MATERIALS AND METHODS

3.1 Chemicals:

The sources of chemicals used in the study are as follows: -

Fungizone, a commercial deoxycholate preparation of amphotericin B was obtained from M/s. Ambalal Sarabhai Enterprises, Baroda, India and was reconstituted with 5% dextrose prior to use. Amphotericin B (AmB) and L - α - phosphotidyl choline from egg. Yolk (EPC) and Dioleoyl phosphotidylcholine (DOPC) Louis, MO, USA. Cholesterol (Chol), Dicetylphosphate (DCP) and stearylamine (SA) were obtained from Centre for Biochemical (CSIR), Delhi, India, β-Cyclodextrin derivatives i.e. hydroxypropyl β-CD (HPBCD) and sulfobutylether β-CD (SBEBCD) were obtained from M/s. Cyclo Lab. Budapest, Hungary.

Sodium metabisulphite and solvents such as methanol, ethanol, diethylether, dimethyl Sulphoxide (DMSO) were obtained from E, Merck Ltd. (Mumbai, India).

Sodium Cyanoborohydride and Ninhydrin reagent were obtained from Fluka Co. (Buchs, Switzerland). All the constituents of fungal culture media and Tween – 80 were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India). Sephadex, G-25 was obtained from Pharmacia fine Chemicals (Uppsala, Sweden). Polycarbonate membranes were from Necleopore (Pleasanton, CA, USA). All other Chemicals used were of analytical reagent grade and reagents were prepared in double distilled water.

3.2 Animals:

Male Balb/c mice weighing (20-25g) were obtained from animal house facility of National Institute of Nutrition, (Hyderabad, India) and maintained in our animals house. The animals were housed in a constant humidity (50%) - temperature (25^{0}C) environment in wire bottom cages with alternating 12h light and dark cycles during the experiment. One day prior to the experiments, food was withdrawn but water was available *ad libitum* during the experiment.

3.3 Culture of *Aspergillus* :

The fungus used in the course of this study was *Aspergillus fumigatus* strain, a clinical isolate obtained from B.J. Medical College, Pune. It was subcultured regularly on sabouraud dextrose agar medium for retaining complete viability. For developing *A. fumigatus* infection, the organism was cultivated for 48 h and spores were harvested. They were suspended in normal sterile saline containing 0.02 % Tween 80 and counted on a haemocytometer.

3.4. Animals model for aspergillosis in BALB/C mice :

The animal model for aspergillosis in BALB/C mice was established as described by Ahmad et.al., (1989). In brief, 1.8 x 10^7 viable *Aspergillus fumigatus* spores suspended in 0.17 ml of sterile saline were injected intravenously into BALB/C mice via the caudal vein and the infection was confirmed by the presence of fungal spores in the lungs.

3.5 Preparation of liposomes:

3.5.1 Hydroalcoholic proliposome method :

1. Formation of inclusion complex:

The present study involved the complexation of amphotericinB (AmB) with different β-cyclodextrin derivatives (HPBCD / SBEBCD) by co-grinding

method (Adhage, et.al, 1998) with modifications. AmB and β-CD. Derivative was mixed in the ratio 1:10 (w/w). This mixture was compacted in a roll compactor at a speed of 5 rpm at 40 tons pressure and allowed the flakes to pass 4 times through roll compactor. The solid inclusion complex was dissolved into hot (60^{0}C) aqueous 0.5% W/W hydroxy propyl methyl cellulose (HPMC) solution (pH 6.0) with continuous stirring so as to obtain ternary complexes. The suspension of drug / βCD derivative ternary complexes was allowed to centrifuged at 25000 rpm for 60 min. at 4^{0}C to yield pellets and clear supernatent. Most of the drug was solubilized in the supernatent (presumably in the form of inclusion complex), with a small amount of insoluble drug present in the pellet. The complex formation was confirmed with thin layer chromatography (TLC) using solvent system butan-1-ol-pyridine: H2O (Ochab, 1970) and verified by Differential Scanning calorimetry (Swarbic and Boylan, 1997). The supernatent contained 0.5ml, approx. 2mg AmB and 5mg β-CD derivative inclusion complex was used for entrapments into liposome, manufactured from different phosphotidylcholine and cholesterol by proliposome method. The β-CD contents were evaluated by dissolving a fraction containing inclusion complex (AmB-βCD) in methanol and assayed the supernatent by liquid chromatography (Altech model, USA) L-8 reserve phase column (4.6 mm × 25 cm) employing filtered and degassed mixture of acetonitrile and water (16:35) as a solvent system, at flow rate of 2.0 ml per minute with refractive index detector.

