LIPOSOME: A NOVEL AEROSOL CARRIER OF DOXOPHYLLINE IN TREATMENT OF CHRONIC ASTHMA & CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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This article is available online at www.ssjournals.com

ABSTRACT

The aim of the present research work is to develop liposomal dry powder inhaler of doxophylline by double hydration method to increase the entrapment efficiency of this hydrophilic drug. In this formulation, PVP coated mannitol and the mannitol were used as a cryoprotectant and a carrier respectively. Doxophylline, a new methylxanthine derivative, chemically designated as 7-(1,3-dixolan-2-yl-methyl) theophylline, is a more potent bronchodilator than theophylline, which is associated with a wide range of adverse effects accounting for poor compliance and high dropout rates. Powder inhaler formulation of the drug was characterized by angle of repose, compressibility index and *in vitro* aerosolization properties using Anderson cascade impactor including fine particle dose (212.9±7.2), fine particle fraction (21.69±1.21), % dispersibility (62.34±3.5) and % emission (72.1±0.13) at a flow rate of 28.3 lit/m for 10 seconds using 10 capsules for each determinations. The optimized formulations were subjected to stability studies at 2-8°C, RT, and 40°C for 3 months. The stability studies of LDPI were determined in terms of their visual appearance (surface characteristics and colour changes) and PDRE. The *in vivo* study was carried out by gamma scintigraphy and that showed better retention of doxophylline in liposomal formulation as compared to the controlled release formulation.

Keywords: Doxophylline; Aerosol drug delivery; liposomal dry powder inhaler; stability; Gamma scintigraphy

1. Introduction

Asthma is one of the prevalent disorders worldwide and better management of asthma is the primary objective of the healthcare team. In the treatment of asthma, inhaled medications are generally preferred as they act directly on the airway surface and airways muscles where the asthma problem initiates. Absorption of inhaled medication into the rest of the body is minimal. Therefore, adverse side effects are fewer as compared to oral medications [1-4].

For the management of asthma, methylxanthines play an important role through the inhibition of phosphodiesterase activities. Doxophylline (fig.1), a new methlyxanthine derivative, chemically designated as 7-(1,3-dioxolane-2–yl-methyl) theophylline, is a more potent bronchodilator than theophylline, and the later one is associated with a wide range of adverse effects accounting for poor compliance and high dropout rates [5]. The clinical effectiveness of theophylline has long been thought to be bronchodilatation by non-selective inhibition of phosphodiesterase enzymes, although it is now known that these drugs also exhibit anti-inflammatory actions by inhibiting NF-kB activity [6,7]. The doxophylline has similar efficacy to theophylline but has less effect on adenosine receptors thereby minimizing cardiovascular and central side effects. Unlike theophylline, doxophylline does not interfere with calcium influx into cells nor antagonizes the action of calcium-channel blockers. Doxophylline has also been shown to have anti-inflammatory activity in a rat pleurisy model and inhibits eosinophil activation by effecting calcium activated K+ channels [8,9].

For the chronic therapy of asthma, nebulizers may be used. The Dry Powder Inhalers (DPI) is most convenient alternative to the pressurized metered dose as they are breath-actuated and does not need any propellants. As the drug is delivered directly to its site of action, a low dose can be used to produce therapeutic response and consequently side effects are minimized and also the needle free route increases patient's compliance [10]. The DPI formulation aims at pulmonary drug delivery having uniform distribution, small dose variation, good flow ability, adequate physical stability in the device

and good performance in terms of emitted dose and its fine particle fraction. The critically importance parameters in the development of DPI products is the evaluation, optimization, and control of flow and dispersion (deaggregation) characteristics of the formulation. These typically consist of drug blended with a carrier (e.g., lactose) and the properties of these blends are a function of the principal adhesive forces that exist between particles, including van der Waals forces, electrostatic forces, and the surface tension of the adsorbed liquid layers [11]. These forces are influenced by several fundamental physicochemical properties, including particle density and size distribution, particle morphology (shape, habit, surface texture), and surface composition (including adsorbed moisture) [12]. Among the novel drug delivery systems, liposomes are currently the most extensively studied biodegradable natural carriers in research for improving established drugs [13]. Liposomal drug encapsulation has been shown to be promising in sustaining the drug residence time within lung, improving therapeutic index, and delaying systemic dilution and thereby, reducing side effects. Delivery of various therapeutic agents along with lipid compositions can be used to treat pulmonary disorders [14,15].

Hence, in the present investigations, liposomal dry powder inhaler of doxphylline by double hydration method is prepared to increase the entrapment efficiency of this hydrophillic drug. Most of the extended release tablet formulations of doxophylline have been developed to provide once-a-day dosage regimen but it is also associated with limitation imposed by first pass hepatic metabolism. To overcome these limitations, present study was aimed to design and develop liposomal dry powder inhaler of doxophylline using PVP coated mannitol as a cryoprotectant and mannitol as a carrier.

2. Materials and Methods

4.1 Materials: Doxophylline was obtained as a gift sample from Cipla Pharmaceuticals, India. Soya Phosphatidylcholine was received as gift sample from Lipoid (Ludwigshafen, Germany). Cholesterol and D-mannitol were purchased from S. D. Fine Chemicals, India and Sigma, UK respectively. PVP (polyvinylpyrrolidone MW 40000) and Rotahaler were procured from Spectrum (Gardena, CA) and Cipla, Mumbai, India respectively. Other chemicals used were of AR grade purchased from local suppliers.

