DISCUSSION

DISCUSSION :

Formation of inclusion complexes: In the present study, drug / β- CD inclusion complexes were formed by mixing the relevant moieties in ratios of 1:1 to 1:2.5 allowing, theoretically to interact one drug molecule at least with one β-CD molecule. Though, in practice such drug / cyclodextrin complexes in solution are in equilibrium with free drug, at the same time it is possible that entities in which two or more cyclodextrin molecules are likely to complex with different groups of the same drug molecule (Uekama, et.al.,1987). Thus, the final drug / cyclodextrin molar ratio in solution of inclusion complex may vary according to the β-CD used (Fig.17a).

The absence of melting peaks (Fig. 19) and data from differential scanning calorimetry above 150° C demonstrate quantitative inclusion of the solubilized portion of the drug AmB used. In view of the aforementioned optimized drug: HPBCD/SBEBCD molar ratios observed for AmB 1:1.8 & 1:1.4 respectively and the inability of HPBCD/ SBEBCD in the solutions to solubilized all the available drug, thereby indicating likely association of more than one HPBCD/SBEBCD molecule per molecule of drug.

Liposome-entrapped inclusion complexes: In the present study, it is demonstrated experimentally that the lipophillic drug AmB can be incorporated into stabilized liposomes derived from proliposome. This approach seems to be

potentially applicable to a wide variety of drugs having differential solubility in water and alcohol. The method is based on simple idea that mixtures of membrane lipids, ethanol and water can be used to form a concentrated proliposome preparation which can be subsequently converted into a stable liposome dispersion simply by dilution with an excess aqueous phase (Fig. 17b). The lipid in the proliposome mixture may be present in the solution either in the form of stacked bilayer sheet or as mixtures of sheet and vesicles (Perrett, el.al.,1991). Under given situation the micelle properties of lipids, the stacked sheets shall themselves can certainly be an integral part of larger closed structures. The relative proportion of sheet and vesicular structure found to be largely depend upon the position of mixture in the three phase organization which is supported by the earlier report (Tinker and Saunders, 1968)

The liposomes produced by the proliposome technique are multilamellar vesicles with diameters centered around 4.17-4.30 μm with EPC liposome contained AmB-HPBCD/ SBEBCD respectively. Whereas 3.44 – 3.54 μm diameter are observed with DOPC liposomes contained AmB-HPBCD/SBEBCD. Liposomes formed from proliposome mixtures containing charged lipids tend to have appreciably larger entrapped volumes per mole of lipid than those formed from neutral lipids.

The effect of β-CDs on the mean vesicle size shown in Tables 3 & 5, it was observed that larger vesicle size were found with sulfobutyl ether β-CD whilst, lowest vesicle size were found with hydroxypropyl β-CD. The present finding clearly demonstrated that an increase in binding cavity is observed with an increase in the degree of substitution for the sulfobutyl ether β-CD, which is in agreement with the findings of Zia, et. al., (1995).

The average diameter of liposomes formed from mixtures containing charged lipids was found to be smaller than that of liposomes formed from neutral lipids. This suggests that this increased entrapped volume reflects an increase in the total number of liposomes formed, and hence a decrease in the average number of bilayer per liposome, when charged lipid is present. This would be consistent with the increased electrostatic repulsion between bilayer surfaces when charged lipids are present (Tables 3 & 5). However, effect of stearylamine as compared to dicetyl phosphate was more with respect to vesicle size and entrapment efficiency. Probably, this is due to the charge of phosphotidylcholine ring.

The essential feature of the proliposome mixture is its ability to rearrange on dilution to form a stable liposomal suspension. Inclusion Complex (AmB-SBEBCD/HPBCD) included in the proliposome mixture is trapped within the liposomes formed by this approach shown to have very high efficiency. The

rearrangement occurring on dilution means that if desired, the drug can be included in the dilution buffer rather than in the original proliposome. Under these conditions, increased drug entrapment can still be achieved by using two-stage dilution procedure involving the initial addition of drug in a small volume of the mixture out of the proliposome region followed by dilution in the usual way.

