Liposomes of terbutaline sulphate: *In vitro* and *in vivo* studies

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In *vitro* studies were conducted to understand the comparative drug diffusion pattern, across artificial membrane, of the drug and of the prepared liposomes of different liposomal membrane composition. *In vivo* studies were carried out to determine the extent and time-course of pulmonary tissue uptake of administered liposomes containing terbutaline sulphate(TER) on rat lungs. *In vitro* studies revealed that the drug released from the prepared liposomes obeys Higuchi's diffusion controlled model. Different loading doses and release patterns of drug from the liposomes can be obtained by altering the PC:CHOL ratio and incorporation of cholesterol was found to reduce permeability of the membrane. Similarly drug absorption *in vivo* in rat's lung following intratracheal instillation, prolonged over 12 hr by liposomal entrapment of TER. The findings of present investigation indicated that liposomally encapsulated TER can be used for pulmonary delivery for maximizing the therapeutic efficacy and reducing undesirable side effects.

Liposomal formulation of β-adrenergic stimulants like terbutaline sulphate (TER) may overcome the altered bronchial hyperactivity that occurs on long term inhalation therapy of pure drug. Aerosolized liposomal delivery of these formulations can improve the therapeutic efficacy of these agents. Most investigations of liposomes as drug-delivery systems have relied on parenteral routes of administration. To achieve targeted delivery to the lung, however, the direct administration of liposomes into the airways has the advantage of circumventing systemic dilution and removal by other tissues and organs. Moreover, extra-pulmonary adverse effects may be minimized. Phospholipids are endogenous to the lung and liposomes composed of naturally-occurring phospholipids, at an appropriate dose, should not pose a toxicological risk to this organ. Direct administration of liposomes into the bronchi and alveoli has been investigated for the treatment of respiratory distress syndrome and for the delivery of antitumor drugs, antimicrobial drugs, and anti-asthmatic drugs to the pulmonary system. Besides, it is known that liposomes are very well taken up by the lung tissue, after their intratracheal administration. Other studies have shown that the lipid composition of the carrier vesicles is an important determinant of the interaction of liposomes with cell and the rate of release of the entrapped agent.

Physiological availability of the drug depends on both, the rate of release from the vesicle and permeability through the lung membrane. *In vitro* methods are valuable for screening procedures and for deducing physicochemical parameters such as fluxes, partition coefficient and diffusion co-efficient. The theoretical disadvantage of such a technique is that the method does not exactly duplicate the behaviour of living tissue *in situ*, particularly with respect to a capricious blood supply and metabolism. The dissolution test is an useful *in vitro* method for assuring batch-to-batch uniformity and bioavailability of the formulation. However, at present, there is no method for determining the *in vitro* release characteristics from a pulmonary product. In the present study, a vertical *in vitro* diffusion apparatus has been designed and validated, and used for *in vitro* studies. In our previous study unpublished, liposomes of TER were prepared, characterized and optimized for process variables: solvent mixture, volume of hydration medium, hydration time and sonication time and formulation variables: molar ratios of drug : lipid and phosphatidylcholine(PC) : cholesterol(CHOL). The optimized formulations (TER A and TER B) were subjected to diffusion studies to short-list the promising formulations for *in vivo* studies.

**Materials and Methods**

*Materials—*Terbutaline sulphate was obtained as a gift sample from Alembic Chemical Works Pvt. Ltd.,
Vadodara, India and complies with IP, 3rd edition monograph. Egg lecithin (PC) and cholesterol AR(CHOL) were purchased from Centre for Biochemical Technology, Delhi, India and S. D. Fine Chem. Ltd., Vadodara, India respectively. Alpha tocopherol was obtained from E. Merck, USA. All the ingredients were used without further purification. All other chemicals used were of analytical grade unless otherwise specified. Cellophane membrane [Mol wt.(cut off) : 12,000] and dialysis tubing were obtained from Sigma, UK. White albino rats, 3-4 months of age were procured from Deep Biolabs, Ahmedabad, India.

Reagents—Phosphate Buffer Saline (PBS; pH 7.4) and sodium borate buffer (pH 9.5) were prepared as described in IP (Indian Pharmacopoeia, 1996).