4.2 Methods

4.2.1 Preparation of liposomes of doxophylline: Liposomes of doxophylline were prepared using Double Hydration Method and the formulation variables such as ratio of phospholipids (50 mg) comprising Soya Phosphatidylcholine (SPC) and cholesterol dissolved in chloroform (different ratio of phospholipids to chloroform) were optimized. The phospholipid was dissolved in chloroform in a pear shaped flask. The flask was attached to a rotary evaporator (BUCHI TYPE EVAPORATOR) under reduced pressure using a vacuum pump. The rotation speed of the rotary evaporator was set at maximum and the water bath was set at 35°C. After 40 min, the negative pressure was released and the flask was detached. A thin lipid film was flushed with nitrogen for 2 min to remove chloroform traces, if any.

Doxophylline was dissolved in NaCl (0.9%) to prepare a solution of 25 mg/ml concentration. Drug solution was added to the thin lipid film at ambient temperature with vigorous hand shaking for 15 min. This was followed by flask immersion in the water bath for 15 min continued by another 10 min hand shaking. Drug free NaCl (0.9%) solution was added to give liposomes having a phospholipids concentration of 10 mg/ml.

Liposomal dispersion thus formed was subjected to sonication in an ice-bath for 10 min and the sonicated vesicles were further stabilized by hydration for 8 hr at ambient temperature. The liposomal dispersion of doxophylline thus obtained was filled in amber colored vials, sealed and stored in refrigerator at 2-8°C until required for further experiments.

***4.2.2 Separation of free drug*:** For the separation of free drug from the solution, liposomal dispersion (5 ml) was centrifuged at 15,000 x g at 4°C for 45 min using ultracentrifuge (REMI) and the supernatant was collected. The liposome pellets in the centrifuge tubes were redispersed in 10 ml NaCl (0.9%) solution to remove drug adsorbed onto liposomes. Centrifugation was repeated for further 45 min and the supernatant was again collected which was added to the first supernatant to comprise the unentrapped fraction of the drug. The pellets comprised of the entrapped drug.

***4.2.3 Preparation of liposomal dry powder*:** The pellets were dispersed in the required quantity of the hydrating medium containing sugars in the ratio of 1:8 having a lipid concentration of 10 mg/ml. Liposomal suspension was frozen over night and then dried under negative

displacement pressure (GYNODAYA lyophyllizer Model–GHTINS 120) for 24 hr. The porous cake thus formed was sized successively through sieve number 120 and 240. The sieved lyophilized liposomal powders were mixed with carrier (D-mannitol (45-75 µm) containing 2% Aerosil in mass ratios 1:0, 1:4, 1:5 and 1:6 and it was blended in mortar and pestle, filled in capsule (size 2) containing 1240 µg of doxophylline. The formulation were packed in vials containing silica bags as dehumactant and stored in a refrigerator (2-8^{o}C) until further use.

4.3. Characterization of liposomes of doxophylline

4.3.1. Photomicrography: All the batches of the liposomes were viewed under Fluorescent (LENOVO) microscope to study their shape and lamellarity.

Scanning Electron Microscopy (SEM) was performed using (JEOLJSM–6380LA) Analytical Scanning Electron Microscope. Powders were deposited on carbon conductive double-sided tape and dusted to remove the excess powder.

4.3.2. Laser light scattering measurement: The vesicle size of sonicated post hydrated liposomes was determined by Malvern laser diffraction size analyzer operating at beam length of 2.41 mm and range of 300 mm. The particle size of the formulation was determined by the volume mean diameter (VMD). The VMD measured using laser diffraction represents the median size of spherical particles which has the same volume as the particles in question.

4.3.3. HPLC analysis of doxophylline: The HPLC system (Shimadzu, Japan) consists of LC-10AT pump, a SPD-10AVP, PDA detector, Inersil C18 (250 mm X 4.6 mm, 5 µm) column and a class LC10/M10A software. The system was run in isocratic mode with mobile phase consisting of 12.5mM ammonium acetate (pH 3) and acetonitrile in ratio of 20:80 (v/v) using C18 column. Mobile phase was duly filtered through 0.22µm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min and delivered at a flow rate of 1 mL/min for chromatographic separation. The injection volume & detection wavelength were 20 µL & 275 nm respectively; PDA analysis was conducted to study the behavior at other wavelengths. The resulting chromatogram is shown in fig. 2

4.3.4. Entrapment efficiency (EE%): The liposome pellets were solubilized using Triton-X 100 (1% w/v) and the released drug profile from the liposomes- entrapped fraction was quantified. The entrapment efficiency (EE%) of doxophylline in liposomes was calculated after quantification of entrapped and unentrapped drug using HPLC (Table 1, 2 and 3).

$$EE\% = \frac{\text{amount of drug entrapped in liposomes}}{\text{(Over all amount of drug in formulation)}} \times 100$$

4.3.5. Determination of percent drug remaining entrapped (PDRE: After lyophilization, the fraction of the powder was redispersed in NaCl (0.9%) solution to give liposomes lipid concentration of 10 mg/ml. The rehydrated liposome dispersion was separated from the leaked drug by centrifugation and analyzed for PDRE as shown in the Table 1, 2 and 3.

