Research Article

COMPARATIVE EFFICIENCY OF FORMULATION TECHNIQUES FOR DEVELOPMENT OF SALBUTAMOL SULPHATE LOADED LIPOSOMES

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ABSTRACT

Present study investigated most efficient technique for the development of salbutamol sulphate loaded liposomes. Salbutamol sulphate is a selective β_2 adrenoceptor agonist widely used in the treatment of bronchial asthma, chronic bronchitis and emphysema. Our work is mainly focused on *in vitro* studies of liposomal formulation encapsulated with salbutamol sulphate which may have high drug entrapment, sustained drug release and effective vesicle size. Drug loaded liposomes were prepared by ethanol injection and thin film hydration techniques using soyaphosphatidyl choline and cholesterol in various molar ratios. Liposomes were evaluated for vesicle size, entrapment efficiency, transmission electron microscopy, zeta potential and drug release parameters. The particle size of drug loaded liposomes prepared by ethanol injection technique was 423-527 nm whereas it was 160-174.2 nm when prepared by thin film hydration technique. Zeta potential of liposomes prepared by thin film hydration technique was enough to stabilize over six months. Optimized batches of salbutamol sulphate loaded liposomal formulations prepared by ethanol injection and thin film hydration technique have shown drug release as 94.59 % and 88.03 %, respectively. On the basis of observed average particle size, percent drug entrapment and drug release profiles, thin film hydration technique was emerged as superior technique over ethanol injection technique.

Keywords: Cholesterol, Drug release, Ethanol injection, Soyaphosphatidyl choline, thin film hydration.

INTRODUCTION

A liposome is a microscopic vesicle consisting of an aqueous core enclosed in one or more phospholipid layers, used to convey vaccines, drugs, enzymes, or other substances to target cells or organs. Liposomes are bilayered structures made of amphipathic phospholipids or cholesterol which spontaneously form closed structures when hydrated in aqueous solutions. In 1961, Professor Bangham, observed the formation of little lipid droplets when phospholipids were placed in water. The hydrated phospholipid film appeared as vesicles in the form of concentric lipid bilayers circumscribing aqueous interior¹. These vesicles were later named as "Liposomes". Scientists from different disciplines have intensively studied liposomes to investigate its potential as a carrier for therapeutic agents. Liposomes are colloidal spherical particles with a membrane composed of phospholipids and cholesterol bilayer². They are regarded as an inert carrier system having lipophilic tails oriented to the middle and polar head directed to inside of the vesicle and its outer surface. These vesicles are able to encapsulate both hydrophilic and lipophilic active ingredient in their structure³. Hydrophilic interactions of the lipid head groups of phospholipids with water results in the formation of multi lamellar and unilamellar vesicles. When materials are encapsulated in a liposome, the oil soluble actives sequester in the phospholipid bilayer, while the water-soluble actives reside in the water core. Most liposome formulations approved for human use contain phosphatidyl choline with fatty acyl chains of varying lengths and degree of saturation as a major membrane building block. A fraction of cholesterol is often included in the lipid formulation to modulate rigidity and to reduce serum induced instability

caused by the binding of serum protein to the liposome membrane⁴. The size of a liposome ranges from some 20 nm up to several micrometers and are composed of one or several concentric membranes, each with a thickness of about 4 nm. Liposomal encapsulation of a drug improves pharmacokinetic properties of a drug, targeting the drug to particular organs and enhances the efficacy of the encapsulated drug⁵. The formulation of an appropriate liposomal system as a carrier for a given drug is dependent on the type of the lipid used and the technique of preparation⁶. According to their size they are known as small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV). If more bilayers are present they are referred to as multi lamellar vesicles (MLV). Many types of liposomal products can be formulated on the basis of lipid composition technique of preparations and the nature of the encapsulated agents. The ideal drug candidates for liposomal encapsulation are those having potent pharmacological action with either high lipid or water solubility. Water soluble drug is encapsulated within the aqueous compartment and its concentration in the liposomal product depends on volume of the entrapped water. Lipophilic drug usually bound to lipid bilayer or dissolves in the lipid phase and more likely to remain encapsulated during storage due to its partition coefficient. Since the lipophilic drug is associated with the lipid bilayers it would not leach out readily to the external water phase. Generally the encapsulation efficiency is higher for lipophilic drugs than hydrophilic drugs⁷. Salbutamol sulphate (SS) is a selective β_2 adrenoceptor agonist widely used in the treatment of bronchial asthma, chronic bronchitis and emphysema⁸. It has been reported that the encapsulation of SS into liposomes significantly improves its therapeutic index by altering pharmacokinetics and pharmacodynamics⁹.