1.1 Differential scanning calorimetry:

Further verification of AmB: β-CD complex formation by differential scanning calorimetry (DSC) was carried out in a Perkin Elmer DSC7 using vented

aluminium pans. Typical conditions were as follows: temperature range, 50-300^{0}C;scanning rate, 10C^{0} /min; sample weight, 4mg. Base lines optimization was performed before each run. All materials were used in the solid state.

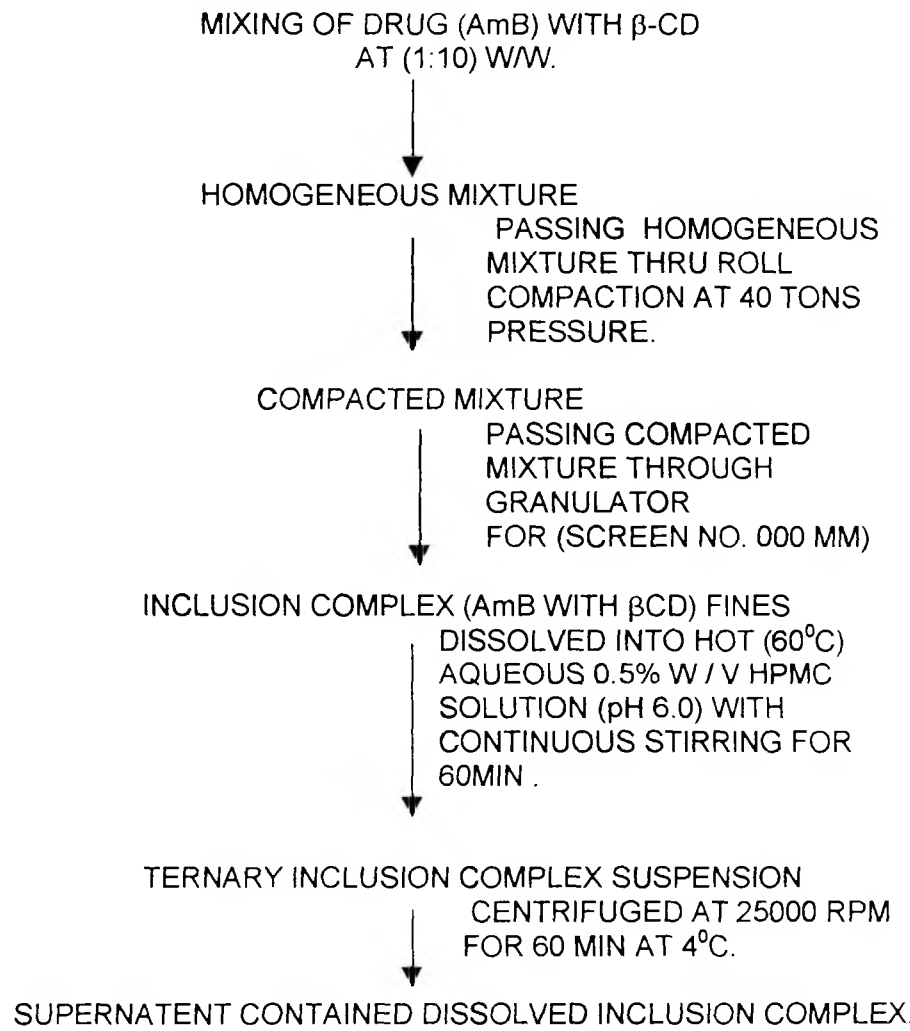
2) Entrapment of inclusion complex into liposomes:

