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Development and Characterization of liposomal aerosols for improved delivery of etoposide to the lungs

Neelima Salvi[*1,2], S.K. Jain[1] and Neelesh Malviya[2]
1, Department of Pharmaceutical Sciences,
Dr. Hari Singh Gour Vishwavidyalaya, Sagar (M.P.) - India
2, Department of Pharmaceutics, Smriti College of Pharmaceutical Education, Indore (M.P.) - India

Abstract

The present study deals with firstly at preparation, characterization and performance evaluation of Etoposide loaded aerosolized liposomes for their selective presentation to lungs, for the treatment of lung cancer. Secondly, to enhance the delivery of drug to the lungs via site specific targeting. Soya phosphatidyl choline and cholesterol based liposomes were modified by coating them with mannose. The prepared formulations were characterized in vitro for vesicle size distribution and percent drug entrapment. Aerosolization was done by air jet nebulizers. *In-vitro* airways penetration efficiency of the liposomal aerosols was determined by percent dose reaching the peripheral airways; it was recorded 1.4-1.6 times higher as compared to plain drug solution based aerosol. *In-vivo* tissue distribution studies on albino rats suggested the preferential accumulation of mannose coated formulations in the lungs. Higher lung drug concentration was recorded in case of ligand-anchored liposomal aerosols as compared to plain drug solution and plain liposome based aerosols. The drug was estimated in the lung in high concentration even after 24 hr. The drug localization index calculated after 6 hr. was nearly 1.42-4.47 and 4.16 fold respectively for plain, galactose and mannose coated liposomal aerosols as compared to plain drug solution based aerosols. These results suggest that the ligand-anchored liposomal aerosols are not only effective in rapid attainment of high drug concentration in lungs and also maintain the same over prolonged period of time.

Key-words: Liposome, Aerosols, Etoposide

Introduction

Lung cancer grows silently, without showing any symptom. Patients with lung cancer often do not develop symptoms until the cancer is in an advanced stage. Since the majority of lung cancer is diagnosed at a relatively late stage, only 10% of all lung cancer patients are ultimately cured. If the patient cannot be cured by surgery at the time the cancer is found, there is a 50% chance that death will occur in less than one year.

*** Corresponding Author**
E.mail: salvi.neelima@gmail.com

Material and Methods

Material

Etoposide was obtained as a gift sample from United Biotech (P) Ltd., New Delhi, India. Soya phosphatidylcholine (PC), cholesterol (Chol), stearylamine (SA), mannose, sephadex G-50, Triton X-100 and phosphotungstic acid were purchased from Sigma, USA. Propellants P_{11} (trichorofluoromethane) and P_{12} (dichlorodifluoromethane) were obtained from Himalaya Refrigeration Co. Ltd. (Bhopal, India). Chloroform and all other chemicals used were of pure analytical grade and obtained from Qualigens, Mumbai, India.

Preparation of ligand-coated MLVs containing Etoposide

Preparation and optimization of MLVs containing Etoposide

Multilamellar vesicles (MLVs) containing Etoposide were prepared, PC and Chol were dissolved in the minimum amount of chloroform: methanol (2:1) mixture in round bottom flask and then methanolic solution (80 µg ml^{-1}) of Etoposide with minimum amount of DMSO was added to it. PC to Chol (7:3

