Research Paper

PREPARATION, OPTIMIZATION, CHARACTERIZATION OF LIPOSOMES CONTAINING SERRATIOPEPTIDASE FOR ORAL DELIVERY

J S Dua, Dr. A C Rana, Dr. Anil Bhandari

Address for Correspondence

[1]Research Scholar, JNU, Jodhpur and Asst. Professor, Shivalik College of Pharmacy, Nangal, Punjab
[2]Director, Rayat institute of Pharmacy, Nawanshahr, Panjab
[3]Jodhpur National University, Jodhpur, Rajasthan

ABSTRACT

The liposome prepared by ethanol injection method by using Phosphatidylcholine and cholesterol at 10:10 ratio shown good % Entrapment efficiency and *In-vitro* drug release study. Optimized formulations were subjected to stability studies at two different temperatures for up to 45 day. No significant changes in drug entrapment efficiency and mean particle size were observed during the course of stability study for formulations stored at 4-8ºC but there was a significant decrease in drug entrapment efficiency for liposome stored at room temperature after 15, 30 and 45 days and the mean particle size were increased at room temperature after 15, 30 and 45 days.

KEYWORDS Serratiopeptidase, liposomes, phosphatidylcholine, Cholesterol, stability studies.

INTRODUCTION

A **liposome** is a tiny bubble (vesicle), made out of the same material as a cell membrane. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases.

Liposomes were first described by British haematologist Dr Alec D Bangham FRS in 1961 (published 1964), at the Babraham Institute, in Cambridge. They were discovered when Bangham and R. W. Horne were testing the institute's new electron microscope by adding negative stain to dry phospholipids. The resemblance to the plasmalemma was obvious, and the microscope pictures served as the first real evidence for the cell membrane being a bilayer lipid structure.

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body. Structurally, liposomes are concentricbleedervesicles in which an aqueous volume is entirely enclosed by a membraneous lipid bilayer. Membranes are usually made of phospholipids, which are molecules that have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water.[1]

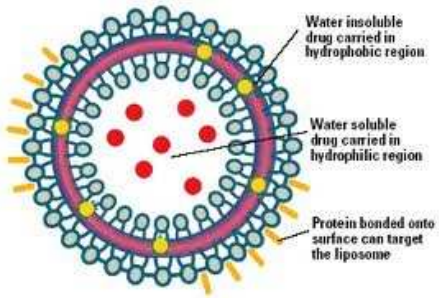

Fig. - 1: Scheme of a liposome formed by phospholipids in an aqueous solution.

In nature, phospholipids are found in stable membranes composed of two layers (a bilayer). In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment, and another layer of heads faces inside the cell, attracted by the water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bilayer.[1]

When membrane phospholipids are disrupted, they can reassemble themselves into tiny spheres, smaller than a normal cell, either as bilayers or monolayers. The bilayer structures are liposomes. The monolayer structures are called micelles.

The lipids in the plasma membrane are chiefly phospholipids like phosphatidylethanolamine and phosphatidylcholine. Phospholipids are amphiphilic with the hydrocarbon tail of the molecule being hydrophobic; its polar head hydrophilic. As the plasma membrane faces watery solutions on both sides, its phospholipids accommodate this by forming a phospholipid bilayer with the hydrophobic tails facing each other.

Liposomes can be composed of naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine), or of pure surfactant components like DOPE (dioleoylphosphatidyl-ethanolamine). Liposomes, usually but not by definition, contain a core of aqueous solution; lipid spheres that contain no aqueous material are called micelles, however, reverse micelles can be made to encompass an aqueous environment.[2]

ADVANTAGES

Some of the advantages of liposome are as follows:

- Provides selective passive targeting to tumor tissues (Liposomal doxorubicin).
- Increased efficacy and therapeutic index.
- Increased stability via encapsulation.
- Reduction in toxicity of the encapsulated agents.
- Site avoidance effect.
- Improved pharmacokinetic effects (reduced elimination, increased circulation life times).
- Flexibility to couple with site specific ligands to achieve active targeting.[1]

MATERIAL AND METHODS

OPTIMIZATION OF PROCESS VARIABLES

Preparation of Liposomes

***Melt Method*[3]**

Table 01: Preparation of Liposome by Melt method

Ingredients (Cholesterol: phosphatidyline)	A1 (2:1) (mg)	A2 (3:1)	A3 (4:1)	A4 (2:1)	A5 (3:1)	A6 (4:1)
Serratiopeptidase	25 mg	25 mg	25 mg	25 mg	25 mg	25 mg
Soya lecithin	200 mg	200 mg	200 mg	-	-	-
Egg phosphatidylcholine	-	-	-	200 mg	200 mg	200 mg
Cholesterol	400 mg	600 mg	800 mg	400 mg	600 mg	800 mg
HPMC K4M	25 mg	25 mg	25 mg	25 mg	25 mg	25 mg
Saline phosphate buffer (pH 7.4)	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml

