Research Article

Development and Characterization of Liposomal Drug Delivery System for Gossypin

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ABSTRACT

Site specific delivery of existing Anti epileptic drugs has led to several remarkable outcomes. One of the outcomes was the use of herbal drugs in the treatment of epilepsy. Gossypin a pentahydroxy flavone glucosidic compound isolated from *Hibiscus Vitifolius* is proven to have anti epileptic activity however the same activity as a formulation is still not provided. Conventional liposomes of Gossypin were prepared by film hydration technique using phospholipids like egg phosphatidyl choline, Soya lecithin, DMPC, DPPC and DSPC. Surfactants were incorporated to increase the flexibility and stability of liposomal vesicle. The concentration of surfactant, its type, drug: lipid ratio and lipid: cholesterol ratio was determined from the preliminary optimization results. Results indicated fast formation of liposome's that possess entrapment efficiency (up to 70%) and vesicle size range of 100-200 µm. Transmission electron and optical micrographs of the gossypin liposome's revealed that they were spherical and some were multilayered. An increase in the release rate of gossypin was observed when prepared with a drug: lipid ratio of 1:3.5 w/w. The release data showed that the highest release rates were obtained for gossypin containing Egg Phosphatidyl choline.

Keywords: Blood brain barrier, Cholesterol, Dipalmitoyl phosphatidyl choline, Distearoyl phosphatidyl choline, Egg phosphatidyl choline, Epilepsy, Gossypin, Liposome, 1-2-dimyristoyl-sn-glycero-3-phosphocholine.

INTRODUCTION

The highly impermeable tight junctions between endothelial cells forming the capillaries and venules in the Central Nervous System of higher vertebrates are thought to be responsible for Blood Brain Barrier.[1,2]

Blood Brain Barrier regulates the passage of the therapeutic proteins as well as drugs from the Cerebrovascular circulation to the brain.[3]

Many strategies have been tried for circumventing this barrier and effectively deliver the drugs to the brain.

Among the several carriers, which have been studied to overcome this problem, liposomes have gained increasing attention as promising strategies for brain-targeted drug delivery.[4]

Liposome's were discovered about 50 years ago by A.D. Bangham and were defined as spherical vesicles with phospholipids bilayer membrane used to deliver drug (s)/genetic material in to cell.[5,6]

Therapeutic efficacy for malignant brain tumors improved by the use of combination therapy of thermo sensitive liposome's and mild hyperthermia. These liposome's released their contents in response to mild hyperthermia.[7] Doxorubicin encapsulation into liposome's enhanced delivery to the tumors and minimized toxicity associated with conventional doxorubicin.[8] The anticryptococal activity of chloroquine was enhanced after incorporation in phosphatidyl serine containing negatively charged liposomes.[9]

The applications of liposomes as a carrier of drugs[10], vitamins[11], enzymes[12] or genetic material require control and prediction of the liposomal dispersion stability.[13]

Flavonoids are Polyphenolic compounds that occur ubiquitously in foods of plant origin. Over 4000 different naturally occurring flavonoids have been described. The flavonoids show various biological activities including antioxidant, anti-inflammatory activity, cardio-protective and antitumor activity.[14]

Gossypin a bioflavonoid (gossypin-8-0 glucoside; 3, 5, 7, 3, 4-pentahydroxy-8-0-glucosylflavone), is naturally occurring in various plants belonging to the family of *Malvaceae.*[15]

Gossypin, a new glycoside of gosypetin, was originally obtained from *Gossypium indicum* but now this bioflavonoid is obtained from *Hibiscus vitifolius* Linn.

Gossypin (10 and 20 mg/kg, p.o) exerts Anti-Convulsant activity against pentelentetrazole, strychnine and maximal electroshock induced seizures in mice.[16] Data from the study suggested that gossypin showed neuro protection /seizure protection probably through GABA (gamma amino butyric acid) aminergic and glycine inhibitory mechanism.

The objective of the present study was to determine the factors influencing encapsulation of gossypin in liposome's with additional advantage of dose reduction and optimize these factors to achieve a suitable liposomal system.

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MATERIALS AND METHODS

Materials

Gossypin was purchased from M/S Chromadex (USA), L-α-Egg Phosphatidyl choline (EPCL), 1-2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and Dipalmitoyl phosphatidyl choline (DPPC) were purchased from Sigma Chemicals. All other reagents and chemicals were of analytical grade.