Tables 4 & 6 showed, higher rate of entrapments of drug within the liposomes were found when inclusion of drug complexed with the sulfobutyl ether β-cyclodextrin in comparison to hydroxy propylated β-cyclodextrin. This may be attributed to the longer the hydrophobic chain linked to the β-CD, the higher association of drug within vesicles (de-chasteinger, 1996). Furthermore, it is reported that the sulfoalkyl βCD derivatives exhibit larger binding constant for neutral forms of drug (AmB) than βCD and HPBCD (Okimoto, et.al.,1995). Subsequently it is demonstrated that the binding potential of the Sulfoalkyl βCD derivatives is not only dependent on both sulfoalkyl chain length and degree of substitution, but also on substrate (phospholipid) properties (Zia, et.al., 1995).

The main difference between multilamellar vesicles formed by the present method and those formed by most other solvent-based method is the absence of toxic solvent residue. Relatively high concentrations of ethanol are present in the proliposome mixture but this can be an advantageous, as it is known to facilitate the storage of the proliposome mixture in a sterile form suitable for the

subsequent in-situ formation of liposome. The proliposome approach described in the present experiments also avoids involving sonication steps and it is eminently suitable for scaling up its production.

The liposome dispersions formed in the present experiment found to consist of study contained 10mg ml^{-1} EPC and 8mg ml^{-1} ethanol for EPC liposome whereas for DOPC liposome it is found to be 10mg ml^{-1}DOPC and 10mg ml^{-1} ethanol. Using partition coefficient value of 0.522 & 0.373 obtained of ethanol for EPC liposomal vesicles (ELV) and DOPC liposomal vesicles (DLV) respectively, which correspond to an ethanol content in the lipid phase of 4.16 μg /mg EPC and 3.70μg /mg DOPC. This means that only 0.52% of the ethanol in ELV system and 0.37% of ethanol DLV system is directly associated with the lipid bilayer. The presence of ethanol at such low levels has shown to elicit little or no effect on the permeability of drug.

Thus, proliposome approach described in the present investigation has shown high entrapment efficiency, employing the two-stage dilution procedure. This procedure seems to be suitable for a wide range of drugs having differential water and alcohol solubility. The method is simple and avoids the use of sonication steps and pharmaceutically unaccepted solvents. This technique is eminently suitable to scale-up for regular commercial production.

Stability studies :

In- Vitro drug release studies : Exposure of liposome entrapped AmB-HPBCD and AmB / SBEBCD inclusion complexes to PBS buffer (pH 7.4) resulted in varying complex dissociation and leakage of drugs into the media (Figure 26-27). Such dissociation of drug presumably depends upon the stability constants of the complexes. In agreement with previous findings (McCormack and Gregoriadis 1996), only 0.36% and 0.28% AmB was released initially with a further modest increase (to 2.16 and 2.04%) from ELV entrapped AmB-HPBCD & AmB-SBEBCD respectively after 6.0h. It is likely that drug displacement from the cyclodextrin cavity is followed by drug accommodation in the lipid bilayer which could then be rendered more fluid (Loftsson and Brewster,1996) and, hence more permeable (Gregoriadis, 1995).

As there is a considerable loss of AmB from DLV in both types of inclusion complexes (AmB-HPBCD and AmB-SBEBCD), even at 30-min. exposure (Fig. 27),it has been suggested (McCormack and Gregoriadis, 1994) that following their displacement, drug localizes at or near the lipid water interface of the vesicles and is hence available for rapid release. This is supported by the earlier findings (Kirby, et.al., 1983) that lipophillic drug entrapped in stable liposomes are

prone to rapid partial loss on exposure to plasma. An additional factor that can augment the release of dissociated drug through the bilayer is bilayer fluidity.

Furthermore, liposomal phospholipids, being weak in acidic environment and hence are easily hydrolyzed by an acidic metabolite released from fungal cells. The results of pH dependent stability experiments clearly demonstrated that liposomes were relatively unstable (in terms of AmB loss) both at acidic and alkaline pH (Fig.28 – 31).