***Ethanol injection method*[4]**

Table 02: Preparation of Liposome by Ethanol injection method

Ingredients (Phosphohatidylcholine: cholesterol) ratio	A_7 (p:c/10:3)	A_8 (p:c/10:5)	A_9 (p:c/10:7)	A_{10} (p:c/10:9)	A_{11} (p:c/10:10)
Serratiopeptidase	10 mg/ml	10 mg/ml	10mg/ml	10 mg/ml	10 mg/ml
Egg Phosphohatidylcholine	1000mg	1000mg	1000mg	1000mg	1000mg
Cholesterol	300mg	500mg	700mg	900mg	1000mg
Methanol:Ethanol (1:4)	10ml	10ml	10ml	10ml	10ml
HPMC K4M	25 mg	25 mg	25 mg	25 mg	25 mg
Saline Phosphate buffer (pH 7.4)	100ml	100ml	100ml	100ml	100ml

***Thin Layer Evaporation Method (TLE)* [5]**

Table 03: Preparation of Liposome by TLE

Ingredients (Phosphohatidylcholine :cholesterol) Ratio	A_{12} (p:c/1:1)	A_{13} (p:c/2:1)	A_{14} (p:c/3:1)	A_{15} (p:c/1:1)	A_{16} (p:c/2:1)	A_{17} (p:c/3:1)
Serratiopeptidase	100 µg/ml	100 µg/ml	100 µg/ml	100 µg/ml	100 µg/ml	100 µg/ml
Soya lecithin	250 mg	500 mg	750 mg	-	-	-
EggPhosphohatidylcholine	-	-	-	250 mg	500 mg	750 mg
Cholesterol	250 mg	250 mg	250 mg	250 mg	250 mg	250 mg
Chloroform : Methanol (9:1 ratio)	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml
HPMC K4M	25 mg	25 mg	25 mg	25 mg	25 mg	25 mg
Saline Phosphate buffer (pH7.4)	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml

Evaluation of prepared liposomes

***Size distribution profile*[6,7]**

The particle size and particle size distribution were determined using Zetasizer

***Drug entrapment studies*[6,7]**

Separation of unentrapped drug from the prepared liposomes can be carried out by mini column centrifugation method. Liposomal suspension (0.2 ml) was placed in Sephadex G-25 column (pre-saturated with empty liposomes) and centrifuged at 2000 rpm for 3 minutes. Elutes containing drug loaded liposomes have to be collected and observed under optical microscope to ensure the absence of unentrapped drug particles. Appropriate amount of elute has been digested with chloroform-methanol (2:1, v/v) and the clear solution thus obtained was analyzed spectrophotometerically (U.V./Visible spectrophotometer, Shimadzu-1601, India) for the drug content estimation at a λ max of 280 nm. Liposomes prepared without drug is treated in similar manner and served as blank for the above study. Percent drug loading (PDL) for the prepared liposomes were calculated as in Eqn.

$$PDL = \frac{\text{Entrapped drug (mg)}}{\text{Total drug added (mg)}} \times 100$$

***Zeta Potential*[6,7]**

The zeta potential of the liposomal formulations are determined using 0.1 M KCl buffer in demineralised water at 4-C ± 2-C, 25-C ± 2-C/60% ± 5% RH, or 40-C ± 2-C/ 75% ± 5% RH for a period of 10, 20, and 30 days (Zetasizer).

Storage - stability studies[6,7]

The ability of vesicles to retain the drug (i.e., drug retentive behavior) has been assessed by keeping the liposomal suspensions at four different temperature conditions, i.e., 4-8°C (Refrigerator; RF), 25±2°C (Room temperature; RT), 37±2°C and 45±2°C for a period of 5 weeks. The liposomal suspensions have kept in sealed ampoules (10ml capacity) after flushing with nitrogen. Samples are withdrawn periodically and analyzed for the drug content, in the manner described under drug entrapment studies.

***In vitro* drug release study:**

In vitro drug release study has been conducted to examine physical stability of liposomal formulation of Serratiopeptidase upon dialysis against PBS. Briefly, 2 ml of reconstituted liposomal Serratiopeptidase sample was placed into the dialysis cassette and then suspended in a temperature-controlled jacketed flask containing phosphate-buffer saline (PBS, pH 7.4) at 37.8^{0}C. At various time intervals, aliquot samples are withdrawn from the flask and subjected to visual inspection and drug content analysis by a stability-indicating UV Visible Spectrophotometer[6,7].

RESULT AND DISCUSSION

Formulation Development

Melt Method

Six different formulations were prepared by melt method by using Phosphatidylcholine and Cholesterol at different concentration level. All the obtained formulation were failed as there were lumps in the formulation visible by naked eyes because soya lecithin and egg phosphatidylcholine are of sticky nature.