The preparation of liposomes derived from proliposome technique (Perrett, et. al., 1991) with modifications (Chakraborty and Naik 1997) was used to entrap inclusion complex (AmB - βCD) into the aqueous phase of liposomes. In brief, proliposome mixture was prepared by transferring lipid (500mg) containing EPC: Chol at molar ratio 7:2, into a round bottom flask. The lipid was thoroughly dried under nitrogen. It was then dissolved in ethanol (400mg) and phosphate buffer – saline (PBS pH 7.4) was added to from a lipid: ethanol: water mixture (500:400:1000). This mixture was heated to 60^{0}C for few minutes and then allowed to cool to room temperature (25^{0} C) yielding a proliposome mixture which was finally converted into a multilamellar liposome (MLV) suspension by dropwise addition of HPMC aliquot (pH 6.0) containing AmB: βCD inclusion complex and subsequently dilution with PBS buffer (pH) to a final Volume of 50 ml. The suspension was vortex mixed throughout this last stage (Chakraborty and Naik 1998,).

Non-entrapped complexes were separated from liposome-entrapped complexes by diluting the MLV suspension with 0.15M phosphate buffer-saline (pH 7.4) and centrifuged at 15000 rpm for 30 min. at 4^{0} C. The pellets were washed with 5ml PBS and centrifuged again as above. The final liposomal pellets with entrapped inclusion complex were suspended in 10 ml PBS.

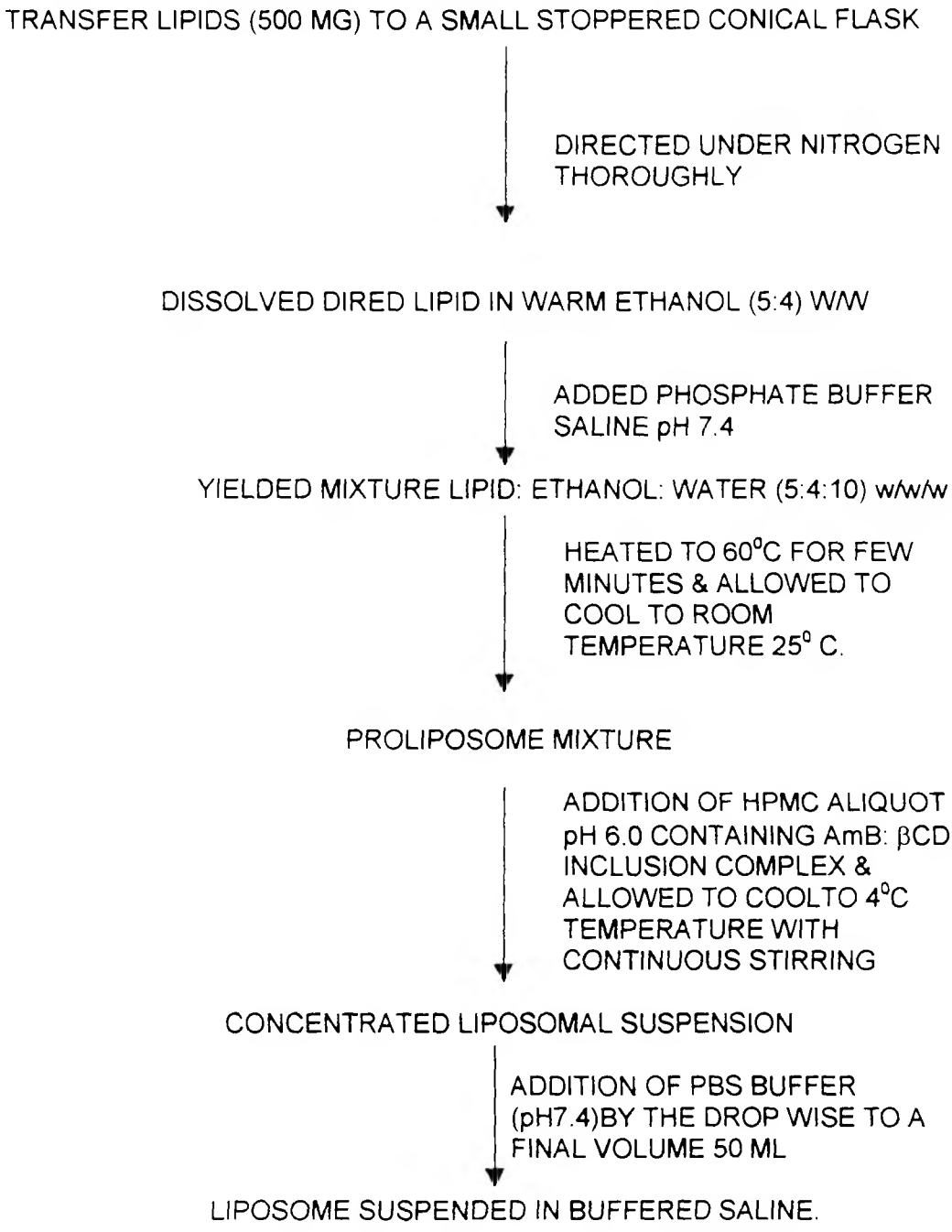
Figure – 17(a)