In- vitro drug leaching studies: The long term drug leaching experiments (Table 7-8) with the EPC liposomal AmB HPBCD/SBEBCD complex revealed a gradual reduction of AmB to 0.93 & 0.69% in proliposome form, after 60 days. Thus, it is conceivable on the other hand that the slow leakage of AmB from vesicles does not entirely reflect its rate of dissociation from SBEBCD or HPBCD but also due to a slow rate of liposome disintegration in-situ.

Product Shelf Life Stability: Stability studies were carried out to determine shelf life of the liposomal preparations. The results (Fig. 32-35) of this study revealed that chemical stability of AmB in EPC liposomal vesicles entrapped AmB–SBEBCD/HPBCD was generally more (404 & 310 days) than observed in other preparation of DOPC liposomal vesicles in concentrated form, which showed a shelf life of 293 & 217 days (Fig. 38-41). However, the binding constants for the vesicles with SBEBCD were significantly superior to those of HPBCD, suggesting

that this complex (AmB–SBEBCD) protect the drug more efficiently in the cavity of this modified β-Cyclodextrin. Experimental findings of Masson, et. al., (1998), indicated that the structure of drug–Cyclodextrin complexes is dependent to a great extent on the charge of the cyclodextrin molecule.

It is interesting to note that, as per finding of Okimoto, et.al., (1995), the degradation constant of AmB-SBEBCD complex was increased when the PBLV carried same charge (Dicetyl phosphate) and decreased when the PBLV had the opposite charge (Stearylamine). This may be due to a clear charge effect wherein attraction of cationic lipid, was observed with SBEBCD leading to drug displacement from inclusion complex.

Stability studies on both types of PBLV formulations in suspension form were discontinued after 1 month, due to leaching of drug more than 10%.

Toxicity and therapeutic efficacy of amphotericinB delivered through proliposome based liposomal vesicles (PBLV) in the treatment of experimental murine Aspergillosis: Aspergillosis model in Balb/c mice was developed by injecting 3.6×10^6 spores of A. fumigatus intravenously. It was observed that the mortality of mice was observed from the 2nd to the 4th day after spore challenge. Since the spores were given intravenously they would be expected to be disseminated immediately to different parts of body and germinate

into hyphae. The resultant hyphae would then invade the tissue and blood vessels causing thrombosis and ischemic necrosis (Walsh and Pizzo, 1994). In humans, the propensity of *Aspergillus* species for voracious invasion may lead to extensive haemorrhagic infarction within 3-4 days thereby leading to death (Meyer, et.al., 1973). Depending on the ability of host defense such as macrophages and neutrophils to prevent the germination and subsequent dissemination of fungus some mice were able to survive for a longer period of time.

In non-treated mice, *Aspergillus* load (cfu) in lung were significantly higher than in those treated with 12mg/kg PBLV. In the non-treated group 100% of mice died within 7 days while in the treated group 90.0% survived during the similar duration of period. This indicated clearly that fungal invasion was the prime cause for high mortality and whenever it was controlled with amphotericin B therapy, there was a significant improvement in the rate of survival of mice. Similar observations have also been reported by Moonis, et.al., (1992) in a murine model of aspergillosis with treatment of liposomal hamycin.

Treatment of fungal infections with AmB_{DOC} is associated with severe side effects like nephrotoxicity and abnormalities in pulmonary function (Medoff, et.al., 1983., Maddux and Barriere, 1980). Therefore, attempts have been made by a number of investigators to develop drug carrier system, which could reduce the

toxicity of amphotericinB while retaining its antifungal activity (de Marie, et.al., 1994; Janknegt, et. al., 1992). For example AmBisome (Alder-Moore, et.al., 1991), ABLC (Clark, et.al., 1991) and ABCD (Fielding, et.al., 1991;Abra, 1989) have been developed and used in animal models and later extended to clinical trials (Ringden, et.al., 1991; Graybill, et.al., 1991). However, in all these preparations the lipid formulation required for delivery of amphotericin B has hitherto not been optimized.

In the present studies, experiments were carried out to evaluate the efficacy of amphotericin B proliposome based liposomal vesicles prepared by the method (Perrett. et.al., 1991; Chakraborty and Naik,1999) on mouse model of aspergillosis.