4.3.6. In vitro drug release studies: The different formulations prepared by varying the process variables were subjected to *in vitro* drug release studies. The release of doxophylline from the liposomal DPI was determined as a function of time in phosphate buffer saline (pH=7.4) using dialysis tube. The samples were withdrawn at suitable time interval (1, 2, 3, 4, 5, 6, 12, 18 and 24 hr) (Table 4). The dissolution medium was replaced with same amount of fresh PBS saline (pH=7.4) solution to maintain the volume upto 40 ml throughout the experiment. The drug content was estimated by HPLC and cumulative % drug release was calculated and plotted against time (t).

4.4. Characterization of liposomal dry powder dry inhaler formulation

4.4.1. Angle of repose: The pile of powder was carefully built up by dropping the powder material through a funnel tip from height of 2 cm. The angle of repose was found to be 26.1^{o}, by inverting tangentially the ratio of height and radius of the formed pile [16,17] (Table 5).

4.4.2. Compressibility index: The compressibility index was determined by tapping the formulation for 500 taps to reach plateau condition [13,16,17] (Table 5).

4.4.3. Water content determination: Water content of the DPI formulation (1 g) was determined in triplicate on 2 consecutive days by Karl Fischer titration (Table 5).

4.4.4. In vitro assessment of aerosol particles: *In vitro* aerosol behaviors of the formulation was investigated in terms of respirable fine particle fraction (FPF) using Andersen 1 non viable cascade impactor [12] (Table 6).

4.4.5. Determination of critical relative humidity: The critical relative humidity (RHo) was determined by measuring the steady-state moisture uptake rate at relative humidity above RHo and then extrapolating the relative

humidity at which the moisture uptake is zero [14]. The saturated solutions of different percentages of various salts [15] were placed in desiccators and maintained at 20°C for creating each respective relative humidity condition. The samples were subjected to respective relative humidity and intermittently weighed for determination of the moisture uptake rate. The results are recorded in Table 5.

4.4.6. Fine particle characterization: The capturing solvent for all the stages of the study was carried out with preseparator and inhalation port and NaCl (0.9%) was used as a solution. Rota haler was used as a delivery device at a flow rate of 28.3 lit/min for 10 seconds and 10 capsules were taken in each batch. The inhaler body, capsule shells, mouthpiece and all the stages and plates were washed 5 times with NaCl (0.9%). Finally, the solution was carefully collected in Petri dishes and analyzed using HPLC.

Amount of drug retained was calculated by quantifying the amount of drug in the inhaler body and capsule shells. The fine particle dose (FPD) was denoted as the quantity (µg) of the particles in all the stages divided by number of doses.

$$FPD = \frac{\sum \text{Stages} \times 100}{\text{No. of doses}} = 0 \text{ to} 7 \text{ stages/10capsules}$$

$$FPF = \frac{\sum \text{Stages} \times 100}{\sum \text{Total}}$$

= 0 to7 stages/preseparator + 0 to 7 stages + filter

Recovered dose (RD) was taken as the total quantity of drug recovered per capsule after each actuation

$$\text{Loaded dose} = \frac{\text{Total recovered from all stages + Preseparator}}{\text{No. of Capsules.}}$$

Emitted dose (ED) was calculated as the percent of total powder mass existing in the inhaler. Percentage emission was calculated as the percentage of emitted dose to total dose. Dispersibility was the percentage of FPD to ED, shown in Table 5. Fine particle fraction corresponds to the respirable fraction reaching the lungs.

4.5. In vitro stability studies: Doxophylline liposomal dry powder inhaler (optimized formulation DOX-XI) was packed in gelatin capsule No. 2 under nitrogen atmosphere in amber colored vials containing silica bags as desiccant. The stability studies of the packed capsules were performed at various temperature (2^{o}-8^{o}C), room temperature (R.T) and 40^{o}C for a period of 3 months. Doxophylline LDPI were sampled at regular time intervals for 3 months and tested for the following attributes at each temperature

a) Surface morphology and color changes

b) Percent drug remaining entrapped on storage

4.5.1 Percent drug remaining entrapped on storage (PDRS): The sample of each batch stored in various storage conditions were withdrawn at definite time intervals, rehydrated with NaCl (0.9%) and analyzed for PDRS within the liposome. Liposomal dispersion was subjected to centrifugation on ultracentrifuge at 45,000 rpm for 45 min. The supernatant was collected and the liposomal pellets were redispersed with appropriate dilution with NaCl (0.9%) solution to remove drug adsorbed onto liposome. Centrifugation was repeated for further 45 min and the supernatant was again collected and added to the first supernatant to comprise the unentrapped fraction of the drug. The liposomal pellets were solubilized using Triton-X 100 (1% w/v) and the drug released was analyzed by HPLC as the liposome entrapped fraction. Entrapment efficiency was expressed at the "Percent drug Remaining Entrapped" on storage. (Table 7 and 8)

PDRS was calculated as follows –

$$PDRS = \frac{\text{(amount of drug entrapped)} \times 100}{\text{(Overall amount of drug in formulation)}}$$

4.6. In vivo studies: *In vivo* lung deposition of liposomal dry powder inhaler was assessed by gamma scintigraphy [18]. *In vivo* studies improve our understanding of aerosol therapy and may contribute to the development of improved delivery system.

***4.6.1. Study design*:** *In vivo* lung deposition of liposomal dry powder formulation was assessed by gamma scintigraphy. Albino rabbits of either sex weighing 2-3 kg were used for the study. Animals were procured from the animal house and had free access to food and water throughout the duration of study. The study was carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on animals), Ministry of Health, Government of India and all the study Protocols were approved by local institutional animal ethics committee. Utmost care was taken to ensure that animals were treated in the most humane and ethically acceptable manner. Gamma scintigraphy and pharmacokinetic approaches, both methods give similar pulmonary deposition estimates.