Preparative Methods

Formulas for preparing liposomes were optimized by "model independent optimization method". Parameters that have significant effect on entrapment efficiency and *in-vitro* drug release were selected to investigate and they were (a) lipid: cholesterol ratio (Table 1), (b) surfactant concentration (Table 2).

The concentration of lipid, type and concentration of surfactant, lipid: cholesterol ratio, to be used was fixed based on the optimization results.

The liposome's were prepared accordingly and evaluated for entrapment efficiency and *in-vitro* drug release (Table 3).

Briefly, the desired amounts of lipid and cholesterol were weighed (including drug) in to 50 mL pear shaped flask and 4 mL of chloroform and methanol mixture (1:1) was added to dissolve lipids.

Organic solvent mixture was then removed under vacuum conditions using rotary evaporator at 60°C for 30 minutes, after which the flask was kept under vacuum overnight to completely remove any residual solvent. Encapsulation of Gossypin in to liposome's was accomplished by the addition of pH 7.4 phosphate buffer saline (PBS) containing 2 % Poloxamer P188. Dry lipids were hydrated with PBS.

Reduction of Liposome Size[17]

Liposomal vesicle size was reduced by subjecting to sonication for 60 minutes. Sonication causes the breakdown of larger vesicles.[2]

Physicochemical Characterization

Microscopic studies[18]

Vesicle morphology of the prepared liposome's with different molar ratios of phospholipids and cholesterol was determined using a photomicroscope.

Mean Particle size analysis[18]

Particle size and size distribution of liposomes were measured by the photon correlation spectroscopy (PCS, Zetasizer Nano S 90 Malvern, England)

TEM[19]

The TEM of the optimized formulation showed uniform spherical appearance of the liposomes.

Encapsulation efficiency[20]

Ultracentrifugation method was used to determine the encapsulation efficiency. In this method 1 mL of the liposomal suspension was subjected to centrifugation at 8000 rpm for 30 minutes at room temperature. Later the supernatant and sediment (concentrate) were collected and diluted with methanol. Lysis of liposomes was achieved with methanol and vortexing for 5 minutes. From the obtained clear solution concentration of gossypin was determined spectrophotometrically at a χ max 280 nm using empty lysed liposomes.

In-vitro release

Gossypin release from the liposomal formulation was evaluated using dialysis method proposed by Xu *et'al*. Briefly, 500 µl of liposomal suspension was taken in to an eppendorf tube assembly and then immersed in 40 mL of release medium (pH 7.4 PBS) while stirring the release medium using a magnetic stirrer, samples (1mL) were collected and replaced with same amount of fresh medium at predetermined time intervals over a 72 hrs period.

At the end of 24 hrs, 40 ml of release medium containing 0.5% Triton X100 and SDS was added to the previous one and 500 µl of the same was added to the liposomal sample in the tube. The collected samples were diluted suitably and concentration determined.

The release data of the final optimized formulation EPCL was subjected to Kinetic analysis (Figure 5).

RESULTS AND DISCUSSION

Optimization of formula

Selection of lipid: Cholesterol ratio

Five formulations (SLC 01 – SLC 05) were prepared having different lipid: Cholesterol concentrations (Table I) in order to optimize for highest drug entrapment and *in-vitro* drug release. It was found that formulation SLC 02 with a lipid: cholesterol concentration of 55:45 mM showed highest entrapment efficiency (87.09 %) and *in-vitro* drug release (87.27%) when compared to other concentrations.

Selection of surfactant

Surfactants like Tween 80, Sodium lauryl Sulphate and Poloxamer 188 were used in the concentration range of 0.025%-0.25% (Table 2). A significant increase in entrapment efficiency was observed with increase in Poloxamer 188 concentration from 0.025% to 0.2 %. The same was not observed with Tween 80 and Span 80. Improper correlation was observed in case of Tween 80 and Span 80.

Preparation of Gossypin containing liposome

Based on the above optimization results 55:45 lipid: cholesterol ratio was fixed as it showed highest *in-vitro* release and entrapment efficiency (Table 1 Formulation

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SLC02). Poloxamer at a concentration of 0.2% was selected for further studies (Table 2 Formulation SLP4).