Although variations and discrepancies amongst data in experimental animals, observed due to the methods of drug preparation and various other factors from different investigators. The present investigations nevertheless attempt to compare the toxicity and therapeutic efficacy of amphotericinB in proliposome -based liposomal vesicles with lipid formulations described earlier by other workers. The in-vitro toxicity of the PBLV was significantly less than that of the AmB_{DOC}. It is known that mammalian cell toxicity arises from the formation of conducting pores in the cell membrane. When AmB binds to cholesterol, a major sterol in mammalian membranes (Khutorsky,1992; Hoogevest,et.al.,1978; Kruijiff,

et.al.,1974; Bittman, et.al., 1974) From experimental results, the transfer of AmB from the EPC and DOPC liposomes to RBCs would be less favorable than that of AmB_{DOC}. Infact, the EPC liposomes entrapped (AmB-HPBCD/SBEBCD) inclusion complexes are more stable than the DOPC liposomes entrapped inclusion complexes in terms of AmB release. Therefore, it is reasonable to assume that the decreased in-vitro toxicity is attributed to decreased in AmB release. Furthermore consistent a diminished nephrotoxicity was observed in all PBLV-treated mice with single dose (2-19 mg/kg). However, insignificant rise in serum creatinine levels over the base line was observed. The comparison of serum creatinine level between PBLV (HPBCD/SBEBCD) and AmB_{DOC} treated groups, it was observed that the latter group showed moderately higher serum creatinine levels(Fig.47).

The LD_{50} value of amphotericin B in PBLV-HPBCD & PVLV-SBEBCD formulation was 17.4 and 18.6 mg/kg respectively which is almost 9 times greater than that of AmB_{DOC} preparation (2.0 mg/kg). However, a significant reduction in the toxicity of amphotericinB has been reported by a number of workers when delivered in the form of liposomal preparation (30 fold) (Alder-Moore, et.al., 1991; Proflitt, et.al., 1991) or ABLC (15-25 fold) (Clark, et al., 1991.)

It is observed that there was 70% and 80% survival upto 7 days after infection when treated with single dose of 0.5 mg/kg. PBLV entrapped

AmB/HPBCD and AmB/SBEBCD. Whereas, the group treated with AmB_{DOC} showed only 20% survival. However, other workers have observed various responses when amphotericin B was delivered through different lipid formulations. For example, Allende, et. al., (1992) have reported a 30% improvement in survival when treated with ABCD used for treatment. On the other hand Gondal, et.al., (1989) have observed 50-60% improvement in survival when liposomal amphotericin B was used for treatment. Similarly around 60% survival has been noted by Allendoerfer, et.al., (1991) with ABLC treatment. Considering all aspects of reported AmB liposomal preparations it is felt that the low cost of PBLV and its inherent stability towards oxidation makes it a more attractive and practical alternative to other lipid formulations for treatment of systemic/deep seated fungal infections.

Considering percentage survival and fungal load in infected mice as the key parameters of therapeutic efficacy it was found that the maximum protection could be achieved with the treatment of 0.5mg/kg EPC liposomal vesicles entrapping AmB-SBEBCD/HPBCD. This dose could not only significantly reduced fungal load as well as increased the percentage of survival of infected mice. It was concluded that in this murine model, PBLV (AmB / HPBCD & AmB/SBEBCD) has a higher therapeutic efficacy than AmB_{DOC} in curing invasive aspergillosis. Our results conclusively suggest that proliposome-based liposomal

vesicles could be an effective alternate delivery vehicle or carrier system for amphotericin B.

There are numerous reports in literature on lipid formulations of amphotericin B and significant reduction in their toxicity profiles without *significant* loss of antifungal activity. All these formulations have altered the pharmacokinetic pattern of AmB as compared to AmB_{DOC} in animal experiments. By careful evaluation of all the aforementioned studies it was proposed that reduction in toxicity of PBLV may be due the rapid elimination of amphotericin B from circulation and increased accumulation in liver resulting in reduced levels in kidneys, the prime target organ of toxicity. Therefore, it was thought worthwhile to study and examine whether altered biodistribution as reported for other lipid formulations like ABLC, ABCD, AmBisome is also a vital factor for reduction in toxicity and increased therapeutic efficacy of PBLV.

