ISSN 0975 - 6299 Vol 2 / Issue 3 / Jul - Sept 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

NOVEL DRUG DELIVERY SYSTEM

DESIGN AND CHARACTERIZATION OF LONG CIRCULATING LYOPHILIZED VESICULAR DRUG DELIVERY SYSTEM FOR ANTINEOPLASTIC AGENTS

ARJUN GOJE,* RAJENDRA C. DOIJAD AND SOMPUR C.K

NIMS University Shobha Nagar Jaipur India.

ARJUN GOJE
NIMS University Shobha Nagar Jaipur India.

ABSTRACT

Gemcitabine is an antineoplastic agent commonly used to treat some types of leukemia as well as cancers. In the present study, long circulating liposomal drug delivery system for Gemcitabine was developed, which will increase the efficacy and reduce the toxicity and target the organs (RES). Long circulating liposomal drug delivery system for Gemcitabine was prepared by thin film hydration method using two different carriers and same coating polymer with different concentrations. Four Formulations were prepared by using DPPC and DSPC as a carrier. All the formulations were compared in terms of particle size, polydispersity index, zeta potential, encapsulation efficiency, invitro release, in vivo tissue distribution and stability. Among those, Formulation F_2 and F_4 show better results. F_2 and F4 were subjected to in vivo studies, where formulation F_2 showed lower targeting efficiency towards liver spleen and heart, when compared to the formulation F4. Stability studies of F_2 revealed that 4^oC is the most suitable temperature for storage of long circulating liposomes.

KEY WORDS

Gemcitabine, leukemia, long circulating liposomal drug delivery, Dipalmityl phosphatidyl choline (DPPC), Distearoyl phosphatidyl choline (DSPC).

INTRODUCTION

The concept of site specific drug delivery for the treatment of localized disease in the body to improve therapeutic index of the drug is considered as a perennial challenge to the formulator in modern formulation design. Constant efforts have been pursued in designing such an ideal drug delivery system which can effectively overcome dose related toxicity and adverse side effects and thus improve patient compliance.[1] One such area which has attracted ever growing attention of pharmaceutical scientists and has shown tremendous potential and promise is colloidal drug carrier system.[2]

The ideal drug carrier with targeted specificity has been a challenge for any passionate scientist for a quite number of years and in the last decade, successful efforts have been made to achieve this goal.[3] The ultimate form of targeted drug delivery system should be realization of Paul Ehrliches "magic bullet concept"[4] which documents the delivery of drug exclusively to a preselected targeted cell type.

Amongst all targeted drug delivery systems, liposomes are recently gaining popularity because of their biological inert nature, freedom from antigenic, pyrogenic or allergic reactions and their enhanced stability.[5] Liposomes are micro particulate or colloidal carriers which form spontaneously and certain lipids are hydrated in aqueous media.[6] Liposomes are composed of relative biocompatible and biodegradable material and they consist of aqueous volume trapped by one or more bilayers of natural or synthetic lipids. Highly lipophilic drugs, with partition coefficient greater than 5 are entrapped almost completely in the lipid bi-layer of liposomes.

Antineoplastic agents used in the treatment of lung cancer, solid tumor, testicular cancer, breast cancer, several types of leukemia, lymphoma, etc. have often been associated with severe toxicities such as bone marrow depression. Consequently, bone marrow depression results in granulocytopenia, agranulocytosis, aplastic anemia, lymphocytopenia and inhibition of lymphocyte function which further leads the suppression of host immunity and etc.[7]

Cancer chemotherapy is generally accompanied by side effects. Cancer could be cured without side effects if an anticancer drug is delivered in the right site, in the right concentration, and at the right time. For such delivering system liposomal formation is thought to be useful since liposomes are essentially non toxic and biodegradable. Moreover, their size, components and modifications with various molecules are easily controlled, and they could deliver a large amount of either hydrophilic or hydrophobic agents.[8]

The delivery of liposomes at the appropriate site, however, is still not achieved for this purpose; both active targeting and passive targeting are considered. Conventional liposomes, however, tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. On the contrary, passive targeting, especially targeting tumor tissues, could be achieved by reducing the RES trapping, since the Vasculature in the tumor tissues is leaky enough to extravasate liposomes and circulating liposomes may accumulate passively in tumor tissues.[8] The development of liposomes containing lipid derivatives of PEG or saturated phospholipids such as DSPC with cholesterol has made targeted liposomal therapy more feasible by reducing the uptake by