Preparation of liposomes—Liposomes were prepared essentially according to lipid film hydration technique reported by Mezei et al. and the process details are shown in Fig 1. TER, PC, and CHOL in the ratios of 1:9:9 (TER A) and 1:12:6 (TER B) were used. These ratios were selected as they were previously optimized for high drug entrapment and desired liposomal size (below 5 μm).

In vitro diffusion studies

Preparation of membrane—Cellophane membrane was previously boiled for 15 min in PBS and soaked overnight in PBS with EDTA (1mM). Hydrodynamic characteristics of the diffusion cell were established using benzoic acid disc method.

Method—Comparative diffusion studies were carried out among pure drug, 250 (μg in 5 ml PBS, and liposomal pellets (TER A & TER B) containing 250 μg of drug, diluted to 5 ml with PBS, using equilibrium dialysis method. A vertical diffusion cell (Fig. 2) was designed comprising donor and receptor compartment, agitated constantly and separated by an artificial diffusion membrane (cellophane membrane). Pulmonary conditions were stimulated by agitating PBS maintained at 37° ± 0.5°C using a water bath in the receptor compartment.

Drug : PC : CHOL (α-tocopherol 1% of PC) in 10 ml solvent mix.
(CHCl₃ : CH₃OH : H₂O) in 250 ml round bottom flask (Quick Fit neck 20/22)

Dry lipid film formation using Rotary Flash Evaporator at 180 rpm., 20 inches Hg vacuum, RT and under nitrogen atm. for 45 min

Hydration of the film using PBS pH 7.4 (5 ml) for 1 hr at RT under nitrogen atm. with gentle shaking

Liposomal suspension sonicated for 15 min in Probe sonicator (minimal speed) using icebath

Hydration of the liposomes in refrigerator for 2 hr (4°C)

Hydrated liposomes were separated from free drug by dialysis through tubing for 5 hr

Purified liposomes are transferred into vials, purged with nitrogen and stored in refrigerator till further use

Fig. 1—Flow diagram showing formulation technique for preparing liposomes of terbutaline sulphate
Solutions of both the compartments were stirred at the rate of 50 (donor compartment) and 100 rpm (receptor compartment) using triple blade plastic stirrer and magnetic stirrer respectively. The drug is highly soluble in PBS, so sink conditions were maintained with 50ml PBS and zero order flux conditions were not violated. One ml of sample from the receptor compartment was withdrawn at definite time interval and equivalent amount of fresh PBS was replaced. Absorbance of the drug in sample was determined using spectrophotometric method\textsuperscript{19}. The results of the analysis were plotted as regressed per cent drug released vs root T (Fig. 3) and histographically plotted in terms of mean flux and diffusion coefficient (Fig.4A & 4B).

**In vivo studies**

**Selection of animal**—Three, albino rats (220-240 g) were used in each group at every time interval for all formulations. In all, 72 rats were used for the study. The rats were fasted overnight, but had access to water ad libitum. These rats were selected randomly without any specificity about their sex. The rats at every stage of experimentation were anesthetized using pentobarbitone sodium (40 mg/kg; ip).

**Method**—A comparative biological study of pure drug with potential liposomal batches (TER A and TER B) was carried out using intratracheal instillation\textsuperscript{8}. The dosing was done by intratracheal instillation following cannulation with 6 cm length of PE-200 polyethylene tubing. The tip of the cannula was positioned approximately at the tracheal bifurcation. This cannula served to guide the introduction of 9.2 cm length of PE-50 polyethylene tubing, attached at one end to a 250 μl glass Hamilton syringe. Each rat received 100 μl of either liposomal suspension or drug solution (PD), followed by 50 (μl of 0.9% NaCl (saline) to rinse the syringe and tubing. Suspension of the solution (100 μl) was adjusted to provide a unit dosing of TER (200 μg). Animals to be killed at 1.5, 3.0, 4.5 and 6.0 hr after administration, had cannula secured with sutures, the incision closed with surgical staples. Rats to be killed after 12 and 24 hr, had the cannula removed and the tracheal incision sutured. Similarly blank liposomes were also instilled at every time interval.