Similariy, attempts were made to develop PBLV vesicles using another phospholipid, DOPC that is similar to egg phosphotidylcholine and found significant reduction in in-vitro and in-vivo toxicity of AmB when delivered through DOPC-PBLV. However, treatment with DOPC-PBLV failed to improve the survival rate of infected mice. Apparently high toxicity of AmB could not be completely circumvented even in DOPC-PBLV. Furthermore, the variability in toxicity and therapeutic efficacy in different batches of DOPC-PBLV added further

complication. Hence, it is felt that different approaches are required to address the problem of high toxicity of AmB when delivered through DOPC-PBLV; where in the release of dissociated drug through the bilayer is bilayer fluidity.

Thus, the present experimental findings are in agreement with previous investigators who have reported increased uptake of liposome intercalated HPBCD (McCormack and Gregoriadis, 1996), suggesting liposome entrapped complexes end up in the liposomal apparatus following endocytosis of the carrier. One of the plausible explanation for an enhanced uptake of stabilized liposome could be due to its increased stability in serum with respect of entrapment (Duchene and Wonessidjewe, 1996) Such retention observed in the present studies, would likely to diminish excretion of inclusion complexes in the urine, protect intercalated drug from displacement by plasma components and, as complex concentration within circulating individual vesicles remains essentially unaltered to prevent inclusion complexes from being dissociated by plasma dilution (Gerloczy,et.al., 1997)

Pharmacokinetics of amphotericin B when delivered through proliposome-based liposomal vesicles in normal and *A.fumigatus* infected mice: It is found that there was rapid elimination of amphotericin B from plasma and accumulation mainly in the liver after administration of PBLV. Because of this

accelerated removal from circulation deleterious unwanted interactions with RBCs are minimized and therefore toxicity is significantly reduced. It is also observed that amphotericinB was not degraded rapidly in liver since nearly 56.4% and 39.13% were recovered in intact form, from liver after 24 and 48 h respectively with EPC (AmB/SBEBCD), whereas 49.3% & 32.85% recovered after similar period in intact form with EPC liposome (AmB/HPBCD). Therefore it appears that the liver serves as a primary reservoir of amphotericin B in the body from where it is slowly released at non-toxic levels to different tissues.

The rapid elimination from the circulation and accumulation in the liver may be attributed to the phagocytosis of PBLV by reticuloendothelial system. The present findings are in complete agreement with the earlier reports dealing with lipid based formulations like ABLC (Clark, et. al., 1991), ABCD (Fielding, et.al., 1991; Wang, et.al., 1995) and AmBisome (Proffitt, et. al., 1991;Lee, et. al., 1994) which are rapidly removed from circulation and mainly concentrated in the liver as compared to AmB_{DOC}.

A number of investigators have shown that amphotericin B formulations like ABLC and AmBisome increase delivery of amphotericin B to spleen as compared to AmB_{DOC} (profitt, et al., 1991; Olsen, et al., 1991). Similarly, in the present investigation it is demonstrated that uptake of amphotericin B in the spleen from EPC liposome entrapping AmB-SPEBCD/HPBCD is slightly higher

than AmB_{DOC} preparation. Thus, the present findings are in agreement with previous investigators who have reported increased uptake of liposome intercalated HPBCD (McCormack and Gregoriadis, 1996), suggesting liposome entrapped complexes end up in the lysosomal apparatus following endocytosis of the carrier. Hence, one of the plausible explanation for an enhanced uptake of stabilized liposome could be due to its increased stability in serum with respect to entrapment (Duchene and Wouessidjewe, 1996). Such retention observed in the present studies, might diminish excretion of inclusion complexes in the urine, protect intercalated drug from displacement by plasma components and, as complex is concentrated within circulating individual vesicles would remain essentially unaltered to prevent inclusion complexes from being dissociated by plasma dilution (Gerloczy, et,al.,(1997).

Some of the formulations like ABLC due to its large size (1.6-6μm) increased the uptake of amphotericin B in lungs because of physical entrapment in pulmonary capillaries (Taylor, et. al., 1982). Similar observation has been reported with the administration of ELV comprised inclusion complex of AmB – SBEBCD/HPBCD. It was also noted that there were significantly lower concentrations of amphotericin B in kidneys after PBLV administration. This is an important observation considering that kidney is the prime target organ of amphotericin B mediated toxicity.