RES system and their by prolonging the circulation time.[9]

Particularly, PEG is useful because of its ease of preparation, relatively lower cost, controllability of molecular weight and likeability to lipids or protein. Including the antibody by a variety of methods, the presence of PEG reduces the binding of serum protein, i.e. opsonins marking the liposomes for clearance by macrophages. We proposed that antibodies should be attached to the distal end of the PEG chains which are already bound to the liposome membrane.[10] In the proposed research, we have selected several functionalized PEG derivatives such as DPPE-PEG-Mal, and DSPE-PEG, in order to prepare long circulating (RES avoiding) liposomes.

In the proposed work an attempt is being made to develop long circulating liposomes (RES avoiding), by attaching functionalized PEG derivatives over the liposome membrane, for site specific delivery of selected anticancer drugs. Long circulating liposomes have the following advantages over the conventional liposomes.

- Reduced dosing frequency
- Increased efficiency and therapeutic index
- Increased stability via encapsulation
- Provide selective passive site targeting
- Reduction of unwanted side effects
- Sustained release of drug

MATERIALS AND METHODS

2.1 Materials:

Gemcitabine was received as a gift sample from Naprod life sciences P.Ltd Thane, India. Di steryl phosphotidyl choline (DSPC), Di palmitoyel glycerol 3 phosphocholine (DPPC), Methoxy polyethylene 2000-distearoyl phosphoethanolamine (DSPE, Sodium salt)- were obtained as gift samples from Genzyme, Switzerland. Cholesterol was received as a gift sample from Loba Chemie Pvt, Ltd., Mumbai-400002, India whereas Di sodium hydrogen phosphate, Potassium dihydrogen phosphate, Chloroform, Sucrose were received as a gift samples from S.D. Fine-Chem Ltd., Mumbai. Water HPLC grade was received from Spectrochem Pvt. Ltd., Mumbai.

2.2 Preparation of Gemcitabine loaded liposomes by thin film hydration Technique:

Thin film hydration technique as described by Bangham et al (1965) was used for the preparation of liposomes and long circulating liposomes. The phospholipids used were Di palmitoyl phosphocholine(DPPC), Di steryl phosphotidyl choline(DSPC), cholesterol and pegylated phospho lipid (for coating of the liposomes). Drug, cholesterol and DPPC or DSPC were taken in the mass ratio of 1:3:25 respectively. Only the concentration of PEGylated phopholipid was changed for all four formulations that are given in the table 1 of formulation plan[11].

Table 1
Formulation plan for Sterically Stabilized liposomes for Gemcitabine

Batch	Composition	Quantity (mg)
F-1	Drug : DPPC : Cholesterol : MPEG 2000 – DSPE Sodium salt	10 : 30 : 250 : 75
F-2	Drug : DPPC : Cholesterol : MPEG 2000 – DSPE Sodium salt	10 : 30 : 250 : 25
F-3	Drug : DSPC : Cholesterol : MPEG 2000 – DSPE Sodium salt	10 : 30 : 250 : 75
F-4	Drug : DSPC :Cholesterol : MPEG 2000 – DSPE Sodium salt	10 : 30 : 250 : 75

2.3 Freeze drying of Liposomes:

Usually liposomes may subject to a series of stability problems such as aggregation, fusion and leakage of the encapsulated drugs in to the storage medium. One of the approaches to resolve these kinds of problems is to freeze drying of the liposomes. A freeze-dried product offers the advantages of improved stability, dosing accuracy and sterility. The liposomal formulations were dispensed in glass containers rapidly frozen at -40^{0}C, freeze-dried for approximately 14 hours under vaccum in a Cuddon freeze dryer.[12]

2.4 Vesicle shape and surface morphology:

Particle shape analysis was done by Scanning Electron Microscopy (SEM). The three dimensional information about macro (0.1-10mm), meso (1-100 mm) & nanostructure (10-1100nm) are often found within the same micrograph. SEM has been used to determine particle size distribution, surface topography, texture and to examine the morphology of fractured or sectioned surface. Particle shape analysis was done by SEM using JEOL JSM-T330A scanning microscope. Cleaned brass specimen studs were used for mounting the samples.[13]

