ABSTRACT :

The objective of this research was to develop a novel hydroalcoholic method for the preparation of liposome entrapping inclusion complex of amphotericin B (AmB) with hydrophilic chemically modified β-Cyclodextrin (Hydroxy propyl β-Cyclodextrin, HPBCD & sulfobutyl ether β-Cyclodextrin, SBEBCD) was developed with a view to achieve reduced toxicity and superior tissue distribution of AmB in -vivo.

The method involves initial preparation of AmB–HPBCD/SBEBCD intercalated proliposome which is subsequently converted into liposome dispersion by single dilution method. The new method for preparation of liposomes is simple that avoids the use of pharmaceutically unaccepted solvents and energy expensive procedures such as sonication.

Feasibility of formation of solid inclusion complex using heavy-duty role compactor has been investigated and verified ratio of AmB HPBCD/ SBEBCD (1:1.8 /1.4) complex formation by differential scanning colorimeter (DSC). Use of heavy-duty roll compactor permitted the inclusion complex formation of tremendous cost effective.

Formulation and characterization of the proliposome based liposomal vesicles (PBLV) using two different phospholipids i.e. EPC & DOPC have been investigated. EPC liposomes derived from proliposome exhibited a superior entrapment efficiency (51.81± 1.24% to 66.7 ± 1.03 %) in the ratio of lipid: ethanol: water (500: 400: 1000) w/w/w as compared to entrapment efficiency DOPC liposome (41.8±1.64% to 46.5 ± 1.26 %) in the ratio of lipid: ethanol: water (500: 300: 800) w/w/w and size (3.44 to 3.5 μm).

The structure organization of proliposome mixture and the final multilamellar vesicles (4.17 to 4.30 μm) were analyzed by electron microscope and ^{31}P-NMR.

Dynamic dialysis has been used to study the drug release profiles of different PBLV prepared under various conditions and compared release rate pattern of drug. The results of long-term drug leaching experiments revealed slow complex dissociation and leakage of drug (1.69%) from EPC liposome comprising of AmB-SBEBCD in concentration form after 60 days period.

Products shelf life determination were carried out based on arrhenious degradation kinetics at different accelerated temperatures i.e. 5^0C, 30^0C, 40^0C. Study revealed the proliposomal products exhibited more stability in the concentration form (404 days).

The in-vitro toxicity of the different PBLV containing varying amounts of AmB was compared with that of the AmB_{DOC} taken as measure 100% erythrocyte lysis. Whereas, the reduced nephrotoxicity of different PBLV was confirmed with lowered creatinine level. The hemolytic ability of the proliposome based EPC liposomes containing AmB-SBEBCD at 37^0C was approx. 50% at maximum of the DOPC liposome (AmB-SBEBCD) at a dose of 118 μg /ml as measured after 1h incubation. The significant reduction in the hemolysis effect may infact be attributes to the reduced rate of drug release from the EPC liposomes.

The acute LD_{50} of AmB_{DOC} as well as different liposomes comprised of AmB-βCD inclusion complex was determined in Balb/c mice by intravenous route. The LD_{50} of AmB is increased from 2.0 mg/kg (AmB_{DOC}) to 18.6 mg/kg in mice, when AmB was administrated with EPC liposome (AmB-SBEBCD). Whereas, LD_{50} of DOPC liposome (AmB-SBEBCD) was 7.8 mg/kg. EPC liposome (AmB-HPBCD/SBEBCD) treated mice exhibited comparatively mild toxic reactions (viz. pyrexia, preconvulsive symptoms etc) as compared to those treated with DOPC liposome (AmB – HPBCD/SBEBCD).

Therapeutic efficacy of liposomal amphotericinB and commercial AmB_{DOC} (fungizone) was evaluated by developing aspergillosis model in Balb/c mice.

Liposomal AmB and AmB_{DOC} were injected in a single dose (0.5mg/kg of AmB) I.V. into mice infected with Aspergillus fumigatus spores.

Yates correction of the chi-squared analysis and Fisher's exact test indicated significant difference when compared between the control and other groups (P>0.001 two tailed) and also between EPC with DOPC liposomes and AmB_{DOC} (P>0.01) on 5^{th} and 7^{th} days after therapy. The difference were found to be insignificant on 3^{rd} day after therapy.

Tissue distribution analysis of AmB by HPLC showed an increase in concentration of the AmB in lung for both free and liposomal AmB in infected animals as compared to normal untreated animals. Use of liposomal AmB (PBLV) increased the concentration of the drug in the disease-affected organs such as liver, spleen. The result clearly demonstrated preferential uptake of L-AmB entrapped inclusion complex (AmB-SBEBCD) by the RES (Reticulo endothelial system). Further analysis of the results clearly indicated a good correlation ship between diminished level of amphotericin B in infected (P>0.05) kidney after 24h as compared to L-AmB (HPBCD) treated group.

Finally, while studying pharmacokinetics an attempt was made for prolonging the retention of AmB in circulation by carrying out saturability of RES macrophages via concurrent increasing lipid infusion. The influence of lipid dose on the plasma pharmacokinetics of amphotericinB (AmB_{DOC}) and liposomal amphotericin B (HPBCD/SBEBCD) in rats were compared. A series of liposomal AmB formulations with varying β-CDs and lipid dose but constant AmB dose (0.5 mg/kg) was compared with free amphotericin B (AmB_{DOC}) in rats. Pharmacokinetic data were analyzed by considering the varying volume of distribution with respect to the varying lipid concentration in blood. These results indicated that L – AmB entrapped inclusion complexes safely achieved higher C_{max} & AUC and demonstrated saturable, non-

linear elimination from plasma via reticuloendothelial organ uptake at higher lipid levels.

Based on our experimental findings the following inferences can be derived:

1. Intravenous administration of proliposome based liposomal vesicles containing AmB-HPBCD/SBEBCD inclusion complexes alter the pharmacokinetics in a fashion, which may be advantageous in the use of water insoluble drugs in therapy. In this respect it is recognized that the drug employed here may not meet the requirement for a preference of the present approach over that of direct accommodation into the lipid bilayers of liposomes, but certainly established an alternative for the delivery of insoluble drugs.
2. The present delivery approach fulfills the requirements viz. inability of drugs to dissolve in solvents required for the solubilization of liposomal lipids, incompatibility of certain drugs with bilayer formation or stability and yield of low drug to lipid mass ratios in the liposomal formulations.
3. The drug incorporated in liposomes as such are released free to assume their rate of metabolism upon vesicle disintegration, a process which is normally independent of vesicle contents.
4. It is observed that drug metabolism in the tissues of animals treated with liposome entrapped inclusion complexes appears to commence only upon drug dissociation from the complex (an event that may or may not depend on previous vesicle disintegration) at rates which vary with individual drugs.

Considering such metabolic situation it is suggested that the present proliposome approach incorporating chemically modified β-cyclodextrin has clearly demonstrated high binding affinity and specificity to accommodate water

insoluble molecules (guest) in the hydrophobic milieu of the cavity present in the modified β-cyclodextrin structure and circumvented the liposomal (in-vitro &in-vivo) stability problem completely in biological environment. The newly developed techniques could be extended to other chemotherapeutically important drugs viz. Immuno-suppressants and anticancer drugs etc. to improve their therapeutic window.