment. But for hydrophobic drug like AMB when purified by ultra centrifugation and analyzed for drug content showed a very high drug entrapment value of 96.24 % (AMB 19). The microscopic examination of this batch showed the presence of pelleted drug crystals confirming incomplete and improper separation of unentrapped drug. Dialysis though time consuming has shown to be better in the terms of drug entrapment (AMK 7; 69.77 %) for AMK. However, in order to verify that the entrapment is not a result of insufficient separation of unentrapped drug, pure dug (10mg) was dialyzed through the same system and it was found that the total drug was emptied in to the receptor compartment within 4 hours.

For AMB, being a hydrophobic drug is unfit for dialysis as precipitated drug crystals cannot cross the dialysis membrane. Protamine aggregation is destructive approach and its use is restricted for the determination of the drug entrapment and cannot be used for the separation of the liposomal dispersion. Also, this method is not suitable for positively charged liposomal systems. Protamine aggregation method is only suitable for negatively charged or neutral liposomes. When liposomal dispersion was centrifuged at a very low speed (4.38 x 103 x g) for 90 seconds, and the supernatant was analyzed for drug entrapment

(AMB 9; 60.32 %); it gave similar entrapment value as that obtained by protamine aggregation method. Liposomes being less dense than drug crystals, will settle fast when centrifuged at low speed. This basic difference in density was exploited for the separation of unentrapped drug from the liposomal dispersion and to pellet the drug. A similar approach of sizing of vesicle drug formulations by ultra centrifugation has already been described by McCracken et al (1987). The entrapment value similar to that obtained with protamine aggregation confirms the accuracy of the process and it was further confirmed microscopically to reassure total drug separation and to check any loss of liposomes. The process was also optimized by altering the time and speed which is sufficient to separate the drug crystals without loss of any liposomes.

4.6.2 Influence of formulation parameters

Formulation parameters 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.

1. Composition of solvent system: The organic solvent system of chloroform: methanol (2:1) was used for dissolving the formulation components like SPC/HSPC, CHOL, SPG-3/SA and α-tocopherol. The solvent system composition should be such that to prevent precipitation of formulation components during solvent stripping process. For AMK being hydrophilic drug, was hydrated later on, solvent system of chloroform: methanol (2:1) is found most suitable. For AMB, chloroform: methanol (1:1) is found more suitable to prevent precipitation of

AMB as AMB was added along with lipid phase & solubility is limited in both solvents and thus combination at 1:1 ratio is more suitable to prevent precipitation of drug crystals.

2. Volume of Solvent system: The organic solvent system chloroform: methanol (2: 1) was used for dissolving the formulation components like PC/HSPC, CHOL, SPG-3/SA and α-tocopherol from 5ml to 30 ml. The optimum solvent system is required for both drugs were 20ml. The lower volume of the solvent system like 5ml has resulted in lesser surface is of the formed film. Thus increase in solvent system is directly related to increase in the surface are of the formed film and an increased surface area resulted into a net increase in the drug entrapment value (Payne et al, 1986). Further increase in the solvent system did not show any increase 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 affects 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 planner bilayers to form the spherical liposomes. For hydrophilic drugs like AMK, 1 ml of hydration volume was found suitable to give uniform spherical liposomes by TFH evaporation method (AMK5, 58.54 %). Further increasing the hydration volume will lead to decreased drug entrapment values may be due to increase in the leakage of water soluble drug. For AMB, increase in hydration volume from 1 ml to 4 ml, no significant change

in drug entrapment was noticed may be due being a hydrophobic drug, only the dilution of liposomes will be possible without any loss of drug by leakage.