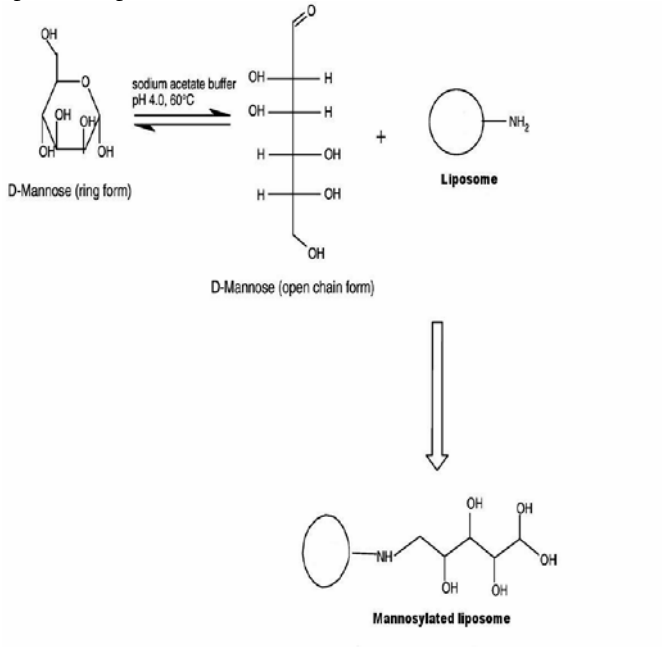
molar ratio) was kept constant, while Etoposide content was varied at different mole percent ratio levels, i.e. (10, 8, 6, 4, 2 and 1 mole% of the total lipids) in different preparations for determining optimum Etoposide content. The organic solvent mixture was removed using a rotary flash evaporator (Stereoglass Rotavap, Italy) under reduced pressure. The dried film was hydrated with 10 ml of PBS (pH 7.4) followed by continuous vortexing of the flask for about an hour to get multi lamellar liposomes. Liposomal suspension was allowed to stand for further 3 to 4 hours in dark at room temperature to allow complete swelling of the vesicles. The suspension was then centrifuged at 2000 rpm for 4 h, and the pellet was resuspended in PBS (pH 7.4).

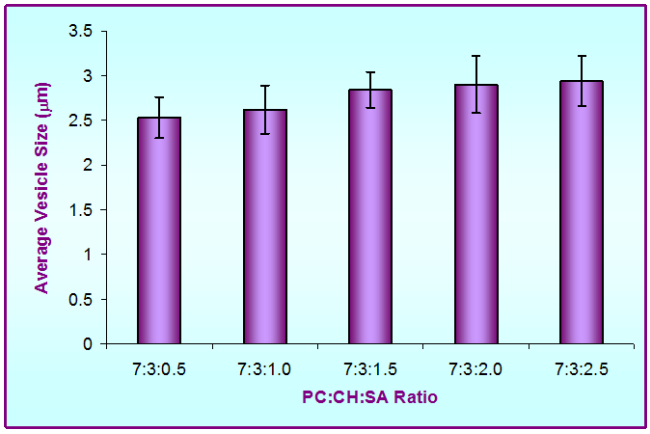
The liposomal formulation were centrifuged through sephadex G-50 mini-column at 2000 rpm for 3 min to remove the free drug and stored in dark at low temperature. The liposomal fraction was added with minimum amount of Triton X-100 (0.1%, v/v), drug content was determined spectrophotometrically at 289 nm and percent drug entrapment was calculated. MLVs with optimum Etoposide to lipid ratio were optimized for optimum PC to Chol ratios (9:1, 8:2, 7:3, 6:4, 5:5 molar ratios) were prepared which was then utilized for preparation of cationic liposomes.

Cationic liposomes containing Stearylamine were also prepared in the same way by dissolving Soya PC, Cholesterol and Stearylamine in minimum quantity of chloroform: methanol (2:1) mixture and further procedure was followed in a similar way as that mentioned for the multilamellar liposomes.

Preparation of mannosylated multilamellar liposomes

The attachment of mannose to Etoposide bearing multi lamellar liposomes was performed using the method described by Jain et al. (2009) with minor modifications. The method involves ring opening of mannose followed by reaction of its aldehyde group with free amino groups present over the surface of prepared liposomes. Mannose was dissolved in 0.1 M sodium acetate buffer (pH 4.0). This solution was then added to liposomes, agitated, and allowed to stand at ambient temperatures for 2 days. The resulting solution was concentrated under vacuum at 50°C. Mannosylated liposomes were purified by dialyzing against double-distilled water in a dialysis tube (12 kDa; Himedia,India) for 24 hours to remove unreacted mannose, salts, and partially mannosylated liposomes followed by lyophilization (Heto Drywinner, Denmark, Germany). The liposomes were characterized by infrared (IR) spectroscopy. IR spectroscopy was carried out using the KBr pellet method after adsorption of a smaller amount of substance on KBr using a Perkin-Elmer IR spectroscope Waltham, Massachusetts.





