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Preparation and evaluation of a liposomal formulation of sodium cromoglicate

M.S. Nagarsenker *, V.Y. Londhe

Department of Pharmaceutics, Bombay College of Pharmacy, Kalina, Santacruz (E), Mumbai 400098, India

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Abstract

Sodium cromoglicate (SCG) is given by inhalation in prophylactic control of asthma. It was encapsulated in liposomes with a view to improve utilization of the drug when given via pulmonary route. The liposomes were characterized for encapsulation efficiency, shape, size and release rate. Liposomal dispersions were freeze-dried using a cryoprotectant. Freeze-dried liposomal dispersion retained 60% of drug upon reconstitution but increase in size of liposomes was noted. Liposomes exhibited good keeping properties when stored at 4 °C. In vivo performance of liposomal SCG was evaluated in sensitized guinea pigs. In one of the studies, differential leukocyte count and total leukocyte count in bronchoalveolar lavage fluid was measured. Liposomal dispersion showed significant inhibition of influx of neutrophils as compared with drug solution at 24 h. However, in the second study, when recovery period required by animal to revert back to normal respiratory pattern from the onset or preconvulsion time was measured, no significant difference was found between drug solution and liposomal dispersion when administered 2 h before allergen challenge.

Keywords: Sodium cromoglicate; Liposomes; Characterization; Inhibition of neutrophil influx

1. Introduction

Inhalation therapy has advantage of rapid onset of action and requirement of small doses for effective therapy. It reduces exposure of a drug to systemic circulation and minimizes adverse effects. Although the onset of action is very rapid, the duration of action is generally short lived as the drug can be quickly removed from the lungs through various clearance mechanisms. Therefore, most patients required frequent dosing. Sodium cromoglicate (SCG) is used for prophylactic control of asthma. It is poorly absorbed from gastrointestinal tract with a reported bioavailability of 1%. It is rapidly absorbed from the lungs; however, elimination half-life is 80 min following inhalation, which necessitates four to eight times administration daily (Parfitt, 1999).

Liposome delivery system has potential to prolong the effect of the drug by preventing rapid clearance and metabolism of drug by lung. One of

* Corresponding author. Tel.: +91-22-612-6284; fax: +91-22-610-0935
E-mail address: mnagarsenker@hotmail.com (M.S. Nagarsenker).

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the major advantages of liposomes over other colloidal carriers of the drug for the lung is that they can be prepared from endogenous materials. In the first study, Juliano and McCullough (1980) reported persistence of liposomal β-cytosin arabinoside in lungs with $t_{1/2}$ of 8 h as compared with 'free' drug which rapidly cleared from lungs ($t_{1/2}$ = 40 min). Taylor et al. reported encapsulation of sodium cromoglycate in liposomes composed of EPC/cholesterol and DPPC/cholesterol in 1:1 proportion and stability of vesicles to jet nebulization process (Taylor et al., 1990a). In view of this information, with the objective to improve utilization of the drug when given via pulmonary route, SCG was encapsulated in liposomes. This paper discusses characterization of liposomes, freeze-drying of liposomal formulation, leakage on storage and in vivo evaluation.

2. Materials and methods

2.1. Materials

SCG was obtained as a gift sample from Unique Chemicals and Cipla Ltd. Mumbai. Phospholipon 90 and Phospholipon 90H were obtained as gift samples from Nattermann Phospholipid GmBH, Germany. Cholesterol and egg albumin was purchased from Qualigens, Mumbai and Himedia Laboratories, Mumbai. All other reagents were of analytical grade. Distilled water was used throughout this study.

2.2. Methods

Aqueous liposomal formulations were prepared by conventional lipid film hydration method.

2.2.1. Preparation of empty and drug loaded liposomal dispersions

A mixture of phospholipids and cholesterol (1:1) in chloroform was taken in round bottom flask with glass beads. A thin film was formed on the inner side of round bottom flask and on the surface of glass beads by evaporating chloroform under vacuum in rotary flash evaporator at 40 °C. The required amount of phosphate buffer saline (PBS), pH 7.4 or drug solution (10 mg/ml in PBS) was added above phase transition temperature and the flask was maintained at that temperature for 1 h to anneal liposome structures. Further the flasks were shaken for 5 h with intermittent sonication using a bath sonicator.