2.5 Vesicle size and size distribution:

The size distributions along the mean volume and diameter of the suspending particles were measured by dynamic scattering particle size analyzer (Nanotrac Particle Analyzer 150, Microtrac Inc., PA. USA) The particle size distribution was characterized using polydispersity index which is a measure for the width of the size distribution. The range of measurement of NPA 150 is 0.8 nm ~ 6.54 µm.[14]

2.6 Zeta potential: [15]

Measurement of the zeta potential of samples in the Zeta sizer Nano ZS is done using a combination of laser Doppler velocity and phase analysis light scattering (PALS) by a patented technique called M-3 PALS to measure the particle electrophoresis mobility. The liposome samples were diluted 1 in 10 with JBS and measurements were carried out at 25°C.

2.7 Determination of drug entrapment efficiency:

The percentage of incorporated Gemcitabine HCL (entrapment efficiency) was determined spectrophotometrically at 268nm. The entrapment efficiency (EE%) could be achieved by the following equation[9]

$$\text{Entrapment efficiency (\%)} = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}}$$

2.8 In vitro drug release studies:

Liposomes equivalent to 10 milligrams of Gemcitabine HCL were taken into a tube with both ends open. One end of the tube is closed with dialysis membrane. Now the tube containing drug loaded liposomes is kept in a beaker containing 50 ml of PBS of pH 7.4. The tube is arranged in such a way that, it just touches the surface of the buffer solution. The whole set up is placed on a magnetic stirrer and rotated at 50 rpm. The temperature of the buffer is maintained at 37^{0}C ± 1°c. One ml aliquots of release medium were withdrawn at time intervals of 0.5, 1, 2, 4, 8, 16, 24 hrs and replaced by the same volume of PBS. These samples were filtered through 0.45µm membrane filter. The Filtrate was diluted suitably and estimated by UV - Vis. Spectrophotometer at 268 nm[17].

2.9 Sterlity test:

The formulation was sterilized by membrane filtration method and was incubated with different culture media like agar medium, thioglycate medium etc., for a period of 14 days and observed for the absence of microbial growth.

2.10 In vivo studies:

This study was carried out after obtaining the due permission for conduction of experiments from relevant ethics committee which is registered for "Teaching and Research on Animals by Nims University Jaipur", a committee for the purpose of control and supervision of the experiments on animals.

This study was carried out to compare the targeting efficiency of drug- loaded liposomes with that of free drug in term of percentage decrease in targeting to various organs of reticuloendothelial system (RES) like liver, lungs, spleen and kidneys. Experiments were performed on Wistar rats.[18, 19]

The individual organs of each rat were homogenized separately by using a tissue homogenizer with 5ml of ethanol and the homogenate was centrifuged at 15,000 rpm for 30 minutes. The supernatant was collected and filtered through 0.45µm filters and analyzed spectrophotometrically after dilution with phosphate butler saline at 268 nm.

2.11 Short term Stability studies: [20, 21]

From the 4 batches of liposomes, formulation F2 was tested for stability studies. Formulation F2 was divided into 3 sample sets and stored at:

- 4^{o}C in refrigerator.
- 30^{o} C ± 2o C/65% RH in humidity control oven (GINKYA IM 3500 Series).
- Ambient temperature and humidity.

The liposomal samples were tested for the following attributes at each temperature after 30 days of storage.

- Signs of sedimentation or creaming.
- Change in particle size, PDI and Zeta potential.
- Change in the encapsulation efficiency.

RESULTS AND DISCUSSION

Vesicle shape and surface morphology:

Scanning electron photomicrographs by Scanning electron micrograph (SEM) of all the four formulations are shown in plates 1, 2, 3 and 4. Magnification of 7.500- 20,000 X was used while taking these photographs. The images confirmed that liposomes were spherical and smooth in nature. Therefore it seems that Gemcitabine encapsulation did not affect the morphology of liposomes.