Characterization of mannosylated multilamellar liposomes

Various liposomal formulations were evaluated for vesicle size and its distribution by laser diffraction particle size analyzer (Cilas, 1064 L, France) and vesicle shape by Morgani 268 Transmission Electron Microscope (TEM). Phosphotungstic acid (1%) was used as a negative stain. Carbon-coated samples were placed over a copper grid and subjected to TEM analysis. Vesicle count was performed by optical microscopy.

Preparation of aerosolized packed liposomes

Liposome aerosols were produced from 10 mL of liposome formulation. 10 mL of the prepared

liposome formulation was filled into the container and the nebulized product was produced by air jet nebulizer. The air jet nebulizer was fixed over the neck of the container and the nebulized product was produced by the actuation of the nebulizer.

Characterization of liposomal aerosols

Various liposome aerosols formulations (PD-plain drug solution, PL_1-uncoupled liposomal aerosols, PL_2-mannose coupled liposomal aerosols) were characterized for parameters like appearance, leak test, internal pressure, amount discharged per actuation, spray pattern, penetration efficiency and vesicle size. The characterization tests were performed as follows:

Appearance of Mist

Appearance of the various liposomal aerosols was observed macroscopically. All the liposomal aerosols were found to be homogeneous.

Leak test and Internal pressure

Each container was completely immersed into a hot water bath maintained at 50°±1°C for 5 min and examined for leakage. Internal pressure measured with the help of a pressure gauge.

Aerosol valve discharged rate and Spray Pattern

An aerosol product of known weight was allowed to discharge the contents for three actuations into a calibrated single stage liquid. Aerosol valve discharge rate was determined with the weight difference of the aerosol container before and after actuations (Jain et al., 2008). Aliquots of receptor fluid from single stage liquid inpinger were collected and average amount of Etoposide delivered per actuation through different formulations was determined. The spray pattern area was based on the color produced on impingement of the spray, over a piece of paper pre-trated with methylene blue-talc mixture, kept at 15 cm distance.

Airways penetration efficiency

The term penetration efficiency from the valve is used to express relative efficiency of the developed aerosols measured as percentage of dose that eventually arrives at lungs. From mouthpiece of the aerosol pack three actuations were fired. The amount of Etoposide deposited at different parts (filter, bronchi, and trachea) was determined.

Results and Discussion

Cationic Multilamellar vesicles were prepared and the optimized formulation was utilized further to produce mannose coupled liposomal formulation. Here the molecule of mannose is liganded to the surface of cationic liposomes through chemical linkage.

Different formulation and the process variables viz. PC: CH ratio, stearylamine to PC:CH ratio and sonication time were optimized to get small multilamellar liposomes with maximum drug entrapment efficiency and maximum vesicle count. The PC:CH ratio was optimized on the basis of vesicle size and drug entrapment efficiency.

Similarly, the concentration of Stearylamine and sonication time were optimized by varying the PC: CH: SA ratio from 7:3:0.5 to 7:3:2. and sonication time from 2 to 8 min to get small vesicles with high drug entrapment efficiency. The prepared liposomes were characterized for vesicle size using laser diffraction based particle size analyzer (Cilas, 1064 L, France) and drug entrapment efficiency.