2.3. Characterization of liposomal dispersion

2.3.1. Determination of encapsulation efficiency

The liposomal dispersion was suitably diluted with buffer and centrifuged at 15 000 rpm for 30 min in R-24 research centrifuge (Remi, India). The supernatant was analyzed for drug content at 327 nm on UV-160A UV–vis spectrophotometer (Shimadzu, USA). Knowledge of total drug in the preparation allowed the amount of the drug associated with the liposomes to be calculated by difference. Encapsulation efficiency (EE) was expressed as mg of SCG entrapped per mM of lipid (mg/mM).

2.3.2. Particle size measurement

The mean particle size and size distribution of liposomal dispersions were studied using SALD-2001 laser counter (Shimadzu, USA). The sample is put in the receptacle of the sampler unit for dispersion and delivery to the flow cell. The collimated laser beam is made incident to the sample particles suspended in the liquid flowing in the flow cell. The intensity signals of the diffracted/scattered light are processed into particle size distribution.

2.3.3. Transmission electron microscopy

A small aliquot of the liposomal dispersion was negatively stained using phototungstic acid (1% w/v). The samples were examined using TM-101 transmission electron microscope (Zeiss, Germany) employing an accelerating voltage of 80 K eV.

2.3.4. In vitro release studies

In vitro drug release from the liposomal dispersions was determined by using dialysis bags (Sigma, USA). The liposomal dispersion was suitably diluted with PBS and centrifuged at 15 000 rpm for 30 min in a R-24 (Remi, India)

research centrifuge. The pellet formed after centrifugation was resuspended in PBS and dispersion was transferred to the dialysis bag, which was tied to the paddle of the dissolution apparatus, Programmable Tablet Dissolution Tester (USP XXI/XXII) TDT-06P (Electrolab, India), which was lowered into a 100 ml beaker containing 50 ml PBS (pH 7.4) as dissolution medium. The contents of the beaker were stirred at 50 rpm at the temperature of 37 ± 1 °C throughout the experiment. Five milliliter samples were withdrawn periodically at the end of 10, 20, 30, 45, 60 min, every hour thereafter for 6 h and finally at 24 h. Every withdrawal was followed by replacement with fresh medium and the samples were analyzed spectrophotometrically using a UV-160A UV–vis spectrophotometer at 327 nm against the samples withdrawn at respective time interval from empty liposomal dispersion treated in a similar manner. In vitro release of plain drug solution of same concentration as in liposomal dispersion was also studied.

2.3.5. Stability of the liposomal dispersions

The empty and SCG loaded liposomal dispersions were stored at 4, 25 and 45 °C for 1 month and were followed by visual and microscopic observation. EE was determined at the end of 15 and 30 days from the date of preparation by using specific, validated HPLC method. HPLC was carried out using Lichrosphere® 100 C-18 (5 μm, 4.6 mm × 22 cm) column connected to Jasco PU-980 Intelligent HPLC Pump equipped with Jasco UV-975 Intelligent UV–vis detector and Spectra-physics SP-4270 integrator. The mobile phase used consisted of 0.0025 M octane sulfonic acid sodium salt monohydrate in acetic acid (1:100) and methanol, acetonitrile (3:2) mixed together in the proportion 8:2 (Segall et al., 1996). Liposomal dispersion was suitably diluted with 10 ml PBS and centrifuged at 15 000 rpm for 30 min in a R-24 research centrifuge (Remi, India). The supernatant was diluted appropriately with mobile phase. The final solution was injected onto the HPLC column in duplicate and the mean peak area was calculated at detection wavelength 325 nm. The content of SCG was then computed from the respective standard curve. This experiment was carried out in duplicate for each sampling point. Particle size distribution of liposomal dispersion stored at 4 °C was studied after 2 months.