Drug deposited in the central airways was detected by scintigraphic method that is not captured by pharmacokinetic method, if the drug is removed by mucocillary clearance to the oral cavity before being absorbed into the blood of experimental animals [19].

4.6.2. Radiolabelled method: Liposome dry powder labeled by adding it to a NaCl (0.9%) solution containing gamma emitting radionuclide Tc^{99m}. Excess solution was removed by freeze drying, leaving the radiolabel attached to the liposomal powder. Then, it was blended with the carrier [19].

4.6.3. Scintigraphy; Rabbits were treated with liposomal dry powder by intrathecal administration using dry powder insufflators. Animals placed on a modified slant board to optimize visual placement of the insufflations cannula into the trachea [20]. Tip of the insufflation's device was placed in the trachea just above the carina. Powder is delivered through the insufflations device by rapidly pushing the bolus of air through the device. Immediately following administration of the radiolabelled aerosol, scintigraphic images were taken showing lung deposition. The result was derived as counts per minutes.

3. Results

3.1 ***Preparation of liposome:*** The doxophylline liposomes were prepared by two steps hydration technique. The characteristics of the prepared liposomes were determined by various methods. A marked reduction in the entrapped aqueous volume was confirmed while the liposomes were prepared by reverse phase evaporation extruded through polycarbonate membrane. Since the entrapment is dependent upon the volume of aqueous phase encapsulated during liposome formation, it may reduce the hydrophilic drug entrapment. However, the EE% of doxophylline in the study is superior due to the application of the two steps hydration protocol used in this study (the thin film hydrated first with drug solution and then again with drug free NaCl (0.9%) solution).

2.2 Size and morphology of liposomes: Photomicrographs (x1000 magnification) were taken before and after lyophilization and are shown in Fig. 3 (a-c). It shows that the size of the formulation was reduced and lamellarity was still maintained after sonication which may be due to incorporation of cholesterol. Cholesterol is known to have important modulatory effect on bilayer membrane which acts as fluidity buffer. The size and morphology of the particle was determined by Malvern particle size analyzer and scanning electron microscopy (Fig. 4) which depict the porous structure of the liposome preparation looking like crumpled paper and possess rough surface, which is very important for the aerodynamic properties of the powder. The Primary particles size of the formulation was determined by Volume Mean Diameter (VMD). The VMD measured using laser diffraction represents the median size of a spherical particle which has the same volume as the particle in question. VMD may be equivalent to Mass median Aerodynamic Diameter (MMAD) for non-volatile aerosols and has a good correlation with pulmonary deposition finding which was found to be 1.36 µ.

The formulation (DOX-XI) that exhibited good flow characteristics is confirmed from the analysis results given in Table 5. Powder inhaler formulation of the drug was characterized by angle of repose, compressibility index and *in vitro* aerosolization properties using Anderson cascade impactor including fine particle dose (212.9±7.2), fine particle fraction (21.69±1.21), % dispersibility (62.34±3.5) and % emission (72.1±0.13) at a flow rate of 28.3 lit/m for 10 seconds using 10 capsules for each determinations.

2.3 Entrapment efficiency and percent drug remaining entrapped (PDRE): It was observed that on increasing cholesterol proportion, the PED and PDRE efficiency has increased (Table 1) which may be due to changing the fluidity of bilayer membrane. The ratio of drug: PVP coated mannitol was varied from 1:4 to 1:10 and when ratio was 1:4 the PED and PERD was 20.13% and 45.14% respectively. On increasing the mannitol ratio, the efficiency of PED and PDRD has increased (at 1:6; 35.21%, 43.34% and at 1:8; 46.14%, 64.23%). The data revealed that highest PDRE was found to be with DOX-XI formulation containing 2.5% w/v PVP coated mannitol.

Mannitol is a monosaccharide alcohol that is easily crystallized and has the tendency of phase separation in the lyophilized cake, which is deleterious to the stability of dry liposome. Crystallization of saccharides may be inhibited in the presence of povidone due to its ability to increase the Tg (glass transition temperature) of the saccharides and the larger molecular weight PVP strongly prevent the crystallization. Therefore, povidone was added to mannitol to optimize the lyophilization process. On further increasing the ratio of mannitol, the efficiency of

PED and PDRE was decreased to 44.21% and 63.14% respectively.

2.4 In vitro release of doxophylline from liposomal dry powder: *In vitro* release of the doxophylline was determined and its release profile is presented graphically in Fig. 5. The optimization study of the formulation revealed that DOX–XI formulation followed the Higuchi diffusion kinetics with cumulative release profile till 24 hr. The control release may be due to the incorporation of cholesterol, which causes strong reduction in the permeability of the liposome system and thus reduce leakage of drug from the liposomes due to increased rigidity of liposomes membrane by increased concentration of cholesterol. The data for cumulative %drug release at particular time interval are given in Table 4. By fitting the *in vitro* drug release data into zero order, first-order and Higuchi model, it was concluded that the release followed Higuchi diffusion kinetics as the correlation coefficient R^2 value was higher than those of the other 2 release models (Fig. 6 and Table 7).