FLOW CHART FOR FORMATION OF INCLUSION COMPLEX OF AMPHOTERICIN-B WITH β-CYCLODEXTRIN



Figure–17(b)

FLOW CHART FOR THE PREPARATION OF LIPOSOMES



3.5.2. Optimization of formulation conditions:

Although the general procedure for the preparation of liposomes has been essentially the same in almost all the reports screened, variations in the formulation conditions have been observed (Table 5 - 8).

Various batches of liposomes were prepared by varying the parameter under study, keeping all the other parameters constant and were evaluated with respect to particle size and size distribution, yield and other associated characteristics (Table 5 - 8).

3.5.3 Incorporation of AmphotericinB into liposomes:

After the formulation conditions were optimized, drug loaded liposomes were prepared by dissolving amphotericin B (AmB) so as to yield approx.2mg of Amp-B per ml of phosphate buffer saline and then proceeding, further as per the method described previously.

3.6 Evaluation of proliposome based liposomal vesicles (PBLV) :

3.6.1 Intercalation efficiency of drug in PBLV :

In order to determine the amount of amphotericinB intercalated, the proliposome based liposomal vesicles were extensively dialyzed against 10 mM Tries HCl. The amount of amphotericin B retained after dialysis was determined by solubilizing an aliquot in methanol and measuring the absorbance at 405 nm. The absorbance obtained was compared with standard curve (Fig. 24) with a blank, which consisted of equivalent amount of lipid dissolved in chloroform-methanol. Phospholipid contents were determined according to the procedure introduced by Vaskovsky et.al. (1995)

3.6.2 Entrapment efficiency of inclusion complex in PBLV :

To determine whether PBLV preparation formed closed structures like liposomes, the entrapment efficiency of the liposomes was examined. The drug complexed with β- cyclodextrin was normally added in the buffer used to form the concentrated liposome mixture. Its concentration in the liposome mixture was adjusted so as to yield a concentration of approx.2mg ml^{-1} in the final diluted liposome preparations. Entrapment efficiencies were determined by centrifuging the liposomes at 15000 rpm at $4^{0}C$ for 30min. to pellet the liposomes, and assaying the supernatant spectrophotometrically at 405 nm for non trapped drug. The trapped fraction was then calculated by difference.

3.6.3. Structural organization of proliposome mixtures:

The organization of lipid: ethanol: water mixtures used for preparation of liposomes was investigated by determining the positions of the boundaries between the clear isotropic, the precipitated bilayer and liposomal region of the

structural organization. The position of the boundary between the former two regions was determined by adding increasing amounts of buffer to different concentrations of lipid in ethanol and noting the point at which precipitation of lipid first took place. The boundary between the liposomal region and the hydrated bilayer region was estimated from measurements of the entrapment of amphotericin B in mixtures containing different ratios of lipid and ethanol as detailed above. The proliposome compositions giving rise to maximum entrapment were taken to mark the approximate boundary between the two regions.