Ethanol injection method

Further, five different formulation (A_7 to A_{11}) were prepared by ethanol injection method by using Phosphatidylcholine and cholesterol at different concentration level. All the formulation were obtained successfully and evaluated for various evaluation parameters.

Thin layer evaporation technique

Six different formulations were prepared by thin layer evaporation method by using Phosphatidylcholine and cholesterol at different concentration level. All the obtained formulation was failed due to sticky nature of soya lecithin and Phosphatidylcholine. They were strongly stuck to the flask of rotary evaporation.

Characterization of liposome:

Determination of size distribution and surface charge

Prepared liposome batches were monitored by Malvern (laser technique)

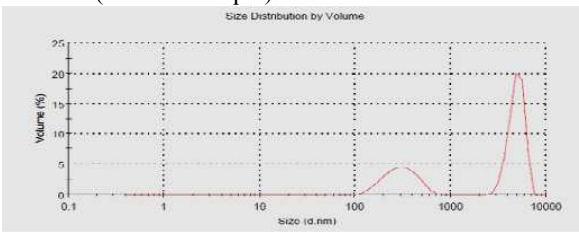

Fig. 02: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 270.3 nm for formulation A7.

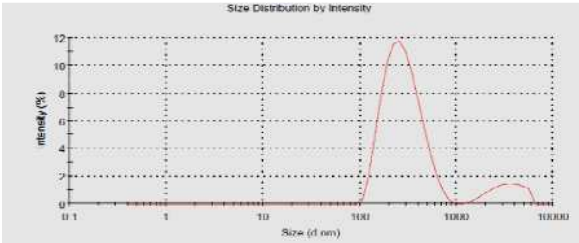

Fig. 03: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 281.1 nm for formulation A8.

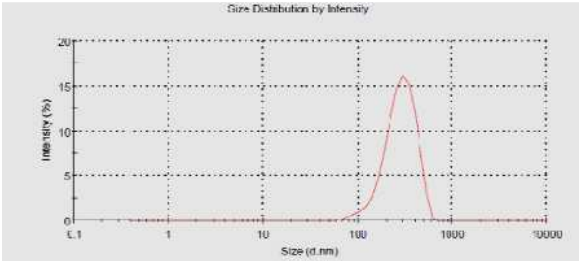

Fig. 04: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 286.7 nm for formulation A9.

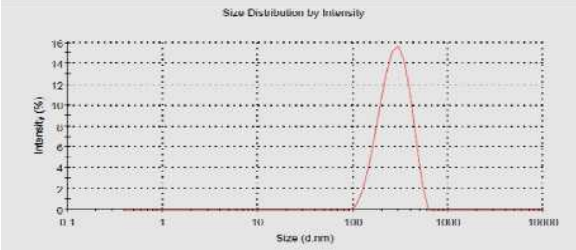

Fig. 05: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 410.4 nm for formulation A10.

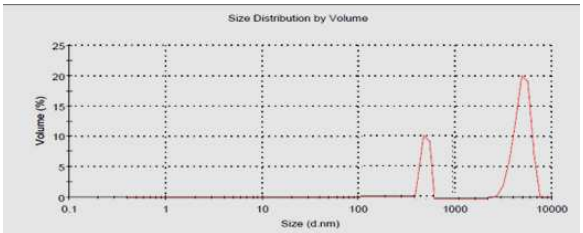

Figure No.06: Size distribution (SD) graph obtained from Malvern by laser technique showing SD of 420.5 nm for formulation A11.

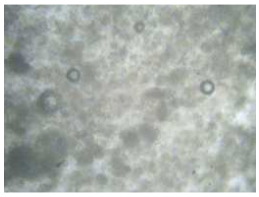

Fig. 07: TEM photohraph of liposome prepared by Ethanol Injection method

% Entrapment efficiency

Estimation of unentrapped drug from the prepared liposome was carried out by mini column centrifugation method and % entrapment was determined for each formulation.

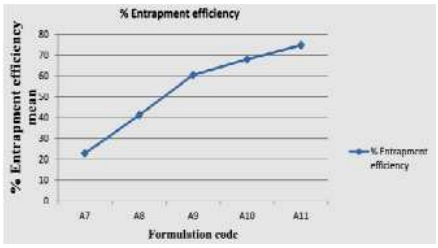

Fig . 08: % Entrapment efficiency

In the above formulation A_{11} have 74.34 % entrapment efficiency, A_{10} found to have 67.38 % entrapment efficiency, A_9 was found to have 62.28 % entrapment efficiency, A_8 have 40.55% entrapment efficiency and A_7 found least entrapment efficiency of 21.60 %.

Amongst all the formulation A_9, A_{10} and A_{11} were found to have good entrapment efficiency which is suitable for further evaluation studies. A_7 and A_8 were found to have least entrapment efficiencies of 21.60 & 40.55 % respectively.