**Preparation of biological sample**—Rats were killed by a blow with an iron rod followed by breaking the spinal junction. The lungs were excised carefully from the rats, to prevent attached skeletal muscle from coming along, and were subsequently homogenized and extracted with sodium borate buffer (pH 9.5). The extract was centrifuged using Remi C-24 centrifuge at 1.7x10\textsuperscript{7} g and -4 °C for 60 min to pellet cells and liposomes. The pellet was washed thrice with 3 ml of buffer pH 9.5 and subsequently centrifuged. The combined supernatant was analyzed for per cent drug released against the combined supernatant of blank for same time interval and the result of it was plotted against time (Fig. 5A). The pellet was resuspended and extracted with 10 ml of triton-X-100(10%)\textsuperscript{20} in sodium borate buffer (pH 9.5). It was gently warmed at 40°C for 15 min to assist solubilization of liposomes while doing so care was taken to prevent any coagulation. It was then spun in a similar fashion in a refrigerated centrifuge at 1.7x10\textsuperscript{7} g for 60 min to pellet cells. The pellet was washed thrice with 3 ml of triton-X-100 in sodium borate buffer (pH 9.5) and subsequently centrifuged. The combined supernatant was subjected to analysis of per cent drug remained entrapped, against the combined supernatant of blank for same time interval, the result of which was plotted against time (Fig. 5B).

**Data analysis**—The results of *in vitro* studies were analysed for mean steady state flux and diffusion coefficient. The flux across the cellophane membrane was calculated using the following formula\textsuperscript{21}:

\[ J = V \frac{dc}{dt}, \]

[where, \( J \) = Flux of the drug across the membrane; \( V \) = Volume of receptor compartment; and \( \frac{dc}{dt} \) = Rate of change of concentration]
Diffusion coefficient of the drug was calculated using the following equation:\(^{22}\):

\[ R = 200 \sqrt{\frac{D}{h^3}} \]

[where, \( R = \% \) drug released; \( h = \) thickness of the membrane (0.04); \( t = \) time (sec); and \( D = \) Diffusion coefficient (cm/\( \text{sec}^2 \)).]

Statistical analysis using ANOVA technique was applied for comparing the data obtained in \textit{in vitro} studies. The differences were found significant at \( P < 0.01 \). The data obtained in \textit{in vivo} studies were also subjected to ANOVA and were found significantly different from standard \( [2.49(f_1 = 2, f_2 = 15) \) at \( P < 0.1] \).

### Results and Discussion

A comparative diffusion study was carried out of pure drug in PBS and liposomal formulations (TER A & TER B) in PBS, in a self designed and validated diffusion cell for a period of 24 hr. The calculated F value (3.01) for validation was less than the table F value at \( P < 0.01 \) indicating that all the six sampling from diffusion cell did not vary significantly in \textit{in vitro} runs. Terbutaline sulphate is highly soluble in water, and so in PBS, sink conditions are maintained when 50 ml of PBS is used as diffusion fluid and hence zero order flux conditions are not violated. Comparison of the mean per cent drug diffused (Table 1), was done using ANOVA technique. The calculated F value (34.79) is higher than the table value \( (5.39, f_1 = 2; f_2 = 27) \) at \( P < 0.01 \) suggesting that the mean per cent drug diffused from various formulations are significantly different from each other.

In these study there are two rate controlling factors, \( K_1 \) the diffusion rate constant of the drug from liposomes and \( K_2 \) the cellophane membrane diffusion rate constant. However, total drug release was observed for both the liposomal formulation (TER A & TER B) prolonged over a period of 24 hr (Table 1). This is suggestive of the fact that the rise in drug concentration in receptor compartment is controlled by \( K_1 \) and not by \( K_2 \). The mean per cent drug released vs root T was plotted for all the formulations (Fig. 3). The regression coefficient of the data of per cent drug diffused vs root T \( (0.986 - 0.991) \) suggested a linear relationship between the two parameters and it obeyed Higuchi’s diffusion controlled model\(^{21}\). Thus the diffusion rate of the prepared liposomal formulations was found to be first order, which is consistent with the findings of Yerushalmi \textit{et al.}\(^{24}\).