3.6.4. ^{31}P-NMR Measurements:

Samples for ^{31}P-NMR measurements contained 0.5 – 1.0g lipid spectra were measured at a scan frequency of 101 – 256 M H_z with 80% phosphoric acid as a standard, using a Bruker WM-250 spectrometer Samples were not spun during the measurement and signal were averaged over 800-2000 scans to increase the signal to noise ratio.

3.6.5 Particle size Analysis:

Particle size analysis of the liposomal vesicles was carried out according to the standard procedure (Ibrahim, et.al.,1983) using ore microscope (MNH – 9, Russia, least count 0.25 μm) at 600 x magnification, A minimum of 500 particles were counted for each batch and the average diameter along with the frequency distribution was calculated.

3.6.6 Transmission electron microphotography :

The structures of the liposomes containing 12 wt % AmB were observed by an electron microscope (Phillips) at a magnification of 115000. Before the

microphotographs were taken, the liposomes were negatively stained with freshly prepared 2-wt % phosphotungstic acid solution.

3.6.7 Partition coefficient measurements:

The partition coefficient of ethanol between lipid and water defined as K = (g ethanol per g lipid) / (g ethanol per g water) was estimated as follows. 100 mg of lipid was first dried in a rotary evaporator. The dried lipid film was then dispersed in 2% ethanol solution. The resulting liposomes were precipitated by centrifugation at 25000 rpm at 20^{0}C for 1 h. The concentration of ethanol in the supernatant fraction was measured using a sigma ethanol determination kit and the concentration of ethanol in the lipid phase was then calculated by difference.

The directly measured values of the partition coefficient were corrected for the presence of non-solvent water associated with the lipid head groups using relationship:

$$K = K' + f$$

Derived by Katz & Diamond (1974) K' is the measured partition coefficient, K the partition coefficient, and f the equivalent weight of water in g (g lipid) that completely excludes solutes.

3.6.8. In-vitro drug release studies:

The stability of liposomes in terms of AmB loss was evaluated by the method of Juliano et. al., (1986). A suspension of liposomes comprises inclusion complex of AmB: β-CD at a concentration of approx.130μg AmB/ml was divided into several portions. One was immediately assayed for the AmB contents, and the others were incubated at 37^{0}C for various period of time. Each sample was centrifuged at 15000 rpm for 45 min, and the supernatent was removed. The remaining pellet was washed twice with phosphate buffer saline (pH 7.4) and was

then resuspended in fresh phosphate buffer saline (pH 7.4), so that the volume of the final suspension was the same as that of original volume. Finally, the amount of residual AmB were determined as described earlier. In order to determine the effect of pH on stability, different buffer systems (Acetate buffer of pH 5.0, tris buffer of pH 7.0 & $NHCO_3/Na_2CO_3$ buffer of pH 10.0) were used in place of phosphate buffer saline.

3.6.9. In -vitro drug leaching studies:

As all the products were stored as suspensions in phosphate buffered saline, in- vitro drug leakage studies were essentially conducted to estimate the extent of drug leaching from the proliposome during storage. A suspension of concentrated and final diluted liposomes comprises inclusion complex of AmB-βCD at a concentration of approx. 2 mg AmB/ml of phosphate buffered saline (pH 7.4) were stored in a refrigerator and sampled at periodic time intervals upto 2 months and analyzed for AmB content after suitable dilution.

3.6.10. Product shelf life determination:

To assess the shelf life of a liposomal product, accelerated studied based on chemical Kinetics were carried out (USP 1995).

A suspension of concentrated and final diluted liposomes comprises of inclusion complex of AmB - βCD of each product along with plain drug at a concentration of approx. 2 mg/ml. of phosphate buffered saline (pH 7.4) were kept in sealed, vials at different temperatures (5^0C, 30^0C and 40^0C). Sampling was done at periodic time intervals (1, 2, 3 and 6 months) and samples were analyzed for drug content as described earlier.