2.3.6. Freeze-drying of liposomal dispersion

Liposomal dispersions were freeze-dried using 1:1 ratio on weight basis of trehalose and lipid. Liposomal dispersions were prepared by hydrating a dry lipid film with aqueous phase containing 43.197 mg/ml of trehalose. Non-encapsulated SCG was removed by centrifugation at 15 000 rpm for 30 min using a R-24 research centrifuge. The pellet was resuspended in aqueous solution of trehalose in 1:1 volume ratio. The mixture was frozen immediately at −40 °C. Freeze-drying was performed in EF03 freeze drier (Edwards, UK) till a dry powder was obtained. The whole drying procedure took 16 h.

The percentage of encapsulated drug (EE) was calculated as fraction of drug in a pellet expressed as percentage of total drug content. The percentage of entrapped drug was determined before ($\%\text{Encaps}_{(0)}$) and after freeze-drying (%Encaps) so that the leakage of the drug was expressed as the percentage of release as follows,

$$\%\text{Release} = \frac{(\%\text{Encaps}_{(0)} - \%\text{Encaps})}{(\%\text{Encaps}_{(0)})} \times 100$$

Empty and drug loaded liposomal dispersions were studied for particle size distribution and transmission electron microscopy before and after freeze-drying. For optical microscopy study of liposomal dispersions, 300–500 vesicles were examined.

2.3.7. In vivo study

This study was carried out in guinea pigs of English strain and of either sex weighing 450–550 g.

2.3.7.1. Method based on cellular leukocyte infiltration (Hutson et al., 1988).

2.3.7.1.1. Method of sensitization and challenge. Guinea pigs were actively sensitized by exposing them for 3 min to aerosolized egg albumin, 10 mg/ml in 0.9% saline. Aerosol was generated in a rectangular perspex chamber of 4.5 l using an

atomizer via a compressed air line operating at a pressure of 10 psi and the animals were placed in the chamber for required time. This procedure was repeated 7 days later. One week later the animals were challenged by inhalation of aerosolized egg albumin, 20 mg/ml, under cover of the histamine H_1-receptor antagonist mepyramine maleate (10 mg/kg) injected intraperitoneally 30 min beforehand.

The animals were divided into four groups of five animals each. Group I was not sensitized but exposed to allergen challenge, Sham Challenged Control (Tarayre et al., 1991). Group II was positive control in which animals were sensitized and exposed to allergen challenge. Aerosolized SCG solution and liposomal dispersion was administered by inhalation for 2 min to group III and IV, respectively, 30 min prior to allergen challenge. The dose of SCG aerosolized was 50 mg. Liposomal dispersion was freshly prepared, centrifuged as described earlier, resuspended in solution of SCG so as to have 50% of the drug in encapsulated form. Under the conditions employed for aerosolization the median particle diameter was 5.6 ± 0.7 μm, determined by Malvern mastersizer particle size measurement system (Malvern, UK). Animals were anaesthetized with phenobarbitone sodium, 50 mg/kg intraperitoneally 24 h after challenge. As it was reported that the greatest degree of bronchial inflammation occurs at 24 h (Tarayre et al., 1991) this time point was selected. A tracheostomy was performed, the trachea was cannulated, and the animals were exsanguinated by severing the carotid arteries. Towards the end of exsanguination, both lungs were lavaged with 3 ml of sterile saline at 37 °C instilled through the tracheal cannula by syringe. After 3 min the lavage fluid was recovered by gentle aspiration. A second lavage was performed with a further 3 ml of saline, and the fluids were collected. The collected fluid was counted for leukocytes on an Erma-PC607 cell counter (Erma Inc., Japan). After spreading on a slide, fixing and staining with lucofine stain, the number of mononuclear cells, neutrophils and eosinophils per micro liter were determined. Student's *t*-test was used to compare differential and total leukocyte count in BAL, of all groups.

2.3.7.2. Method based on recovery from micro anaphylactic shock (Lakdawala et al., 1982).

2.3.7.2.1. Method of sensitization and challenge. Guinea pigs were sensitized using suspension of aluminium hydroxide gel and egg albumin. The suspension was prepared by mixing 20% aluminium hydroxide gel with 0.1% egg albumin in saline. Each animal received injection of 0.5 ml of this suspension subcutaneously. After 21 days, the sensitized guinea pigs were placed in perspex chamber of 4.5 l exposed to 0.5% egg albumin aerosol, which was generated via a compressed air line operating at a pressure of 10 psi.