2.5 In vitro aerosolization properties: Mannitol with 2% Aerosil was used as carrier for the in vitro aerosolization property determination of the formulations and the results obtained by the addition of 2% Aerosil proved the concept that FPF can be increased. The carrier of choice for DPI products is currently lactose monohydrate. Nearly all DPI products already in market are relying on lactose as a carrier. The advantages of lactose monohydrate are its well investigated toxicity profile, its broad availability and relatively low price. However, for some drugs lactose monohydrate may not be the carrier of choice, due to its reducing sugar function that may interact with functional groups of the drug. In addition, lactose monohydrate is produced from the additives of bovine source so that the Transmissible Spongiform Encephalopathy (TSE) discussion is still an issue for this compound (EC statement 2002) also, discussion regarding endotoxin content and the necessity for a specification limit are ongoing (FDA draft guidance for Industry, 1998). Several sugar alcohols are evaluated for the potential use in dry powder inhalation formulation. Mannitol proved most promising candidate for this application. Difficulties arising from their hygroscopicity are overcome by adding an ultra fine hydrophobic excipient to the powder blend [21].

DPI were taken in liposome: carrier ratio of 1:0, 1:4, 1:5, 1:6 with maximum fine particle fraction with 1:5 (Table 6) carrier and no further increase in FPF was reported by increasing carrier ratio, optimum concentration of carrier is required to achieve detachment of liposome drug from carrier molecule. The capsule size 2 is filled with 150 mg powder containing 1240 µg of doxophylline with liposome: carrier ratio of 1:5.

2.6 Stability studies: Stability studies on optimized formulation (DOX–XI) were performed at 2-8^{o}C, R.T and at 40^{o}C for one month and analyzed by considering following parameters:

2.6.1. Visual appearance and observation (Surface characteristics and color changes): The surface characteristics and colour changes of DOX–XI formulation is shown in Table 7. During one month of accelerated storage period, caking and discoloration (cream colour) was observed under storage conditions (40^{o}C) at the end of the month (Fig. 7).

2.6.2. Percent drug entrapment on storage of DOX–XI formulation: The percentage drug entrapment of DOX–XI formulation is given in Table 8. The results of PDRS of DOX–XI shows that maximum amount of drug loss was observed at 40^{o}C i.e. 5.43%, 17.67% and 27.69% after average sampling for 7, 15 and 30 days respectively, while at room temperature the formulation shows 1.07%, 2.55% and 3..88% loss of doxophylline after storage of formulation for 7, 15, 30 days respectively. It was found that formulation placed at 2-8^{o}C shows very less amount of drug loss following rehydration after storage of formulation for 30 days which indicates that formulations are more stable at lower temperature.

2.7 In vivo studies: Results relating to the fates of formulation DOX-XI in the rabbit lungs are shown in Fig. 8-10. The intensity of liposomal formulation was more after 2 hr, while the intensity of control formulation (Asthmatic) faded out after 2 hr. The results supported the role of liposome in enhancement of drug permeation through alveolar epithelium by altering physicochemical properties of the drug rendering the drug hydrophobic. Liposomal encapsulation of DOX also acted as a biodegradable reservoir that prolonged pulmonary residence time of doxophylline.

4. Discussion

In this study, unilamellar liposomal vesicles loaded with doxophylline were successfully prepared and stabilized by lyophilization in DPI formulations with one month shelf life. The *in vivo* studies by gamma scintigraphy showed better retention of doxophylline in liposomal

formulation as compared with the controlled release formulation. Hence, the developed liposomal DOX formulated as DPI offers exciting possibilities of liposomal delivery in the anhydrous state. However, the role of DOX-DPI in clinical practice can only be justified after *in vivo* studies on further species of animals followed by extensive clinical trials. Further research in these formulations is ongoing in the laboratory for clinical practice.

Acknowledgement

The authors acknowledge the School of Pharmaceutical Sciences, Rajiv Gandhi Technological University, Bhopal for successful implementation of the research project. Authors are thankful to Prof. A.N Mishra, Department of Pharmacy, M.S. University, Baroda for providing the facility of Cascade Impactor. We also thank Dr. N.K. Mathur, Head, Department of Nuclear Medicines, Jawaharlal Cancer Hospital, Bhopal for *in-vivo* studies.

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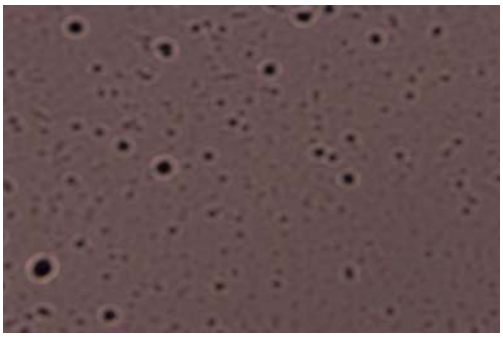
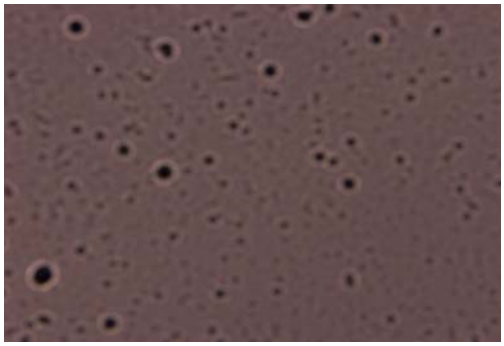
Fig. 1 (a) Photomicrograph showing coarse liposomal dispersion



(b) Photomicrograph showing Purified dispersion before lyophilization



(c) Photomicrograph showing Liposomal dry powder upon immediate Suspension in water showing the initiation of rehydration.