3.6.11. Toxicity Studies:

3.6.11.1. Determinations of in-vitro toxicity of free amphotericin B and after intercalation in proliposome based liposomal vesicles.

A hemolysis assay was employed to monitor the reduction in toxicity of amphotericin B to erythrocytes when delivered as PBLV entrapped inclusion complex (Mehta, et. al., 1989, Forster, et. al., 1988). In brief, RBCs were drawn from Balb/c mice in the presence of heparin (50 units/ml) and washed three times with phosphate buffer saline. Next, 0.8 ml. Of 0.1 % RBCs (vol./vol.) were mixed with 0.2 ml of buffer containing varying amounts of AmB_{DOC} and PBLV entrapped inclusion complex. The mixture was incubated at 37^0C for 1 h and spun at 2500 X g for 2 min. The amount of haemoglobin released in the presence of 0.3 % Triton X – 100 was taken as a measure of 100% lysis.

3.6.11.2 Determinations of in-vivo toxicity of free amphotericin B and after intercalation in proliposome based liposomal vesicles:

A) Nephrotoxicity of free amphotericin B and PBLV entrapped inclusion complex:

Serum creatinine levels of mice treated with AmB_{DOC} and different doses of PBLV-AmB/ βCD were monitored to evaluate nephrotoxicity associated with the administration of AmB_{DOC} and PBLV-AmB/BCD (Patterson, et. al., 1989). Serum obtained from mice was mixed with equal amounts of buffer, 5% sodium hydroxide and 2 N ammonium sulfate to precipitate proteins. The supernatant was separated, picric acid and sodium hydroxide were added (which forms a colored complex with creatinine) and color developed was measured at 620nm.

B) Determination of LD_{50}:

AmB_{DOC} (0.4 – 4 mg/kg) and various liposomal formulations intercalated amphotericinB (2.5 – 20mg/kg) were injected intravenously into Balb/c mice

weighing 20-30 g (10 animals in each group) via caudal vein and survival was checked for a period of 7 days. Median lethal dose (LD_{50}) were calculated by Litchfield & Wilcoxon (1949).

3.6.12 Evaluation of therapeutic efficacy:

Comparative studies on the therapeutic efficacy of various liposome preparation of AmB on the aspergillosis mice model:

Forty male Balb/c mice were infected with 1.8×10^7 viable. *A. fumigatus* spores via the caudal vein. After 24h of spore challenge, the mice were randomly divided into groups of ten. The first group was the untreated control group whereas second, third, and fourth groups received free AmB, solvent and proliposome based liposomes respectively. The therapy was started with different liposomal preparations of AmB (dose 0.5mg/kg-body). The survival of the animals after therapy was observed for 7 days . Infected and treated mice were observed everyday till 7 days post infection. They were observed for any loss of gait or convulsions. They were also observed for any loss of gait or convulsions. The pathogen load was determined by culturing the processed left lung from killed animals on sabouraud dextrose agar plates and then counted for colony forming units (CFU) after 48 h in incubation at 37^0 c (Ahmad, et.al.,1989).

3.6.13. <u>In-vivo</u> tissue distribution studies in normal and infected mice:

3.6.13.1. Tissue Distribution of AmB-Incorporated in Different liposomes in Mice:

AmphotericinB content was assayed in mice after sacrificing at 1 different time interval (1 – 48h) following intravenous administration of 2 mg /kg. AmB_{DOC} or 12mg /kg liposomal AmB preparations. For tissue distribution studies, groups of 6-8 mice were sacrificed and various organs (lung, liver, spleen kidney and

serum) were removed and frozen until analysis, Pooled organs (0.5g) were homogenized in 2 ml methanol, centrifuged and the supernatent was analyzed by HPLC. For serum analysis 2-ml methanol was added to 0.5 ml pooled serum and centrifuged at 8000 rpm for 15 minutes. The supernatant was analyzed by HPLC. The amount of AmB estimated in various organs was multiplied by recovery factor. In the case of liposomal AmB, owing to low value of injected drug, and internal standard of AmB (6.25mg/ml) was added while homogenizing the tissue samples. The peak area of internal standard was substracted from the final peak area obtained from various tissues and serum after HPLC analysis.