The other important observation of present findings indicate that there was a significant enhancement in uptake of amphotericin B from liposomal vesicles comprising inclusion complexes (AmB-SBEBCD/HPBCD) in infected tissues like liver, spleen and kidneys as compared to tissues of normal mice. Therefore, it appears that slow complex dissociation rather than rate of vesicle disintegration or rate of elimination of AmB content from these tissues following the initial 24h period in mice injected with proliposome based liposome (McCormack and Gregoriadis, 1996). A number of investigators have also reported an increased uptake of amphotericin B from lipid based formulations in infected tissues (Lopez-Berestein, et.al., 1984; Ahmad, et. al., 1989, 1991). The higher uptake of amphotericin B in infected organs has been attributed to damaged capillaries of various organs (Ahmad, et.al., 1989, 1991) and delivery of drugs to the infected site via circulating monocytes and macrophages which have the capability to phagocytose amphotericin B containing lipid vesicles in circulation and have tendency to migrate to the infected site (Morgan, et al., 1985). Therefore it appears that in case of aspergillosis, circulating monocytes or macrophages phagocytose PBLV and subsequently deliver them to infected sites thereby increasing the local concentration of drug resulting in improved therapeutic efficacy. Since the circulating macrophages capture the PBLV immediately after their I.V. administration, the RBCs and other sensitive cells are

not exposed to the drug resulting negligible lysis in red cells and decreased toxicity. Mehta, et.al., (1994) have also confirmed that circulating phagocytes could take up liposomal amphotericin B and transport it to the sites of inflammation and infection.

The preferential uptake of liposomal vesicles entrapped inclusion complex (AmB-HPBCD) by the RES (reticuloendothelial systems) may partly explain minimal disposition of liposomes derived from proliposome in infected and non infected kidney which would result in reduced nephrotoxicity (Ahmad, et.al., 1989; Joly, et. al.,1989). The reduced rate of nephrotoxicity from proliposome based liposomes might be related to the diminished efflux of decreased filtration of free amphotericin B into the tubules, alternatively a decreased transfer and accumulation of amphotericinB into renal tubular cell membranes. However, this needs further confirmation especially during chronic treatment of liposomal AmB, which has a greater clinical significance.

In conclusion, these pharmacological studies demonstrated that PBLV has significantly altered biodistribution as compared to AmB_{DOC}. Increased uptake of amphotericin B in liver with concurrent decreased levels in kidneys and plasma, and enhanced localization at infected sites may likely to lead to higher therapeutic efficacy of EPC liposomal vesicles (AmB/SBEBCD).

Influence of lipid dose on plasma pharmacokinetics of PBLV (AmB-HPBCD/SBEBCD). The concluding part of the study involves the evaluation of the effect of different chemically modified β-cyclodextrin (β-CDs) which might affect the in-vivo stabilization of liposomal preparation derived from proliposome via amphotericinB / β-CDs inclusion complexes with varying lipid dose. Pharmacokinetic data were analyzed considering the varying volume of distribution with respect to the varying lipid concentration in blood.

The pharmacokinetic characteristics with single I.V. dose of L-AmB were conversely different with that of free AmB after the administration of increasing lipid dose, as compared to without the lipid dose.

The Cmax and AUC of L-AmB elevated proportionately, as lipid dose was increased. This is possible due to saturability of the reticuloendothelial system (RES) (Dave, et.al., 1986). The normal route of elimination of liposomal AmB from plasma is via phagocytic uptake by the RES, which is most likely to be saturable process. Therefore, keeping in mind the above mentioned hypothesis it is possible that at low lipid dose (80 mg per animal), L-AmB might be taken up easily by RES macrophages, whereas at higher lipid dose, these sites might become saturated (i.e. too many vesicles for a limited number of macrophages or limited capacity to bind and engulf the vesicles). Furthermore, this concept is in complete agreement with findings of Proffitt and coworker (1983). Thus, the

vesicles might remain in circulation for longer period resulting to higher Cmax and AUC. The higher Cmax and L-AmB (Proliposome based) is known to entrap different inclusion complexes in which AmB is tightly bound with β-CD derivatives and these vesicles containing inclusion complex are found to be quite stable in plasma (Chakraborty and Naik, 1998,2000a, & 2000b). Probably, these β-CDs bound in L-AmB would slow down the rate of transfer of the amphotericin-B from the vesicles to erythrocytes and other tissues and also its subsequent removal from plasma, resulting in higher Cmax and AUC of amphotericin-B.