In this study the SS encapsulated liposomes were prepared by ethanol injection and thin film hydration techniques. In addition to numerous advantages of liposomes as carrier to deliver SS over its free form, our work is mainly focused on *in vitro* studies of liposome-encapsulated SS for evaluation of best technique of SS liposome formulation which may have high drug entrapment, sustained drug release and vesicle size.

MATERIALS AND METHODS

Salbutamol sulphate was a gift sample from Cipla Pharmaceuticals Pvt. Ltd. Mumbai, India. Soyaphosphatidyl choline (SPC) was gift sample from Lipoid Germany. Cholesterol was purchased from Qualigens Fine Chemicals, Mumbai, India. Sephadex® G50 was purchased from Sigma Aldrich, India. Stearic acid was purchased from Research Lab, Mumbai, India. Ethanol (AR), methanol and chloroform were purchased from Merck, India.

Preparation of salbutamol sulphate liposomes

SS liposomes were prepared by ethanol injection and thin film hydration technique. The compositions of formulation with varying amounts of lipid and cholesterol are given in Table 1.

Ethanol injection technique

It has been reported that ethanol injection technique is suitable for preparing SUV or LUV³. Different molar ratios of SPC: CH was dissolved in 2 ml ethanol and 10 mg stearic acid. Ethanolic solution was kept in sonicator bath for about 2 minutes to dissolve entire contents in ethanol with the end point as clear solution. This solution was rapidly injected in aqueous 10 ml SS solution (0.1 %w/v) with continuous stirring at 500 rpm using Teflon coated magnetic bead. The ethanol was removed from solution by evaporation with continuous stirring for 45 minutes¹⁰.

Thin film hydration technique

Preparation of SS liposomes by thin film hydration technique was performed using various molar ratios of SPC: CH. These two components were dissolved in chloroform to obtain 60 mg/mL lipid phase. This solution was transferred to rotary flask and attached to a rotary evaporator under nitrogen vacuum for 1 h, while the flask was immersed in water bath at 40°C rotated at 30 rpm. The thin film formed was hydrated by adding phosphate buffer saline (PBS) solution (pH 6.4) containing 0.1 %w/v SS followed by hand shaking for 10 minutes to yield a liposome dispersion having lipid concentration 10 mg/ml. The liposomes were sonicated for 15 minutes to reduce their size to form small and uniform vesicles. The dispersion was left undisturbed at room temperature for 1–2 h for complete vesicle formation followed by storage at 4°C in an inert atmosphere for 24 h¹¹.

Particle size analysis

Mean vesicle size and size distribution of empty and drug-loaded liposomes was determined using Malvern 2000 SM which works on the mechanism of photon correlation spectroscopy. Particle size analysis (n = 3) was carried out for 100 sec at room temperature by keeping angle of detection at 90°.

Drug entrapment efficiency

Drug entrapment was determined by mini-column centrifugation technique. In brief, 10 % w/v aqueous solution of Sephadex® G50 was prepared and kept undisturbed for 48 h for complete swelling. The mini-column was prepared by inserting Whatman filter pad in 1 mL syringe. The swollen Sephadex® was added carefully to avoid air entrapment in the column. Excess water was removed by centrifugation of the column at 2000 rpm for 3 minutes. Accurately measured 100 µL liposomal SS suspension was slowly added on column and centrifuged at 5000 rpm for 3 minutes. This procedure was repeated once again by replacing suspension with 100 mL water. The remaining free drug bound to the gel, while liposomes passed through the pores within the gel and were collected from the first and second stage of centrifugation. The eluted liposomes were ruptured using ethanol. The percent encapsulation was determined using UV-Visible spectrophotometer as total amount of SS present in 100 µL of liposomes using eq. (1). The technique was validated using free drug instead of liposomal dispersion. The free drug was analyzed by UV-Visible spectrophotometer^{12,13}.

$$\text{Encapsulation efficiency} = (Q_e / Q_i) \times 100 \quad \dots (1)$$

Where Q_e is the amount of encapsulated salbutamol sulphate and Q_i is the amount of SS in 100 µL of liposomal suspension

Transmission electron microscopy studies

Vesicle morphology of liposomes obtained by both techniques was analyzed using Hitachi S-7500 transmission electron microscope (TEM). The liposomes were dispersed in water and a drop of the dispersion was placed on a 200-mesh carbon coated copper grid. The photographs were taken at 30,000 × magnification and 100 Kv¹¹.