In-vitro drug release study

Evaluation of *in vitro* drug release from encapsulated liposome was done by dialysis method. The output obtained by the dialysis method provided a correlation with the *in vitro* release.

Stability studies:

Serratiopeptidase liposomes (A_9, A_{10}, &A_{11}) were selected and their stability was evaluated at two different temperatures for up to 45 day. No significant changes in drug entrapment efficiency and mean particle size were observed during the course of stability study for formulations stored at 4-8ºc but there was a significant decrease in drug entrapment efficiency for liposome stored at room temperature after 15, 30 and 45 days and the mean particle size were increased at room temperature after 15, 30 and 45 days.

Table No. 03: Cumulative In-vitro drug release study

Time	Free drug	A9	A10	A11
30	10.54	4.39	2.49	2.5
60	21.12	9.05	8.14	6.21
90	64.42	17.55	15.44	10.52
120	85.44	28.45	24.78	15.44
150	88.77	38.4	31.25	22.98
180	89	52.41	43.65	28.22
210	89.11	64.58	54.19	32.91
240	89.11	78.45	68.18	38.48
270	89.22	82.45	77.98	47.05
300	89.22	89.45	84.11	57.14
330	89.23	92.88	90.18	67.1
360	91.31	94.1	92.13	73.15
390	94.91	94.18	93.44	83.24
420	94.92	94.8	94.91	94.92

Table no. 04: Drug entrapment efficiency of liposome formulation during Stability study

Formulation code	% Entrapment efficiency						
	Freshly prepared	At 4-8 ^{0}C			At room temperature (25 ^{0}C)		
		After 15 days	After 30 days	After 45 days	After 15 days	After 30 days	After 45 days
A9	72.55±0.41	72.23·02	71.56 ±1.13	72.25 ±0.88	64.19± 0.86	63.36±0. 62	60.45± 1.04
A10	53.23±071	54.15±1·2 7	58.14 ±1·37	57.63 ±0.63	56.23± 0.62	53.89±0. 22	502.23 ±0102
A11	61.58±053	64.32±0·7 8	65.58 ±0·76	60.45 ±086	60.78± 0.63	58.78±0. 62	52.95± 0.68

Table No. 05 : Mean particle size of liposome formulation during stability study

Formulation code	Mean Particle size						
	Freshly prepared	At 4-8 ^{0}C			At room temperature (25 ^{0}C)		
		After 15 days	After 30 days	After 45 days	After 15 days	After 30 days	After 45 days
A9	341.26± 3.04	367.98 ±4.84	367.05 ±3.34	380.14 ±6.04	389.0± 6.44	412.43± 4.44	447.0± 6.64
A10	342.23± 3.43	359.85 ±4.83	369.25 ±6.04	373.45 ±44.60	387.0±. 34.6	371.20±2 .64	436.33.± 8.28
A11	346.36± 3.61	340.0± 3.4.3	352.53 ±5.34	368.32 ±8.34	366.64± 6.48	390.21± 6.60	418.62± 4.04

CONCLUSION:

A11 formulation shown better release profile than A9 and A10.

ACKNOWLEDGEMENT

I am very thankful to Dr. D. N. Prasad, Principal, Shivalik College of Pharmacy, Nangal, Punjab for providing the necessary research facility for conducting my work. And I am very thankful to Dr. Amit K Jain, Principal, Guru Nanak Institute of Pharmacy, Dalewal, Hoshiarpur for compilation of my work.

REFERENCES

1. Kimball's Biology Pages, "Cell Membranes." Stryer S. Biochemistry, 1981, 213.
2. Vyas S.P. & Khar R.K. “Targeted & controlled drug delivery: Novel carrier system”. CBS publishers and distributors, 2007.
3. Yan Chen, Qingqing Wu, Zhenghai Zhang, Ling Yuan, Xuan Liu and Lei Zhou “Preparation of Curcumin-Loaded Liposomes and Evaluation of Their Skin Permeation and Pharmacodynamics “ Molecules 2012, 17, 5972-5987.
4. Deamer D. and Bangham A.D. *Biochim. Biophys. Acta.;* 1976; 443: 629.
5. Gomez-Hens, A., Fernandez-Romero, J.M. “Analytical methods for the control of liposomal delivery systems”, Trends Anal Chem, 2006, *25,*167–178.
6. Perrett S, Golding M, Williams WP. “A simple method for the preparation of liposomes for pharmaceutical applications: characterization of the liposomes. “ J Pharm Pharmacol. 1991 Mar;43(3):154-61.
7. Bhalerao SS, Raje Harshal A. “Preparation, optimization, characterization, and stability studies of salicylic acid liposomes” Drug Dev Ind Pharm. 2003 Apr;29 (4):451-67.