Scanning electron micrographs

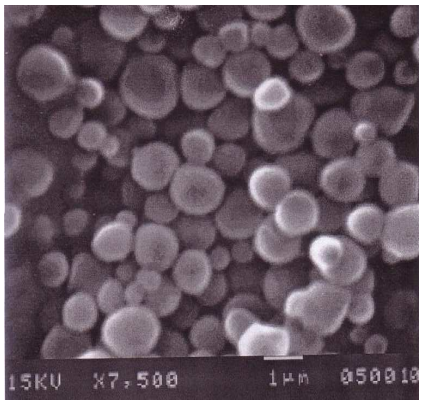


Plate 1

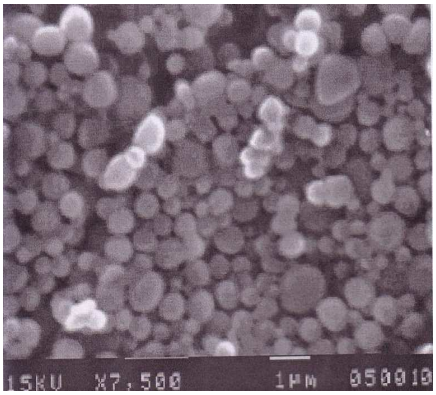


Plate 2

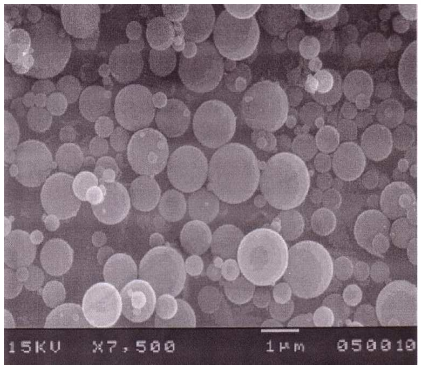


Plate 3

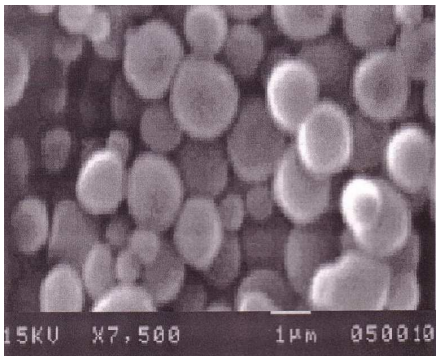


Plate 4

Figure 1
Scanning electron micrograph of F1 (Plate 1), F2 (Plate 2), F3 (Plate 3), and F4 (Plate 4)

Vesicle size and size distribution:

Formulations F1 to F4 are extruded through filters with pore size 0.4 and 12µm before analysis. Average particle size of Gemcitabine long circulating liposome was found to be 321 ± 4,806 nm, 244 ± 1.760 nm, 393 ± 11.5nm, 227 ± 3.00 nm and for FI, F2, F3 and F4. Particle size was in the order of F4< F2< FI < F3 (Table 2).

The polydispersibility index values in table 2 shows the presence of homogenous particle population in F 1, F2 and F4, where as F3 contains heterogeneous liposome population.

Zeta potential:

PEGylated liposomes, which are slightly negatively charged, are moderately stable. MPEG2000-DSPE is the coating polymer used to prepare the long circulating liposomes that prevents particles to come together and give stable formulations. So there is no need to use the charge inducing agents in case of long circulating liposomes. Zeta potential of all formulated long circulating liposomes is presented in table 2, which indicates that they are moderately stable.

Drug Entrapment Efficiency:

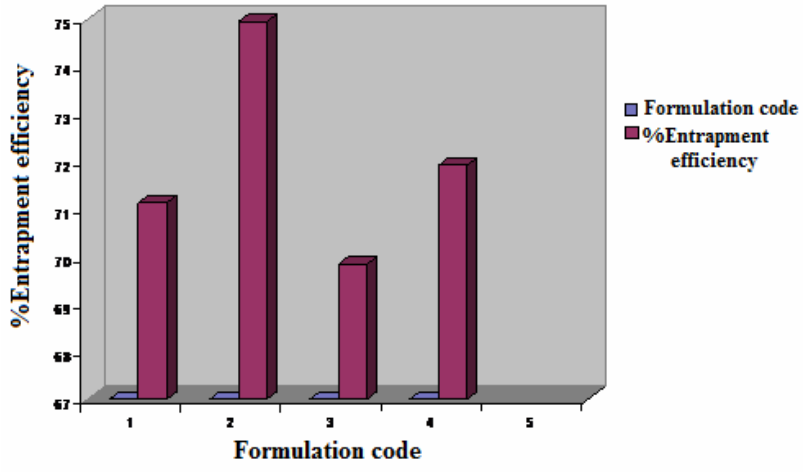
The change in phospholipids and the concentration of PEGylated phospholipids had a significant effect on entrapment of hydrophilic drug, Gemcitabine. This can be explained as the DPPC are more water dispersible, the hydrophilic drug gets encapsulated as well as partitions into vesicle membrane. But, the DSPC are partial water dispersible. Hence the drug gets encapsulated in the aqueous core only. A slight decrease in the encapsulation was found mainly due to increase in the size of vesicle with high concentration of MPEG2000-DSPE. The drug encapsulation efficiency of all four formulations was shown in table 2 and graph 1.