The administration of proliposome based liposome-entrapped AmB. AmB/HPBCD and AmB/SBEBCD inclusion with increasing lipid dose (>80mg) to rat resulted in varying complex dissociation and leakage of drug in plasma which is shown to depend solely on the stability constants of complexes (McCormack and Gregoriadis, 1996). The lower value of Vd in the case of liposomal AmB-SBEBCD may be due to the very low AmB dissociation, where complexed AmB has a very low elimination phase, which is in complete agreement with the findings of de-Chasteign (1996), suggesting that the longer hydrophobic chain linked to the β-CD, the higher the association of drug within vesicle. However, it has also been demonstrated that the binding potential of the sulfobutyl ether β-CD derivatives is not only dependent on both sulfobutyl chain length and degree of substitution, but also on the substrate (phospholipid) properties (Zia, et.al.,

1995). The binding constants for neutral forms of the drug were always greater with SBEBCD then with HPBCD. Furthermore, Masson and his coworkers (1998) have reported that degree of substitution of these β-cyclodextrin is in the order of SBEBCD > HPBCD. Therefore it appears that slow complex dissociation rather than rate of vesicle disintegration accounts for the most of the high Cmax and low volume of distribution at higher lipid level, following the liposome entrapped complex.

Documented findings, clearly supports our hypothesis, that in the presence of lipid matter AmB blood concentration is elevated. Furthermore, it facilitate -

-- L-AmB to achieves higher CmaxS and $AUC_{0-\infty}S$ in serum at single doses of 5 and 10 mg/kg. (Lee, et.al., 1994).This has been attributed that AmB associates predominantly with high density lipoproteins ,HDL (Wasan, et. al., 1993).It has also been stated that phagocytic upake by RES is a saturable process (Lee, et. al., 1994).Therefore, it is possible that saturable elimination of L-AmB from plasma via clearance by the RES is consistent with the Saturability of RES uptake of small unilamellar liposomal vesicles which is supported by the findings of Profit, et.al. (1983).

-- It is reported that continuous infusion of 5 % intralipid for 5 days results in an increase in total serum cholesterol and HDL Cholesterol without altering LDL Cholesterol or total serum triglycerides (Wasan, et.al., 1994).

-- The increase in HDL Cholesterol without altering LDL cholesterol might increase the interaction of AmB with Cholesterol and Cholesteryl esters of HDL and contribute to the observed decreased clearance of AmB from the blood stream, (Wasan, et.al., 1994).These authors have also observed that AmB association with HDL increased by incorporating the drug into negatively charged liposome .

-- PK (Pharmacokinetic) analysis of single iv doses of AmB resulted in higher concentration of AmB in serum in rats receiving 5% intralipid than the concentration in rats receiving the normal saline infusion by a factor of 2 (Wasan, et.al., 1994).

In conclusion, incorporation of chemically modified β-Cyclodextrin has clearly demonstrated high binding affinity as well as specificity to accommodate water insoluble molecules ("guest") in the hydrophobic milieu of the cavity present in β-Cyclodextrin structure and effectively circumvented liposomal in-vivo stability problem in biological environments such as serum.

The altered pharmacokinetic behavior at higher lipid doses of the present liposomal AmB preparation by proliposome approach might enhance the

therapeutic window of AmB in clinical medicine. The high C_{max}S and $AUC_{0-\infty}$ S is achievable with this approach in plasma, especially at lipid doses of 85 to 110 mg/kg, further underline the potential use of PBLV for the treatment of disseminated fungal infections, specially intravascular infections. However, it remains to be seen whether elevated plasma AmB concentration is pharmacodynamically significant or rapid penetration into the tissues is the critical determinant(s) in achieving antifungal therapeutic efficiency.