4. Drug: lipid ratio: Increase in the lipid proportion relative to drug led to the increase in the drug entrapment from 19.30 % (AMK1, 1:2) to 53.71 % (AMK4, 1:10), from 50.95 % (AMB1, 1:10) to 73.60 % (AMB3, 1:30). 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 (AMK 1:3 and AMB 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 (AMK2, 1:3:0, 34.58 % to AMK5, 1:2:1, 58.54 % and AMB1, 1:10:0, 50.95 % to AMB6, 7:3:0, 58.70 %). 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 in drug entrapment for both drugs (AMK5, 1:2:1, 58.54 % to AMK6, 1:1.5:1.5 35.81 % and AMB6, 7:3:0, 58.70 % to AMB8, 5:5:0, 45.22 %). It may be due to drug and CHOL both competes with PC molecules for orientation into the bilayer membranes and results in decrease in entrapment. Also more probable reason may be due to increased CHOL proportion leads to increase the fluidity of membrane and thus loses the packing of bilayer thus leakage of drug observed in the bilayer near the

phase transition temperature (Betageri et al, 1993). Since AMK is more hydrophilic drug, it leaked more in comparison to AMB being hydrophobic drug (AMK6, 1:1.5:1.5 35.81 % and AMB8, 5:5:0, 45.22 %).

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 (AMK5, 2:1:0, 58.54 % to AMK8, 2:1:0.05 (SPG-3), 76.84 % & AMK11, 2:1:0.05 (SA), 78.94 %). The presence of charged lipids also reduces the likelihood of aggregation following the formation of MLVs (Betageri et al, 1993). As AMK is hydrophilic, % increase in drug entrapment was more compared to AMB due to increase in interlamellar distance between two lamellae of MLV led to more increase in aqueous compartment and thus increase AMK entrapment.

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 representing a possible risk for human health and can lead to inadequate stability of the vesicles (Cortesi et al, 1999). Use of other organic solvents like ethyl acetate and ethanol can solve this 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 leading 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: When aqueous phase to organic phase ratio was raised from 1:3 to 1:5, marked increase in the PDE was observed i.e. AMK27 (SPG-3) 76.07 % to AMK31 (SPG-3) 96.71 % and AMK28 (SA) 77.93 % to AMK32 (SA) 98.54 %. Similarly for AMB also; AMB29 (SPG-3) 78.47 % to AMB37 (SPG-3) 95.84 % and AMB30 (SA) 76.90 % to AMB38 (SA) 87.92 %. Further increase in the organic phase did not result in to increase in PDE. 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.

   REV method for the preparation of AMK and AMB liposomes were selected because of REV method has produced more drug entrapment than TFH method. 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 REV method was further employed for the liposome preparation containing HSPC in the bilayer composition as phosphatidylcholine component.

4.6.3 Characterization of liposomes

4.6.3.1 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. Trapped volume is proportionally related to the entrapped material if the drug is water soluble (AMK) and present predominantly in the inner aqueous compartment. For AMB being hydrophobic, it is only a parametric study before going for the study of liposome behavior in physical or biological 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 0.739 to 0.763 µl per µ mole, which is almost 60 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 we have concluded that the large majority of the vesicles produced by extrusion are still multilamellar (Table 4.6).

4.6.3.2 Laser light scattering measurement

The vesicle size before extrusion and after extrusion was determined by laser diffraction using Mastersizer 2000 (Malvern Instruments Ltd. Ver 5.1 UK) operating at a beam length of 2.40 mm and range of lens at 300 mm. 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 4.7). The

Table 4.6 Trapped volume of liposomal dispersions prepared with optimal process and formulation variables

Batch No.	Trapped Volume * (µl / µ mole)
Amikacin Sulphate	
AMK31	0.739 (0.014)
AMK32	0.763 (0.022)
Amphotericin B	
AMB 37	0.692 (0.015)
AMB 38	0.670 (0.021)

* Mean ± SEM (n=6)

Table 4.7 Particle size range of liposomal batches at various stages of preparation