Mean flux and Diffusion coefficient values of pure drug (PD) and of both the formulations (TER A & TER B) were calculated (Fig 4A & 4B). Both these values of PD were found to be almost three times higher than those of liposomal formulations (TER A & TER B). This reduction in mean flux and diffusion coefficient of liposomally-encapsulated drug has already been explained by Margalit \textit{et al.}\(^{25}\). Mean flux and diffusion coefficient of TER B was found higher as compared to TER A, which may be attributed to higher cholesterol content in batch TER A. These values are governed by the release of drug from the

| Table 1—Diffusion of drug across cellophane membrane
| [Values are mean ±SE of 3 observations]
<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Root T</th>
<th>Mean per cent drug diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>1.00</td>
<td>27.82 ±0.085</td>
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<td>07</td>
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<tr>
<td>10</td>
<td>4.89</td>
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Fig. 3—Mean per cent drug diffused vs root T
liposomal formulation and CHOL incorporation retards the drug release by reducing the permeability of liposomal membrane as already been reported by Betagari et al.26. Before the onset of diffusion, a larger initial flux was observed (Table 1) and then over a period of time a steady state was attained. This was true for both the formulations except that the initial flux and later steady state was governed by the cholesterol concentration in liposomal membrane. When cholesterol concentration was higher, a comparatively smaller initial flux was observed.

Comparative in vivo studies of liposomal batches (TER A & TER B) and pure drug (PD) was carried out by intratracheal instillation technique. Three albino rats were used at each sampling time points separately for all the formulations. The per cent drug released (Fig. 5A) and per cent drug remained in the liposomes (Fig. 5B) were estimated. Performing bronchoalveolar lavage to remove the free drug from the lung but not within the lung tissue, was not possible. Hence, the per cent free drug i.e., the drug

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**Fig. 4**—Histographic representation of mean flux (A) and diffusion coefficient (B) of the drug diffused across the cellophane membrane

**Fig. 5**—Drug released (A) from liposomes following intratracheal instillation and drug remained entrapped (B) in liposomes
that has been absorbed by the lung tissue including the drug released from the liposomes but yet to be absorbed, was analyzed after sacrificing the animal. It was assumed that the lung cell barrier has negligible role to play in the absorption of drug. Hence, once the drug is released from the liposomes, it is believed to be absorbed by the lungs immediately and is available for pharmacological response. Discussion in this study is based on this basic assumption. The drug balance between per cent drug released and per cent drug still in the liposomes was found to be close to 100%. It is believed that the amount of the drug which could not be accounted for, may have either metabolized or systemically absorbed or both.

At the first sampling time point (1.5 hr), the drug release was estimated to be 96.92, 11.29 and 12.13% in case of PD, TER A and TER B respectively. The drug released in the lung was also estimated at 3, 4.5, 6, 12 and 24 hr intervals. In case of PD administration, the per cent drug released in the lung gradually decreased and reduced to 3.79% after 24 hr. While, in case of liposomal batches, the drug release increased gradually up to 12 hr (45.16 and 46.32% respectively for liposomal batches TER A and TER B). Afterwards, the drug concentration in the lung tissue gradually reduced to 11.32 and 9.79% respectively at 24 hr for these batches. Thus, liposomal formulations of TER were found to significantly prolong the drug release into the lung compared to pure drug and are expected to prolong the drug action.

On comparing the data of liposomal batches, it was observed that increase in the concentration of cholesterol in liposome (TER A) further prolonged the release of the drug in the lungs. This increase in the biological half life of liposomes following inclusion of cholesterol has been previously described by Gregoriadis et al. The drug release in vivo was found higher at each sampling time point compared to the drug release in vitro studies. Diffusion of the drug across the liposomal membrane is not the only mode which governs the drug release in vivo, but also there are many other modes of drug release as reported by New. This includes engulfment of liposomes in the lung tissue and subsequent drug diffusion from liposomal membrane into the lung tissue directly or engulfment of liposomes by the lung tissue and digestion of liposomal membrane by pulmonary cells of enzymes to release all the drug entrapped at a time.

From the study, it may be concluded that liposomal encapsulation of TER has significantly prolonged the drug release and succeeded in maintaining sufficient drug concentration in the lungs for a longer period. Hence, liposomal products of TER of this investigations are expected to maximize its therapeutic efficacy and may reduce some of its undesirable side effects.

Acknowledgement

Financial assistance by UGC, New Delhi is gratefully acknowledged.

References

11. Joshi MR & Mista AN, Indian Drugs, in press (accepted for publication).