Zeta potential

The formulations obtained by two techniques were optimized on the basis of drug entrapment efficiency and vesicle size. Zeta potential of optimized liposomes obtained by ethanol injection and thin film hydration technique was determined using Zeta sizer 300 HSA (Malvern Instruments, Malvern, UK).

Drug release study

In-vitro drug release from optimized drug loaded liposomes and pure drug was evaluated in a customized and validated diffusion cell across cellophane membrane (12,000 MCO) for 12 h in 200 ml of PBS solution (pH 7.4) as diffusion medium. The rate and extent of drug release from different samples was compared by placing 5 ml (≈ 5 mg) of each sample in donor compartment, separately, and stirred at 50 rpm. The diffusion medium in receptor compartment was stirred at 100 rpm. Accurately 3 ml aliquot was withdrawn from the receptor compartment at definite time intervals and equal amount of fresh medium was replaced in to the receptor compartment. Samples were assayed to determine amount of drug released by UV spectrophotometer. Per cent drug diffused was determined by substituting data in the formula given in equation (2)¹⁴.

$$\text{Percent drug diffused} = \frac{C_r V_r}{C_d V_d} \quad \dots (2)$$

Where, C_r is concentration of drug in receptor compartment, V_r is volume of the receptor compartment; C_d is concentration of drug in donor compartment and V_d is volume of donor compartment

Table 1: Composition of salbutamol sulphate loaded liposomes

Formulation code	SPC: CH molar ratio	Drug (mg)	Stearic acid (mg)
L1	3:1	10	10
L2	1.5:1	10	10
L3	1:1	10	10
L4	3.5:1	10	10
L5	3.5:2	10	10
L6	3.5:3	10	10
L7	4:1	10	10
L8	2:1	10	10
L9	4:3	10	10

Table 2: Effect of formulation techniques on physical characteristics of liposomes

Formulation code	Vesicle size (nm)		%Drug entrapment	
	Thin film hydration method	Ethanol injection method	Thin film hydration method	Ethanol injection method
L1	160.0 ± 0.041	423.2 ± 0.023	77.37 ± 1.131	45.64 ± 0.44
L2	165.4 ± 0.085	427.2 ± 0.066	75.52 ± 0.932	53.26 ± 0.41
L3	167.2 ± 0.17	449.6 ± 0.012	80.68 ± 0.743	59.04 ± 0.89
L4	162.8 ± 0.032	434 ± 0.12	77.17 ± 1.242	48.70 ± 0.72
L5	168.6 ± 0.016	438 ± 0.037	73.22 ± 1.481	52.29 ± 0.52
L6	170.2 ± 0.085	483.4 ± 0.21	69.98 ± 0.639	48.07 ± 0.66
L7	168.8 ± 0.14	428.0 ± 0.058	80.05 ± 0.589	62.34 ± 0.43
L8	170.2 ± 0.062	449.1 ± 0.045	72.79 ± 0.842	50.21 ± 0.44
L9	174.2 ± 0.031	527 ± 0.091	71.42 ± 1.172	57.93 ± 0.36

*(Mean ± S.D., n = 3)

Figure 1: Particle size distribution in liposomal formulation (L7) containing SPC: CH (1:1 molar ratio) prepared by ethanol injection technique

Figure 2: Particle size distribution in L7 formulation of film hydration technique

Figure 3: TEM image of L3 formulation of ethanol injection technique

Figure 4: TEM image of L3 formulation of thin film hydration technique

Figure 5: Zeta potential distribution of L7 of ethanol injection technique

Figure 6: Zeta potential distribution of L3 formulation prepared by thin film hydration technique

Figure 7: *In vitro* drug released from salbutamol sulphate loaded liposomes

Figure 8: Extent of drug leakage from optimized L3 formulation of liposome at different storage temperature

Stability studies

The ability of the liposomes to retain the drug was assessed by storing the liposomal suspension at 4-8°C, 25 ± 2°C and 37 ± 2°C for six months¹⁵. Stability study samples were withdrawn periodically and analysed to determine drug content by UV-visible spectroscopy.