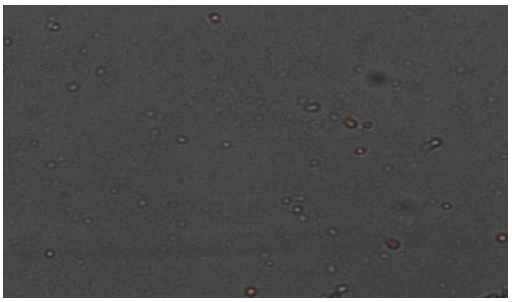


Fig. 2 SEM images of typical particles from formulation DOX XI at different scale bars.

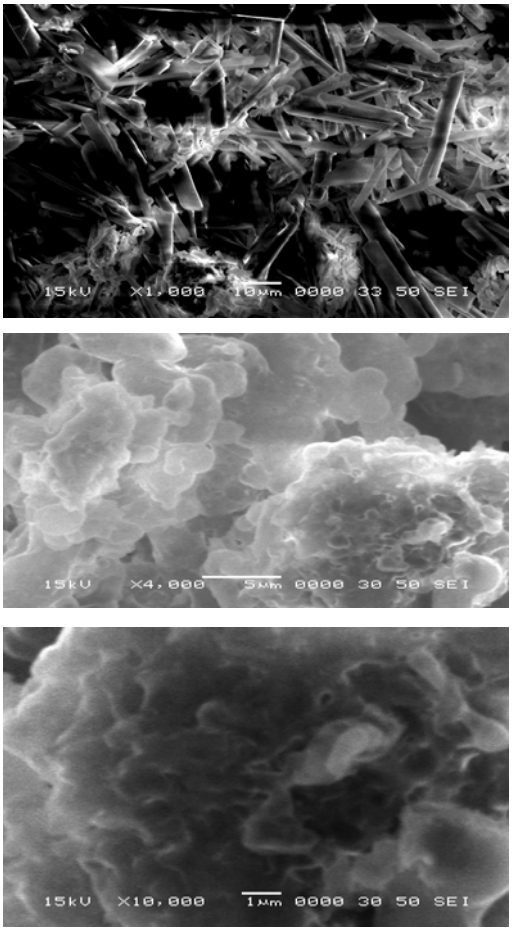


Fig. 3 *In Vitro* Cumulative % drug Release of DOX- XI in PBS saline (pH=7.4) solution.

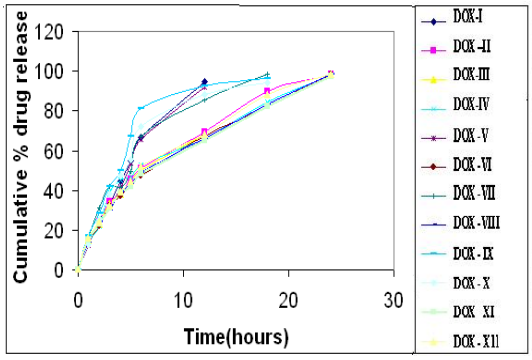


Fig. 4 Higuchi Diffusion Plot.

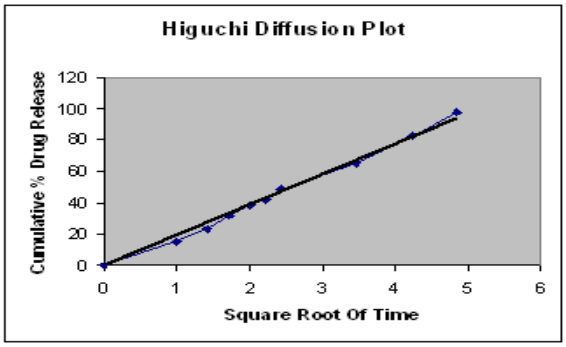
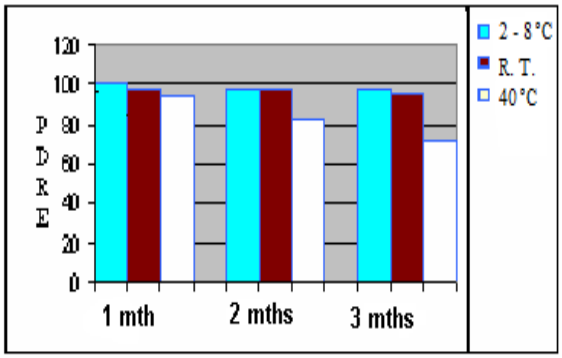


Fig. 5 Effect of storage condition on PDRS of DOX-XI formulation.



R. T. indicates room temperature.

Fig. 6 Dynamic View of the animal given liposomal formulation (DOX – XI).