Batch No.	Pre-extrusion Mean size [size range] (μm)	Post-extrusion Mean size [size range] (μm)
Amikacin Sulphate		
AMK31	12.59 [0.9 – 101]	2.00 [0.5 – 28]
AMK32	11.82 [0.9 – 95]	1.94 [0.5 – 26]
Amphotericin B		
AMB 37	9.59 [0.9 – 68]	1.83 [0.5 – 32]
AMB 38	10.64 [1 – 75]	2.04 [0.5 – 35]

* Mean ± SEM (n=6)

mean liposomal size prior to extrusion decreased with the increased proportion of cholesterol whereas after extrusion, it increased with the increase in the proportion of cholesterol. This may be due to the presence of higher concentration of amphiphilic PC as compared to cholesterol that would have resulted in the formation of liposomes with greater number of lamellae and more inter lamellae distance, thus proportionate increase in mean liposomal size before extrusion. However, incorporation of cholesterol would have imparted rigidity to the bilayer membranes and hence prevented it from downsizing by extrusion.

4.6.3.3 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 4.5 for AMB37. 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).

Thus reverse phase evaporation technique yielded spherical multilamellar liposomes with high drug entrapment. Liposomal dispersion of composition AMK 31, AMK32, AMB37 and AMB38 were selected for the further development of liposomal dry powder inhaler formulations.

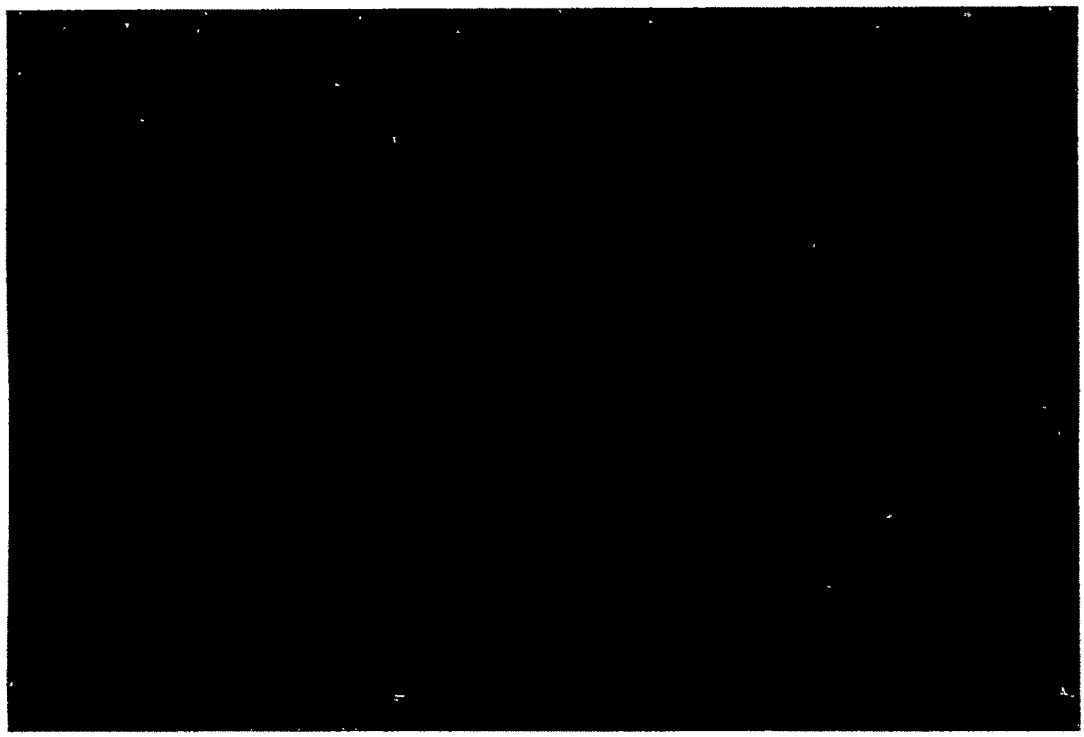

Figure 4.5 Photomicrographs showing liposomal vesicles under normal light for AMB37.

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