Table 1: Characterization of mannosylated liposomes

S. No.	Formulation Code	Ligand: lipid ratio(w/w)	Average size (nm)	Zeta Potential (mV)
1.	$PL\text{-}M_1$	0.10:1	145±0.27	8.0±0.26
2.	**$PL\text{-}M_2$***	**0.20:1**	**174±0.40**	**5.2±0.12**
3.	$PL\text{-}M_3$	0.30:1	130±0.32	-1.3±0.10

*Data are shown as mean ± SD (n= 3)

Entrapment efficiency was determined and expressed as the ratio of experimentally measured amount of drug in dispersion and initial amount used for entrapment. Vesicles (free of unentrapped drug) were lysed by adding 1.0 mL of 0.1% (v/v) Triton X-100, and liberated contents were analyzed for Etoposide content spectrophotometrically at 289 nm.

The zeta potential of samples was measured using Zetasizer 3000 HS (DTS Ver. 4.10, Malvern Instruments, England). Mannosylated liposomes were assessed for *in vitro* ligand-specific activity by mannose-binding concanavalin A (Con A) as reported by Copland et al., (2003) with slight modification.

The in-vitro drug release profile of Etoposide was studied for cationic and coupled liposomal formulations.

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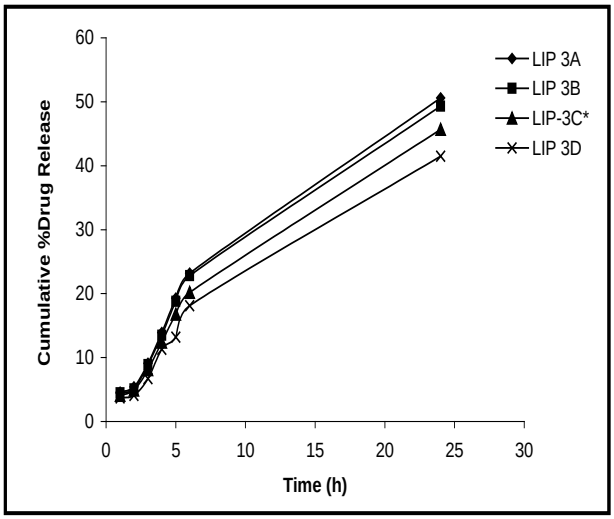
Table 2: Characteristics of Liposome aerosols

S.No.	Parameters	Formulation Code		
		PD	PL_1	PL_2
1.	Appearance	Homo.	Homo.	Homo.
2.	Leak Test	Passed	Passed	Passed
3.	Internal Pressure (psi)	20	32	34
4.	Amount discharge/ actuation (mg)	79.8	88.5	98.3
5.	Spray Pattern area at 15 cm (cm^2)	8.1	15.2	16.1
6.	Penetration Efficiency	37.2	56.4	57.2



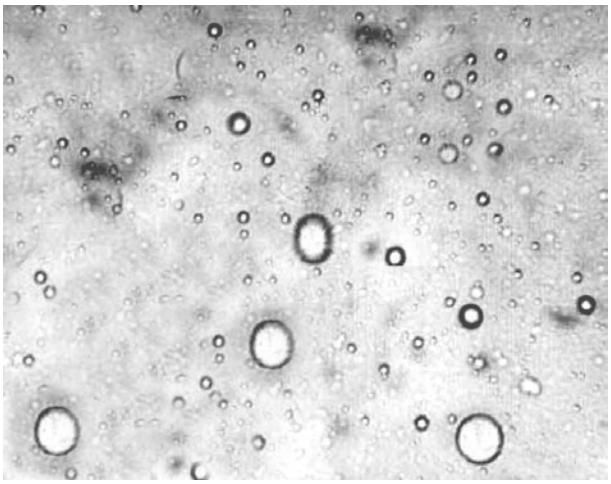


Fig. 1: Prepared formulation

Conclusion

The results of this study suggest that the modification of the liposome surface by the specific ligand mannose improvised their stability and administration of these developed formulations by nebulization aerosols, could affect target drug delivery to the lungs. This leads to better targeting of the anticancer drugs. Furthermore, the encapsulation of anticancer drugs in ligand coupled liposomes resulted in the reduction of toxic side effects due to non specific low level distribution of drug to other organs.

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