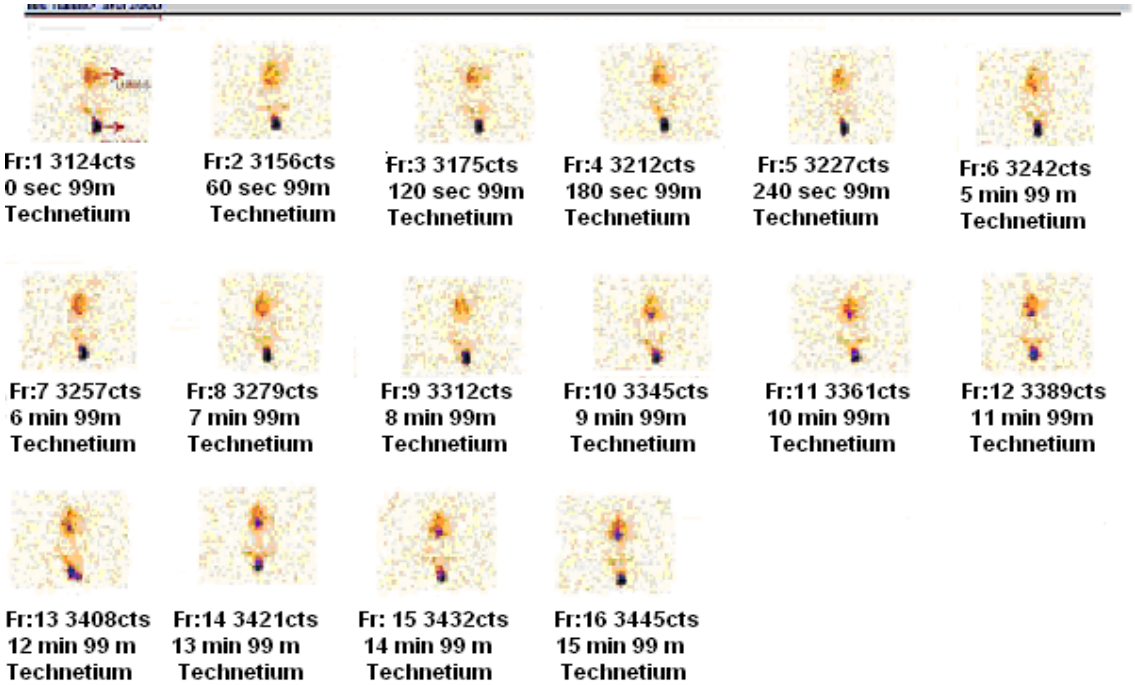


Fig.7 Static view of whole body given liposomal preparation.

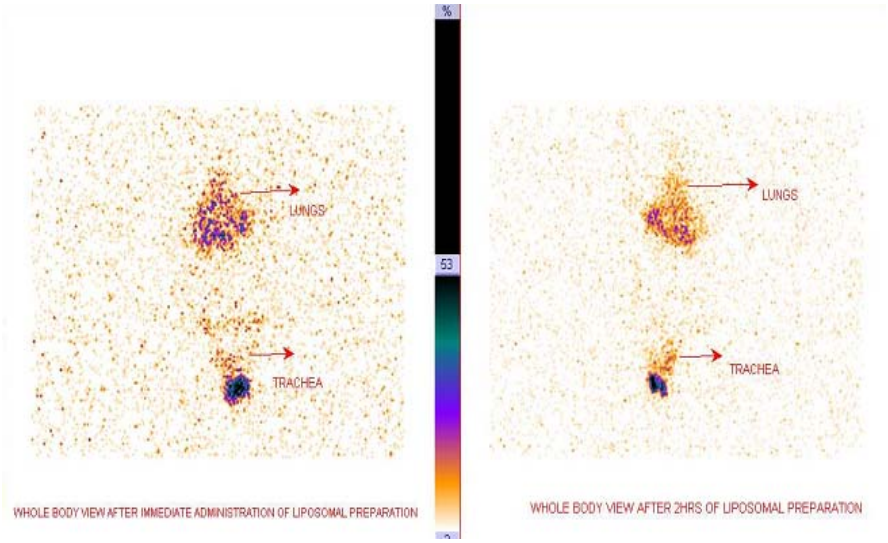


Fig. 8 Static view of whole body given control drug.

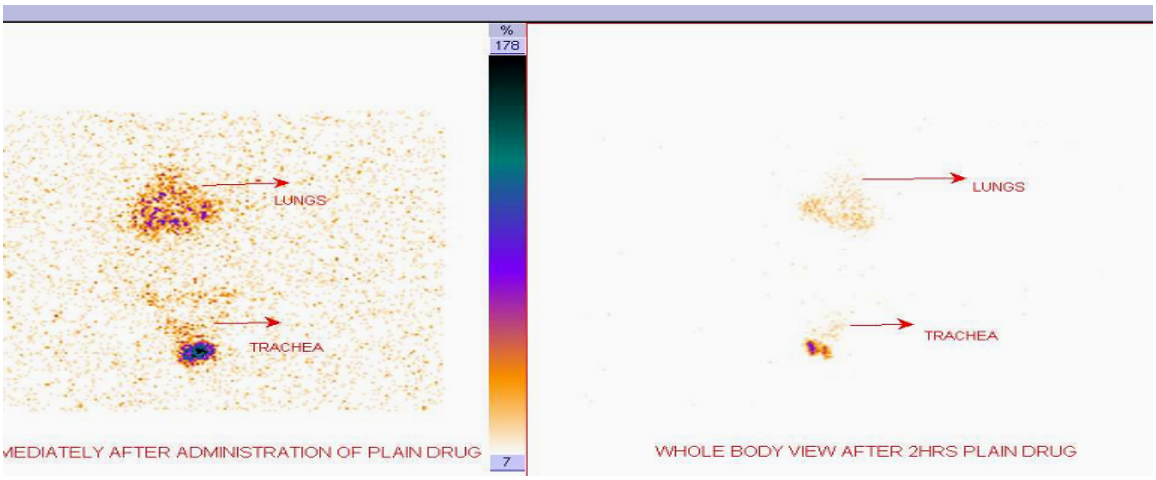


Table 1: Effect of ratio of Soyalecithin to Cholesterol

S.No	Formulation Code	Drug: Lipid	soyalecithin: cholesterol	PDE	PDRE
1.	DOX - I	1:1	1:0	40:12%	55.58%
2.	DOX-II	1:1	3:1	41.23%	60.25%
3.	DOX - III	1:1	2:1	41.64%	60.24%
4.	DOX - IV	1:1	1:1	44.24%	63.12%

PDE indicates percent drug entrapped; PDRE, percent drug remaining entrapped.