The animals were divided into four groups of five each. Group I included animals, which were not sensitized but challenged and kept as a control. For remaining three groups, indomethacin (10 mg/kg) suspended in 0.5% carboxy methylcellulose was administered intraperitoneally 30 min prior to the egg albumin aerosol (challenge). Group III and IV inhaled aerosolized drug solution and liposomal dispersion, respectively, 2 h before allergen challenge.

Recovery period, the time in minutes that the animal took to revert back to normal respiratory pattern from the onset or preconvulsion time, was determined. Student's *t*-test was applied to compare recovery time between different groups.

3. Results and discussions

EE of liposomes was 25.09 ± 3.44 mg/mM. This value is comparable with EE of vesicles prepared by lipid film hydration method. However, it is lower than the value of EE (11.17 mg/ml%), reported for REVs containing SCG (Taylor et al., 1989). The mean particle size of empty and drug loaded liposomal dispersions was 2.92 ± 0.2 and 2.90 ± 0.28 μm, respectively, indicating that incorporation of drug in liposomes did not have significant effect on mean particle size. Empty and drug loaded liposomal dispersions showed majority of spherical vesicles, many of which appeared unilamellar with some bilamellar ones.

In in vitro release studies, the liposomal dispersion was found to release about 3% of drug within first 30 min. At the end of 8 h, 21% SCG was

released. Insignificant increase in release was observed at the end of 24 h as only 24% of SCG was released. In order to understand the barrier presented by dialysis membrane, in vitro release study of plain drug solution of same concentration was carried out in a similar manner. Within first 10 min, 15% drug was detected in dissolution medium, which was 7-folds higher than that of liposomal dispersion and 100% drug was found to permeate through the dialysis bag at the end of 5 h.

These results are in line with earlier reports in which inclusion of higher proportion of cholesterol in liposomes resulted in prolonged drug retention (Taylor et al., 1990b; Peschka et al., 1998). It is also reported that incorporation of cholesterol into liposomes having phospholipids above their phase transition temperatures decreases efflux of cytarabin (Ganapathi et al., 1980). Thus, cholesterol plays an important role in giving stability and reducing permeability of lipid bilayers and liposomal entrapment of SCG can significantly prolong in vitro release of SCG.

Microscopic observation at all temperatures did not show significant change in vesicle size or aggregation for 1 month. Liposomal preparations showed creaming but no crystals were observed and were easily dispersible on shaking. Empty and drug loaded liposomal dispersions showed no significant change ($P < 0.05$) in mean particle size and particle size distribution range even after storage at 4 °C for 2 months in comparison with initial pattern, as shown in Table 1.

Empty liposomal dispersions stored at different temperatures were treated in a way similar to that of drug loaded liposomes. The supernatants when evaluated by HPLC showed no peak at the retention time corresponding to that of the drug. Chromatograms for empty liposomes, initial and after 1 month storage at 45 °C were similar. The percentages of the drug retained in drug loaded liposomes were 100, 91.93 and 83.03% after 15 days storage at 4, 25 and 45 °C, respectively. After storage of 1 month, 100, 89.29 and 71.17% of drug retention was observed in liposomes stored at 4, 25 and 45 °C, respectively, as shown in Table 2. Maximum reduction in EE was observed following storage at 45 °C as shown in Table 2 whereas EE was almost unaffected during storage at 4 °C. This stability of formulation can be attributed to 50 mol% cholesterol included in formulation.

Lyophilization has a great potential as a method to solve long term stability problems of liposomes. Empty liposomal dispersions when freeze-dried without cryoprotectant gave rise to pasty mass. Use of trehalose gave free flowing powder. Trehalose, a carbohydrate commonly found at high concentrations in organisms capable of surviving dehydration, is reported to be an excellent cryoprotectant for liposomes (Harrigan et al., 1990). It may work by stabilizing the bilayers, especially at their phase transition temperatures, during both freezing and thawing (Crowe and Crowe, 1993).