Table 2
Vesicles size distribution, Zeta potential, Entrapment efficiency Gemcitabine long circulating liposomes

Formulation Code	Mean Vesicle Size (nm)	Polydispersity index	Zeta-potential	% Entrapment efficiency
F1	321 ± 4.806	0.212 ± 0..0155	-22.66 ± 2.69	71.13
F2	244 ± 1.760	0.245 ± 0.008	-10.63 ± 4.3	74.94
F3	393 ± 11.54	0.753 ± 0.012	-8.2 ± 1.4	69.84
F4	227 ± 3.00	0.213 ± 0.011	-7.9 ± 0.5	71.92

Graph 1

Entrapment efficiency of Gemcitabine long circulating liposomes



In Vitro Release Studies:

The release data obtained for formulations F 1, F2, F3 and F4 are tabulated in table 3. The burst release in the first hour can be attributed to the drug loaded on the surface of liposomes and due to the imperfect liposomal surface area. The prolonged release in the latter stage can be attributed to the slow diffusion of the drug through lipid bilayer.

These results indicate that formulation FI to F4 appear to fit in **Higuchi-matrix model** as shown in table 4 and graph 2

Table 3

Comparison of In Vitro Release Studies of four formulations

Time	% Drug release profile for formulation F1	% Drug release profile for formulation F2	% Drug release profile for formulation F3	%Drug release profile for formulation F4
0.5	20.17	20.77	17.87	20.77
1	30.52	36.32	30.37	33.42
2	34.89	43.89	34.75	40.84
4	53.08	59.90	46.98	53.66
8	66.62	71.12	57.04	70.25
16	75.21	76.95	65.19	76.08
24	81.18	85.83	76.54	82.05

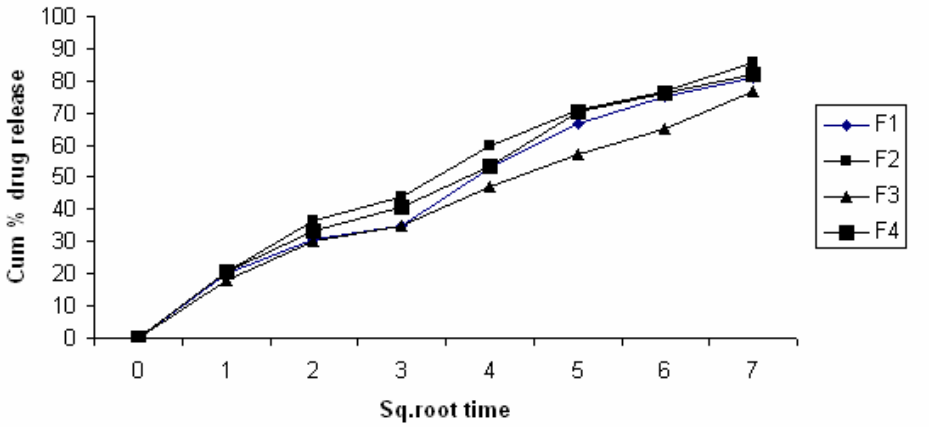
Table 4

Model Fitting for drug release profile of formulations F1-F4

FORMULATION CODE	MATHEMATICAL MODELS(KINETICS)						
	Zero order	First order	Higuchi matrix	Peppas model	Hixson crowell	"n" Value	Best fit model
F1	0.9100	0.9846	0.9945	0.9899	0.9763	0.4769	Higuchi matrix
F2	0.8502	0.9783	0.9995	0.9900	0.9609	0.4665	Higuchi matrix
F3	0.8814	0.9622	0.9885	0.9840	0.9515	0.4625	Higuchi matrix
F4	0.8745	0.9839	0.9964	0.9930	0.9676	0.4663	Higuchi matrix

Graph 2

Comparative in vitro release profile of Gemcitabine long circulating liposome's (F1-F4) according to Higuchi-matrix order kinetics



Sterlity test:

In order to ensure the sterility of the finished product, the optimized formulation F2 was subjected to sterility test. The formulation sterilized by membrane filtration method and incubated with different culture media like agar medium; thioglycate medium etc., for a period of 14 days of incubation shows no growth of organism on the culture medium. This indicates that the formulation is sterile and passes the test for sterility.