Table 2: Effect of Cryoprotectant selected

S.No	Formulation Code	Drug: Lipid	Cryoprotectant	Observation and Inference
1.	DOX - V	1:1	Mannitol	PDRE less and crystallization on storage
2.	DOX-VI	1:1	Sucrose	PDRE comparatively and on crystallization.
3.	DOX - VII	1:1	Lactose	PDRE less
4.	DOX - VIII	1:1	2.5 % w/v PVP coated Mannitol.	PDRE more and on crystallization.

Table 3: Effect of amount of Cryoprotectant selected

S.No.	Formulation Code	Soya lecithin: Cholesterol	Drug: 2.5% w/v PVP coated mannitol	PDE	PDRE
1.	DOX- IX	1:1	1:4	20.13%	45.14%
2.	DOX - X	1:1	1:6	35.21%	43.34%
3.	DOX - XI	1:1	1:8	46.14%	64.23%
4.	DOX - XII	1:1	1:10	44.21%	63.14%

Table IV: *In Vitro* Cumulative % drug Release in PBS saline (pH=7.4) solution.

S. No	Time (Hrs)	Formulation Code											
		DOX-I	DOX-II	DOX-III	DOX-IV	DOX-V	DOX-VI	DOX -VII	DOX -VIII	DOX- IX	DOX- X	DOX-XI	DOX-X11
1.	1	13.34	15.45	15.21	13.21	12.31	14.21	16.25	14.25	17.12	13.12	15.15	16.21
2.	2	25.25	24.45	23.31	22.14	24.65	22.21	31.22	24.12	28.31	25.21	23.12	24.21
3.	3	32.66	34.41	33.24	39.31	31.62	32.22	42.21	30.21	42.21	38.12	31.23	32.33
4.	4	44.42	38.35	38.21	45.21	41.32	37.12	41.25	39.21	50.21	47.12	38.54	39.21
5.	5	54.71	45.62	44.92	46.89	53.69	42.81	49.25	42.81	67.21	55.21	41.87	44.54
6.	6	66.72	51.25	49.91	52.75	65.51	47.57	67.21	49.22	81.34	72.14	48.57	52.14
7.	12	94.54	69.42	68.31	66.23	92.14	67.21	85.62	66.21	92.41	88.14	65.12	68.12
8.	18	-	89.92	88.37	84.42	-	82.36	98.22	83.34	96.21	94.19	82.34	88.21
9.	24		98.45	98.21	98.12	-	98.34	-	98.51	-	-	97.58	98.21

Table V: Effect of liposome: carrier ratio on fine particle fraction.

S.No	Liposome : carrier ratio	FPF
1	1:0	10.22%
2.	1:4	14.23%
3.	1:5	21.69%
4.	1:6	21.70%

Table VI: Characterization of Optimization Liposomal Batch (DOX – XI) formulation with liposome: carrier ratio (1:5) (Mean ± S.D, n=3)

S.No.	Variable studied	Formulation DOX - XI
1.	Mean size of Liposomes (µm) (post sonication)	1.4± 0.2
2.	Volume Median Diameter (VMD)	1.36± 0.13 µm
3.	Tapped Density	0.32± 0.02 g/cm^3
4.	Angle of Repose (θ)	26.1 ± 1.4
5.	Carr's Index	14.26 %
6.	Fine particle Dose (FPD) µg	212.9 ± 7.2
7.	Fine particle Fraction (FPF) %	21.69 ± 1.21
8.	% Emission	72.1± 0.13
9.	% Dispersiblity	62.34± 3.5
10.	Critical Relative Humidity	71.21± 1.21

Table No.VII Regression Coefficients Values for various Kinetic Models

Kinetic Model	r^2
Zero Order	0.7515
First Order	0.9411
Higuchi	0.9924

r^2 indicates regression coefficient.

Table VIII: Effect of storage condition on surface characteristics and colour changes

S.No	Formulation Code	Surface characteristics											
1.	DOX-XI	Initial			1 month			2 months			3 months		
		2-8ºC	RT	40ºC	2-8ºC	RT	40ºC	2-8ºC	RT	40ºC	2-8ºC	RT	40ºC
		-	-	-	-	-	-	-	-	-	-	-	+
- no change + changes i.e; caking occurs													
S.No	Formulation Code	Colour changes											
1.	DOX -XI	Initial			1 month			2 months			3 months		
		2-8ºC	RT	40ºC	2-8ºC	RT	40ºC	2-8ºC	RT	40ºC	2-8ºC	RT	40ºC
		-	-	-	-	-	-	-	-	-	-	-	+

- no change + Changes i.e.; discoloration occurs (Cream color)

Table No IX: Effect of storage condition on Percent Drug Entrapped on Storage (PDRS) DOX – XI Formulation.

Time (Days)	Temperature	PDRS
Initial	2- 8ºC	100
	RT	100
	40C	100
1 month	2- 8ºC	100
	R.T	98.93
	40ºC	94.57
2 months	2- 8ºC	98.53
	RT	97.45
	40ºC	82.31
3 months	2- 8ºC	98.11
	RT	96.12
	40ºC	72.31