3.6.13.2 HPLC analysis of AmB in liposomes & in various organs:

HPLC analysis of AmB content in liposomes and in various organs was carried out by the method described by Nilson-Ehle, et.al., (1977). The system used for analysis of AmB consisted of an Altech model (USA) C-18 Reserve phase column (4mm x 25 cm) employing 0.005M of EDTA-methanol (3.7v/v) as a solvent system at flow rate of 0.1 ml/minute with UV detector at 345nm.

3.613.3 Recovery of AmB in various tissues & serum:

Group of 6-8 mice were sacrificed and various organ were removed (Harindran, et.al.,1999), pooled organs (0.5g) and pooled serum (0.5ml) were homogenized in 2 ml methanol containing 10 mg AmB. The samples were centrifuged at 8000 rpm for 15 minutes and the supernatent obtained was analyzed by HPLC, 20 ml of the supematent was injected into the C-18 column and peak areas obtained were compared with standard AmB to determine recovery efficiency in tissue and serum.

3.6.14. Influence of lipid dose on plasma pharmacokinetics:

This study was carried out in two series of experiments .In the first series of experiment, influence of different β-CD was investigated. Total lipid dose was 40 mg /kg for all liposome types. Whereas, for the second series of experiments aiming at the assessment of the influence of lipid dose on the PK of AmB liposomes consisting of β-CD were used exclusively. Total lipid dose was varied.

Albino rats of wistar strain (150 – 180g) of either sex were selected, using 6-8 rats for each group (total 5 group in each series experiment) tested. Each group of rats received 1 mg/kg of Amphotericin B (AmB_{DOC}) or 6 mg/kg of different liposomal AmB by intravenous route. In all cases amphotericin B dose was adjusted to 6-mg/kg for calculation purpose.

The serum concentration of AmB was determined by drawing blood samples (0.5ml) from each animal directly from heart under light ether anesthesia at regular time intervals like 0h, 0.5h, 1h, 2h, 3h, 4h, 8h, 12h, 18h, 20h, and 24h after drug administration. In order to understand the influence of lipid dose on the pharmacokinetic, AmB liposomes entrapping AmB-HPBCD and AmB-SBEBCD inclusion complexes were used exclusively. Though total lipid dose was varied, AmB dose for all experiments was kept constant 6mg/kg.

3.6.14.a. Pharmacokinetic Analysis:

Single dose pharmacokinetic estimates were derived by non-compartmental technique (Gibaldi and Parrier, 1982). A nonlinear least square regression program was applied to the post distributive phase of the concentration time curves to generate the estimate of the elimination rate constant (k_{el}). The area under the plasma concentration time curve (AUC) was calculated by using the linear trapezoidal rule from time zero to the final concentration measured (C_{last}). The remaining area was calculated by the

relationship C_{last}/k_{el}. Plasma clearance (CL) was calculated as dose/AUC. The volume of distribution (VD) was determined by applying the formula area under moment curve (AUMC), such that VD =CL. (AUMC/AUC). Half–lives ($t_{1/2}\beta$) were calculated as $t_{1/2}\beta = \ln (2) k_{el}$. Peak (Cmax) concentration in serum was defined as the concentration at 5 min. after the infusion. All calculations were performed on the data obtained for each rat, after which the appropriate arithmetic and harmonic mean values for each dosage were determined.

3.6.15. Statistical analysis:

LD_{50} values were calculated by the method described by Litchfield and Wilcoxon (1949). CFU and Tissue disposition values AmB was calculated and statistically analyzed by the method of analysis of variance of one way classification with unequal frequencies as suggested by Snedecor and Cochran (1968). For all tests, a two tailed P value of less than 0.05 was considered significant. The survival data were analyzed by using the chi-square with Yates correction and by Fisher's exact test (1981).