Composition of final liposomal formulations and their % EE and drug release

Gosssypin containing liposome's were prepared using different phospholipids viz. dipalmitoyl phosphatidyl choline (PPCL), distearoyl phosphatidyl choline (SPCL), Egg phosphatidyl choline (EPCL), 1-2-dimyristoyl-sn-glycero-3-phosphocholine (MPCL) and were evaluated for entrapment efficiency and *in-vitro* drug release

Formulation EPCL (Table 3) containing egg phosphatidyl choline showed highest entrapment efficiency of 89.29 % and an *in-vitro* drug release of 83.441 %

EPCL was selected as the final optimized formulation.

Table 1: Effect of Lipid: cholesterol ratio on % Entrapment efficiency and drug release

Formulation Code	Lipid:Cholesterol conc. (mM) {Soya lecithin : cholesterol}	Drug (mg)	P188 conc. % w/v	%EE	*In-Vitro* drug release
SLC01	50:50	10	0.2	82.11	88.72
SLC02	**55:45**	**10**	**0.2**	**87.09**	**87.27**
SLC03	60:40	10	0.2	55.59	47.42
SLC04	65:35	10	0.2	60.61	50.85
SLC05	70:30	10	0.2	32.31	51.20

Table 2: Selection of Surfactant (SLT with Tween 80, SLS with Sodiun Lauryl Sulphate, SLP with Poloxamer 188 as surfactant)

Formulation	Surfactant Conc.(% w/v)	Drug (mg)	Phospholipid (mg) [Soya Lecithin}	Cholesterol (mg)	%EE	*In-Vitro* drug release
SLT1	0.025	10	37.28	17.40	99.29	54.10
SLT2	0.05	10	37.28	17.40	92.38	64.07
SLT3	0.1	10	37.28	17.40	95.89	85.50
SLT4	0.2	10	37.28	17.40	70.66	70.45
SLS1	0.025	10	37.28	17.40	41.04	92.44
SLS2	0.05	10	37.28	17.40	58.71	90.35
SLS3	0.1	10	37.28	17.40	41.04	85.43
SLS4	0.2	10	37.28	17.40	34.33	83.30
SLP1	0.025	10	37.28	17.40	91.03	83.56
SLP2	0.05	10	37.28	17.40	59.47	94.66
SLP3	0.1	10	37.28	17.40	89.23	90.39
SLP4	0.2	10	37.28	17.40	98.52	85.01

Table 3: Final liposomal compositions

Formulation	Drug (mg)	Phospholipid (mg)	Cholesterol (mg)	P 188 Conc. (% w/v)	%EE	*In-Vitro* drug release
EPCL	**10**	**41.80**	**17.40**	**0.2**	**89.29**	**83.41**
MPCL	10	37.28	17.40	0.2	42.06	54.37
PPCL	10	40.37	17.40	0.2	98.48	64.80
SPCL	10	43.45	17.40	0.2	82.45	60.00
SLL	10	37.28	17.40	0.2	98.68	66.96

Characterization of Liposome's

Vesicle Size

Size of the drug loaded liposomes was carried out using Master sizer 2000 (Table 4).

Formulation EPCL showed a vesicle size of 104.35 nm (Figure 1).

Table 4: Vesicle size of final liposomal compositions

Formulation	Vesicle Size (radius in nm)
EPCL	104.35
MPCL	172.16
PPCL	28.33
SLL	33.00

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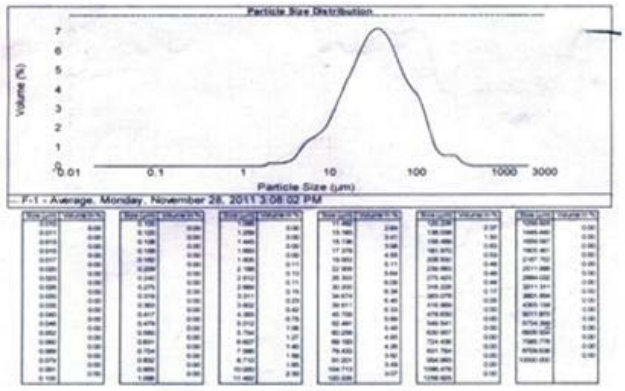

Figure 1: Vesicle Size for EPCL

Vesicle Shape

Vesicle Shape of the liposomes was analyzed by optical microscopy. Pictures of liposome's (Figure 2) were taken at a magnification of above 44 times by Nikon camera. The Liposome's were spherical and bilamellar in case of EPCL.