In Vivo Drug Targeting Studies:

Formulations F2 and F4 with optimal particle size, Entrapment efficiency and satisfactory in-vivo release were selected for in-vivo drug targeting studies. The comparison between the amount of drug targeted from Long circulating liposome's and free drug in various organs is presented in graph 3

The results reveal that the drug loaded Long circulating liposomes F2 and F4 showed preferential drug targeting to liver followed by spleen, lungs, and kidneys. As compared to pure drug, higher concentration of drug was targeted to the organs after administering the dose in the form of long circulating liposomes except in the heart. Drug levels in the heart are closely related to the inherent cardiac toxicity of Gemcitabine. Therefore using liposomal Gemcitabine formulation could reduce the cardiac toxicity of Gemcitabine

It was also revealed that compared to F4, the concentration of F2 liposomal formulation was significantly lower in liver and spleen.

Short term Stability studies:

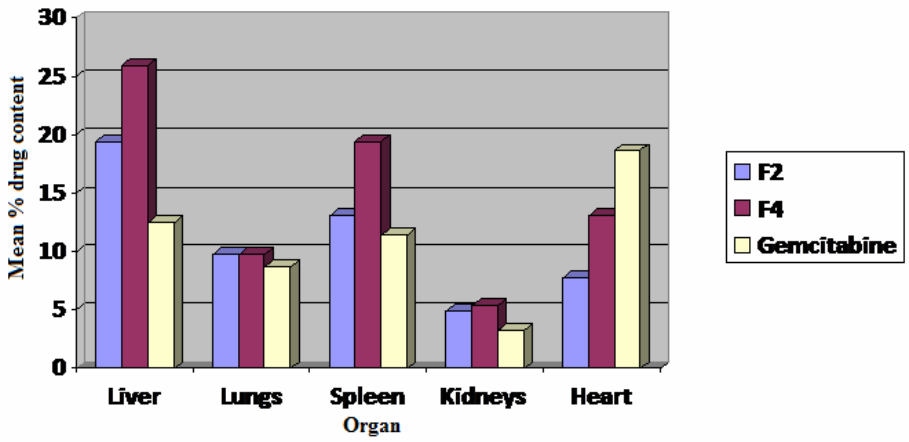
Formulation F2 shows no signs of sedimentation at 4^{o}C whereas slight sedimentation was observed at room temperature and 37^{o}C after 30 days. There was no significant change in the particle size and PDI, ZP, and EE even after 30 days of storage at 4^{o}C. The particle size and PDI were increased considerably at room temperature and 37^{o}C. Maximum reduction in ZP and EE was observed following storage at 37^{o}C.

The details are as shown in table 5

Table 5
Stability studies of formulation (F2) at various temperatures for 30 days.

Parameter	4°C	RT	37±2°C and 65% RH
Sign of sedimentation or creaming.	No sedimentation or creaming	Sight sedimentation or creaming	Sight sedimentation or creaming
Z-average(nm)	255	300	2150
Polydispersity index	0.262	0.565	0.763
Zeta potential	-9.53	-4.34	-2.01
Encapsulation efficiency	71.24	62.25	49.35

Graph 3
In Vivo Drug Targeting Studies



CONCLUSION

Long circulating liposomes of Gemcitabine were successfully formulated, characterized and evaluated in vitro. The DPPC-DSPE sodium (30:25) liposomes were of optimum particle size, Zeta potential, Entrapment efficiency and satisfactory cumulative percent drug release. The average targeting efficiency of drug loaded liposomes was found to be in liver followed by spleen, heart, lungs and kidneys respectively. Stability studies indicated that 4°C is the most suitable temperature for storage of long circulating liposomes of Gemcitabine. This drug delivery is endowed with several exclusive advantages and hence holds potential for further research and clinical application.