The data related to the entrapment of SCG before and after freeze-drying are given in Table 3. The release of drug, i.e. the amount of drug leaked was 40.5% during freeze-drying indicating that

Table 2
EE after storage of 15 days and 1 month at various temperatures

Initial EE: 24.47±1.71			
	4 °C	25 °C	45 °C
After 15 days	24.99±5.61	22.50±2.54	20.32±0.22
After 1 month	24.81±3.27	21.85±1.07	17.42±0.29

EE values are expressed as mg of drug per millimole of lipids.

Table 1
Effect of storage on mean particle size and range of liposomal formulations

Type of liposomal dispersion	Mean particle size (μm) (initial)	Range (μm) (initial)	Mean particle size (μm) (after 2 months)	Range (μm) (after 2 months)
Empty	2.92±0.2	0.2–11	2.92±0.29	0.2–9
SCG loaded	2.90±0.28	0.2–9	2.61±0.31	0.2–9

Table 3
SCG content of the liposomes before and after freeze-drying

SCG content before freeze-drying[a]			SCG content after freeze-drying[b]			Percent release[c]
Total	Free	Percent encapsulated	Total	Free	Percent encapsulated	
651.4	201.5	69.06±1.05	651.4	383.7	41.08±0.01	40.51±0.02

[a] SCG content is expressed as μg of drug per milliliter of liposomal dispersion. 'Total' refers to the concentration of SCG, which is still present in the pellet after centrifugation 'Free' refers to the concentration of unentrapped drug separated by centrifugation.
[b] SCG content determined after freeze-drying and rehydration of liposomes.
[c] Percent of drug released during freeze-drying.

about 60% of encapsulated drug was protected due to presence of trehalose. This is in agreement with earlier studies, which have shown that even under best circumstances like use of saturated lipids and incorporation of cryoprotectants, a portion of the water soluble marker such as carboxyfluorescein, is lost on reconstitution (Crommelin and Van Bommel, 1984). On the other hand, liposomes can retain greater than 90% of lipid soluble drugs, such as Doxorubicin, on reconstitution (Van Bommel and Crommelin, 1984). The amount retained depends on the use of cryoprotectants, lipid composition, liposome type and loading dose (Lasic et al., 1998). The mass ratio between the sugar and lipid is important, not the molar ratio.

Table 4 gives mean particle size of liposomal formulation before and after freeze-drying. Reconstitution of freeze-dried powder showed increase in mean particle size to 7.39±0.21 and 8.08±0.01 μm from initial particle size of 3.22±0.1 and 3.06±0.45 μm, respectively, for empty and drug loaded liposomal dispersions. Optical microscopy of same formulations showed aggregation and mean size was found to be around 5.08 and 4.65 μm, respectively. Transmission electron microscopy revealed vesicles, some of which were spherical but many of them appeared to have slight angular contours.

Table 4
Mean vesicle size before and after freeze-drying

	Type	Mean particle size (μ)
Before freeze-drying (by laser diffraction)	Empty	3.22±0.1[a]
	Drug loaded	3.06±0.45[a]
After freeze-drying (by laser diffraction)	Empty	7.39±0.21[a]
	Drug loaded	8.08±0.01[a]
After freeze-drying (by optical microscopy)	Empty	5.08
	Drug loaded	4.65

[a] Each value is mean of three determinations.

In vivo study was based on evaluation of inhibition of influx of neutrophils at 24 h by SCG formulation. Disordered airway function in asthmatic responses is associated with an infiltration into bronchial lumen of neutrophils (Hutson et al., 1988).

Recovery of lavage fluid was consistently between 70 and 80% of that instilled. Preliminary studies were carried out in the laboratory to determine the effect of egg albumin on cell numbers in sham challenged control and animals exposed to 0.9% sodium chloride solution. Studies revealed no significant differences in the various types of leukocytes obtained from BAL.

Sensitization of the animals by inhalation using egg albumin as the antigen and subsequent challenge was found to significantly augment the cell numbers in the BAL as compared with sham challenged animals ($P < 0.001$). At 24 h after allergen challenge, there was a significant increase in number of neutrophils ($P < 0.001$), eosinophils ($P < 0.001$) and mononuclear cell ($P < 0.001$) population as compared with sham challenged animals as seen in Table 5.