The TEM of EPCL formulation also showed the presence of spherical particles (Figure 3).

Figure 2: Microscopic picture of formulation EPCL

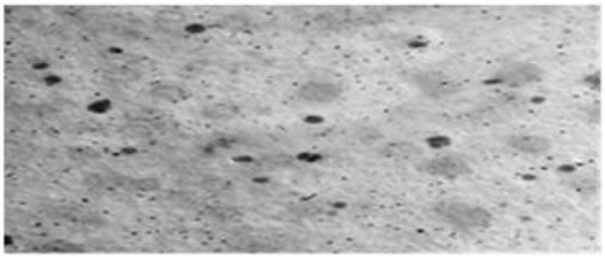

Figure 3: TEM picture of formulation EPCL

***In-Vitro* Drug release studies**

For assessing the drug release from the prepared liposomal formulations, a two stage dialysis method was followed, as proposed by Xu *et'al*.

First stage was for 24 hrs in which it was postulated that the drug release in this stage was only due to free drug. Also this stage mimics that before reaching the target site liposomes circulate in the blood for 24 hrs. Whereas in stage second, it was postulated that liposome's reach the target site by 24 hrs and therefore the drug release after 24 hrs must be taken in to consideration. The drug release after 24 hrs was determined as the actual drug released from the liposomes at the target site (Figure 4).

To mimic this in *in-vitro*, surfactants like Triton X -100 and 2 % SDS was added to both the liposomal sample and also the release medium.

The final Optimized EPCL formulation showed 83.41 % drug release at the end of 72 hrs and other formulations viz. MPCL, PPCL, SPCL & SLL showed 54.77, 64.80, 60 & 61 % drug release at the end of 72 hrs (Figure 4).

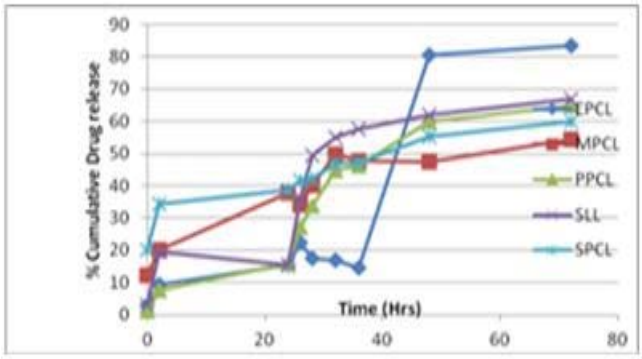

Figure 4: Drug Release profile of liposomal formulations

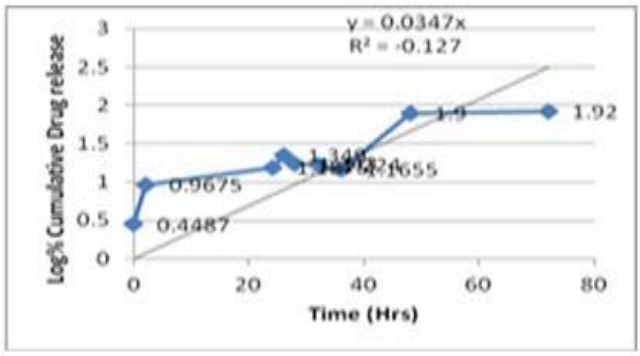

Figure 5: Best fit kinetic model for EPCL

CONCLUSION

The work mainly dealt with the development of gossypin liposomes. The liposome's, with synthetic and/ naturally derived phospholipids as the major component (55 mole %) was applied.

In summary, *In-Vitro* dialysis experiments showed that cholesterol (45mloe %) and 0.2 % surfactant incorporation lead to increased gossypin release from liposome's and higher drug-to-lipid ratio 1:3:6 resulted in faster release. The Improved therapeutic index of gossypin and drugs similar to gossypin are anticipated to be achieved with application of liposomal drug delivery system

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