REFERENCES

1. Chien yw. Novel drug delivery system 2nd Ed. New York: Marcel Dekker Inc; 43 – 137, (1992).
2. Crommelin DJA, Schreier H. Liposomes, In : Kreuter J, Editor. Colloidal drug delivery system. Vol 9. New York, Marcel Dekker Inc; 73 – 190, (1994).
3. Justin MS, Ananth A, Jayanesh UN, Raviv B. Controlled targeting of liposomal doxorubicin via folate receptors In vitro, J. controlled release; 92 : 49 – 67, (2003).
4. Vyas S.P, Khar. R. K. Targeted and controlled drug delivery: Novel carrier systems, New Delhi, CBS Publisher: 304 – 52, (1997).

5. Jain SK, Jain NK, Liposomes as drug carriers, In: Jain NK, Editor. Controlled and Novel drug delivery, New Delhi : CBS Publishers: 304 – 52, (1997).
6. V. Ravichandiran, K. Masilamani, B. Senthilnathan, Liposome- A Versatile Drug Delivery System, Der Pharmacia Sinica, 2 (1): 19-30 (2011).
7. http://www.clinicaloncology.com/download/pg0816_ca_support_WM.pdf
8. Naoto Oku, Anticancer therapy using glucuronate modified long-circulating liposomes, Advanced Drug Delivery Reviews, Volume 40, Issues 1-2, Pages 63-73, (1999).
9. Yan-Li Hao, Ying-Jie Deng,Yan Chen, Ai-Jun Hao, Yong Zhang, Ke-Zhan Wang, In-vitro cytotoxicity, in-vivo biodistribution and anti-tumor effect of PEGylated liposomal topotecan, *Journal of Pharmacy and Pharmacology*,Volume 57, Issue 10, pages 1279–1287, (2005).
10. Kazuo Maruyama, Osamu Ishida, Tomoko Takizawa, Kunikazu Moribe, Possibility of active targeting to tumor tissues with liposomes, Advanced Drug Delivery Reviews, Volume 40, Issues 1-2, Pages 89-102, (1999).
11. LiangG ,Jia-Bi.Z,feix,Bin N.Preperation and charracteristion and pharmacokinetics of N-palmitoyl chitosin anchored docetexal liposomes. Jornal of Pharma pharmacol; 59;661-7, (2007).
12. Tesconi S, Scpassik,Freeze dryingabove room temperature .Journal of pharma Sciences; 88(5):501-05, (1999).
13. Jacob Js .Characterization delivery systems, Microscopy .In: Mathiouitz E.editor encyclopedis of controlled drug delivery Vol 1 John willey and sons,Inc.Newyork, 242-43, (1999).
14. Desai TR, Finely WH.Nebulisation of Niosomal all-trans-retonoic acid:an inexpensive alternative to conventional liposomes .Int J.Pharm; 241(2):311-7 (2000).
15. MolpecersJ,aberturas Mr ,GuzmanM.Biodegradable nano particles as a delivery system for cyclosporins preparation and characterization. J.Microencap; 17(5):599-614 (2000).
16. Nagarshanker MS,Condhe Vy.Preperation evaluation of liposomal formulation of sodium chromroglycate.Int J.Pharm; 251:49-56 (2003).
17. Agarwall R.Ktare op,Vyas SP,preparation and in vitro evaluation of liposomal Neosomal delivery system for antipsoriatic drug dithramol.Int.J.Pharm; 28:43-52 (2001).
18. Vyas SP,Gowswami Sk,singhR,Liposomes based nasal delivery systems of nifedipine.Development and characterization .Int .J.Pharm; 118;23-30 (1995).
19. Pregoc ,torres D,Megia FE,Caballal NR,Quinoa E,Alonsomj,chitosan –PEG nano capsules as new carrires for oral pepetides delivery effect of chitosan pegylation degree.J.controll Rel; 111;299-308 (2006).
20. The European Agency for the evaluation of medical products. Stability testing Guidelines: Stability testing of new drug substances and products. London: ICH – Technical Coordination, EMEA. [Online]. 2003. Available from: URL: http://www.emea.eu.int.
21. Bhalerao SS, Harshal AR, Preparation, Optimization, Characterization and stability studies of Salisylic acid liposomes, Drug DEV Ind. Pharm; 29(4): 457-67 (2003).