RESULTS AND DISCUSSION

Particle size analysis

The particle size of drug loaded liposomes prepared by ethanol injection technique was 423-527 nm whereas it was 160-174.2 nm when prepared by thin film hydration technique (Table 2). The smallest particle size 423 nm for formulation L7 containing SPC: CH at 4:1 was obtained when prepared by ethanol injection technique (Figure 1). On the contrary it was 167.2 nm for formulation L3 containing SPC: CH at 1:1 when prepared by film hydration technique (Figure 2). Overall smaller particles were obtained when thin film hydration technique was used. Probably smaller particles obtained may be due to the gravitational force acted upon film when flask was rotated.

Drug entrapment

The percent drug entrapment in liposomes by ethanol injection and thin film hydration techniques was 45.64 % – 62.34 % and 69.98 % – 80.68 %, respectively (Table 2). Overall drug entrapment in liposomes prepared by thin film hydration technique was higher. Increasing SPC: CH molar

ratio only slightly increased SS entrapment in liposomes prepared by both these techniques. The effect was more pronounced for liposomes prepared thin film hydration technique. In this technique there was a trend towards maximum entrapment efficiency at a SPC: CH molar ratio of 1:1. Improved drug entrapment attributed to cholesterol may be because of increased stability of the liposomal membrane during hydration. It has also been reported that cholesterol increases rigidity of the bilayer^{16,17} and decreases permeability of liposomes¹⁸. Formulations were optimized on the basis of results of particle size analysis and drug entrapment. The optimized formulations used for further studies were L7 and L3 prepared by ethanol injection technique and thin film hydration technique, respectively.

Transmission electron microscopy studies

Morphologies of the formulation L3 and L7 in PBS solution, pH 6.4, were observed by TEM. Drug loaded liposomes prepared by thin film hydration technique showed smooth and spherical vesicles (Figure 4). Diameters of vesicles measured by TEM of formulation L3 prepared by thin film hydration technique are smaller than formulation L7 prepared by ethanol injection technique because film form during rotary evaporation was slowly hydrated. No vesicles were clearly detected by TEM in formulation L7 (Figure 3).

Zeta potential

A zeta potential (ζ) $> \pm 30$ mV is essential for effective stability and to inhibit aggregation.¹⁶ In the present study the ζ for drug loaded liposome formulations L7 and L3 prepared by ethanol injection technique and thin film hydration technique was 8.96 mV (Figure 5) and 9.74 mV (Figure 6), respectively. The results of ζ values of formulations under study showed sufficient charge to inhibit aggregation of vesicles. Highest ζ exhibited for formulation prepared by thin film hydration technique indicates greater stability^{19,20}.

Drug release study

The drug release from liposome in PBS solution, pH 7.4, was studied using diffusion cell. Drug release after 12 h from liposomes prepared by ethanol injection (L7) and by thin film hydration technique (L3), shown in Figure 7, was 94.59 % and 88.03 %, respectively. However, it was 94.63 % within 4 h for pure drug. Liposomes prepared by thin film hydration technique (L3), sustained drug release for more duration compared to (L7) liposomes prepared by ethanol injection technique and that of pure drug.

Stability Studies

The optimized drug loaded liposomal formulation (L3) prepared by thin film hydration technique was tested for its physical and chemical stability by storing for 6 months at 3 different storage conditions as described earlier. The results of stability study indicating % drug leakage is shown in Figure 8. There were no significant changes in percentage drug content for the formulations stored at 4-8°C and $25 \pm 2^\circ\text{C}$ but considerable reduction in drug content was observed for formulation stored at $37 \pm 2^\circ\text{C}$.

CONCLUSION

An attempt has been made to comparatively evaluate efficiency of formulation techniques for development of salbutamol sulphate loaded liposomes. Studies indicate successful preparation of salbutamol sulphate liposomes with the potential for sustained drug release. The highest entrapment efficiency was achieved with thin film hydration technique, prepared with a lipid: cholesterol molar ratio of 1:1, using phosphate buffer saline solution of pH 6.4 as a hydration medium for the lipid film. Presence of optimum amount of cholesterol improved drug entrapment efficiency may be because of increased stability of the liposomal membrane by increasing rigidity of the bi layer and decreasing permeability of liposomes. Zeta potential of liposomes prepared by thin film hydration technique was enough to avoid particle aggregation. The stability results revealed stability of charged liposomes when stored at room temperature as diluted aqueous dispersions in terms of physical stability. Results of particle size analysis, percent drug entrapment and drug release indicate superiority of thin film hydration technique.

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