Liposomes containing SCG caused inhibition of neutrophil influx and eosinophil influx ($P < 0.05$) as compared with positive control as shown in

Table 5
Differential Leukocyte Count in BAL fluid (±S.E.M.) after 24 h

Groups	Neutrophils	Eosinophils	Mononuclear cells
I (Sham challenged)	170±118.02	19.33±11.39	110.67±70.75
II (positive control)	1443.75±147.99[A]	254.25±7.49[A]	852±129.45[A]
III (SCG treated)	718±107.80[C]	136.33±44.9	779±166.32
IV (liposome treated)	291.75±30.53[C,D]	81.25±22.46[C]	327±104.53

Values are expressed as the mean±S.E.M. of five observations. Significant values are analyzed by Student's t-test are as, [A], $P < 0.001$; [B], $P < 0.005$ (as compared with Sham control); [C], $P < 0.05$ (as compared with positive control); [D], $P < 0.05$ (as compared with SCG treated group).

Table 5. SCG solution was found to inhibit neutrophil influx significantly as compared with positive control ($P < 0.05$) but failed to inhibit eosinophil influx. Liposomes significantly inhibited neutrophil influx as compared with the drug solution at this time interval. Mononuclear cell counts did not show significant difference in any of these groups.

Thus, liposomes were found to inhibit significantly neutrophil influx at 24 h not only when compared with the positive control but also in comparison with the drug solution ($P < 0.05$). Pharmacokinetic studies reported in humans wherein liposomal formulation of SCG was compared with solution, showed that the drug was at detectable level in case of liposomal formulation after 24 h following inhalation (Taylor et al., 1989).

Table 6 gives results of recovery time from microanaphylactic shock for various groups. The time for recovery for Group I and II was observed to be 56±8.19 and 104±0.99 min, respectively.

Table 6
Time of recovery phase of microanaphylactic shock in guinea pigs (±S.E.M.)

Groups	Recovery time (min)
I (control)	56±8.19
II (control with indomethacin)	104±0.99[A]
III (SCG treated)	63.75±5.15[C]
IV (liposome treated)	50.5±4.17[B]

Values are expressed as the mean±S.E.M. of five observations. Significant values are analyzed by Student's t-test are as, [A], $P < 0.02$ (as compared with Group I); [B], $P < 0.001$ (as compared with Group II); [C], $P < 0.006$ (as compared with Group II).

Indomethacin is reported to prolong recovery phase of microanaphylactic shock of guinea pigs. The increase in recovery phase was significant between Group I (Control Group) and the Group II, treated with indomethacin ($P < 0.02$). The prolongation in recovery phase was due to inhibition of cyclo-oxygenase pathway, which diverts arachidonic acid metabolism into the lipoxygenase pathway. This in turn enhances the formation of leukotreines which are SRS-A, leading to prolongation of recovery phase. Group III and IV showed recovery phase of 63.75±5.15 and 50.5±4.17 min, respectively. SCG was found to inhibit induced prolongation of recovery phase as it has tendency to inhibit the release of SRS-A. Therefore, no significant difference was found between recovery period of Group I and SCG solution/liposomal dispersion (Group III/Group IV). The recovery phase was significantly reduced by liposomal dispersion ($P < 0.001$) and SCG solution ($P < 0.006$) as compared with indomethacin treated control group (Group II). However, the difference between recovery period of SCG solution treated group and liposome treated group was found significant at $P < 0.09$. Probably experimentation at a time point later than 2 h could have given more probability of distinguishing between performance of SCG solution and liposomal dispersion.

4. Conclusion

Process of 'lipid film hydration' resulted in formation of spherical liposomes of SCG, which exhibited prolonged drug retention in in vitro

release studies. Liposomes exhibited good keeping properties when stored at 4 °C. Freeze-dried liposomal dispersion retained 60% of drug in presence of cryoprotectant upon reconstitution. Liposomal dispersion showed significant inhibition of influx of neutrophils as compared with drug solution at 24 h in BAL when evaluated in guinea pigs.

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